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S. N. ANANCHENKO

USSR Academy of Sciences, Shemyakin Institute of Bioorganic Chemistry, Moscow, USSR

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INTRODUCTORY SPEECH

Yu. A. Ovchinnikov

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow 117988, GSP-1, USSR

Ladies and Gentlemen,

On behalf of the USSR Academy of Sciences I have the great pleasure of welcoming you to the International Symposium on "Frontiers in Bioorganic Chemistry and Molecular Biology" under the auspices of the USSR Academy of Sciences and the Uzbek SSR Academy of Sciences and sponsored by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry. This major scientific forum devoted to a discussion of the physico-chemical basis of life processes has brought together leading scientists from throughout the world actively working in the most prospective areas of bioorganic chemistry and molecular biology.

The programme of the Symposium reflects the results and the future in the study of peptides and proteins, nucleic acids, polysaccharides, and other biopolymers, and in the study of low molecular regulators, including steroids, alkaloids, antibiotics etc. The programme also includes discussion of the latest achievements in the study of genetic structures and of cellular protein synthesizing systems of the molecular basis of enzymic catalysis and of bioenergetic processes.

Whereas a Symposium on the frontiers of a science is necessarily theoretical in scope, it is keenly felt that fundamental discoveries in bioorganic chemistry and molecular biology have a decisive bearing on the solution of practical problems in modern medicine and agriculture.

The Symposium is taking place in the year when one of the founders of Soviet physico-chemical biology, Mikhail Shemyakin, would have celebrated his seventieth birthday anniversary. It is only fitting that in Moscow the responsibility for organizing the Symposium has been assumed by the Institute bearing his name - the Shemyakin Institute of Bioorganic Chemistry of the USSR Academy of Sciences and in Tashkent by the Institute of Bioorganic Chemistry of the Uzbek SSR Academy of Sciences.

The organizers of the International Symposium on "Frontiers of Bioorganic Chemistry and Molecular Biology" wish to express their sincere gratitude to all its participants for giving it their time and thought, and cherish the hope that this meeting, furthering international scientific contacts, will make its contribution to the cause of peace and understanding between all peoples.

INDICATIONS OF A COMMON REACTION MECHANISM OF ENZYMES CATALYZING THE HYDROLYSIS OF PYROPHOSPHATE BONDS*

C. Carlsson and L. Ernster

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

Abstract - As earlier reported from this laboratory, tris-bathophenanthroline-Fe^{2+} (BPh_3Fe^{2+})** and other octahedral BPh-metal trichelates are powerful inhibitors of both membrane-bound and soluble mitochondrial F_1-ATPase, and the inhibition is relieved by uncouplers of oxidative phosphorylation. In this paper it is shown that BPh_3Fe^{2+} and related chelates also inhibit the following enzymes: F_1-ATPase from bacteria and chloroplasts, Ca^{2+}-ATPase of sarcoplasmic reticulum, Na^+K^+-ATPase of plasma membrane, actomyosin-ATPase, microsomal nucleoside tri- and diphosphatases, yeast PPiase and a bacterial PPiase. In all cases except the yeast PPiase, the inhibition is relieved by uncouplers. No inhibition by BPh_3Fe^{2+} was found with the following enzymes: yeast hexokinase, liver pyruvate kinase, liver AMPase, glucose-6-phosphatase, muscle aldolase, adenylate kinase, and intestinal alkaline phosphatase. It thus appears that the inhibition by BPh_3Fe^{2+} reflects a common mechanistic feature of enzymes catalyzing the hydrolysis of pyrophosphate bonds. Possible mechanisms of this inhibition and its relief by uncouplers are discussed.

INTRODUCTION

The pyrophosphate bond plays an important role in biology. It is the main chemical form in which energy is transmitted in living cells. There is a great variety of enzymes that catalyze the cleavage of pyrophosphate bonds. These enzymes can be divided into two categories: transferases, which catalyze the transfer of one of the cleavage products to another ligand; and hydrolases, which catalyze the hydrolysis of a pyrophosphate bond. Hexokinase, pyruvate kinase, adenylate kinase are examples of the first category; and myosin, Na^+K^+-ATPase, Ca^{2+}-ATPase, inorganic pyrophosphatase are examples of the second category. The proton-translocating ATPases of mitochondria, chloroplasts and bacteria also belong in the second category, even though the physiological function of these enzymes is the synthesis, rather than the cleavage, of ATP.

In earlier work (1-3) we have shown that both membrane-bound and soluble mitochondrial ATPase (F_1) is inhibited by the Fe^{2+} trichelate of bathophenanthroline (BPh_3Fe^{2+})**, and that the inhibition is relieved by uncouplers of oxidative phosphorylation. Similar effects were briefly noted with bacterial (2) and chloroplast (4) ATPases as well as the Na^+K^+-ATPase of plasma membrane (5,6) and the Ca^{2+}-ATPase of sarcoplasmic reticulum (5).

In this paper it is shown that these effects are shared by a number of additional hydrolases acting on pyrophosphate bonds. On the other hand, BPh_3Fe^{2+} does not inhibit a number of transferases that catalyze the cleavage of pyrophosphate bonds. A number of hydrolases acting on phosphate esters and other non-pyrophosphate bonds are also unaffected by BPh_3Fe^{2+}. The results suggest a common feature in the mechanism of enzymes catalyzing the hydrolysis of pyrophosphate bonds.

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* Paper presented at the International Symposium on Bioorganic Chemistry and Molecular Biology, Moscow and Tashkent, USSR, September 25 - October 2, 1978.

** Abbreviations: BPh, bathophenanthroline (4,7-diphenyl-1,10-phenanthroline); BPh-sulf., bathophenanthroline sulfonate; DPBP, 4,4-diphenyl-2,2-bipyridine; OPh, orthophenanthroline (1,10-phenanthroline); FCCP, carbonylcyanide-4-trifluoromethoxyphenyl hydrazone; S-13, 2',5-dichloro-3-tert-butyl-4'-nitrosalicylanilide; TTFB, 4,5,6,7-tetrachlorotrifluoromethylbenzimidazole; 1799, 2,6-dihydroxy-1,1,1,7,7,7-hexafluoro-2,6-bis (trifluoromethyl)-4-heptaone; PPiase, inorganic pyrophosphatase; NDPase, nucleoside diphosphatase; NTPase, nucleoside triphosphatase.

MATERIALS AND METHODS

F_1-ATPases from beef heart mitochondria (7), lettuce chloroplasts (8) and Rhodospirillum rubrum chromatophores (9) were prepared and assayed as described in the references indicated.

Myosin and actin were obtained in a pure state from rabbit skeletal muscle according to the procedure of Nauss et al. (10) and Spudich and Watt (11), respectively. Actomyosin was prepared by mixing myosin and actin in a 4:1 weight ratio (12).

Membrane vesicles of sarcoplasmic reticulum from rabbit skeletal muscle were prepared by the method of de Meis and Hasselbach (13). The vesicles were opened by incubation in 7 % diethylether at a protein concentration of 10 mg per ml (14) before being added to the assay system.

Na^+K^+-ATPase from rat kidney was prepared and assayed according to Jørgensen (15). Rat liver microsomes were prepared as described by Ernster et al. (16). Treatment of microsomes with Ultra Turrax Blender was carried out according to Ernster and Jones (17).

Muscle adenylate kinase, muscle aldolase and yeast pyrophosphatase were purchased from Sigma; alkaline phosphatase from calf intestine, yeast hexokinase and muscle pyruvate kinase were obtained from Boehringer.

Nucleoside di- and triphosphatase, inorganic pyrophosphatase, AMPase and glucose-6-phosphatase activities were measured by determining the amount of P_i liberated using the isobutanol-benzene extraction method as described by Lindberg and Ernster (18). The conditions employed in the individual experiments are specified in the figure and table legends.

Adenylate kinase activity was assayed by coupling the reaction to the hexokinase and glucose-6-phosphate dehydrogenase systems and measuring $NADP^+$ reduction spectrophotometrically at 340 nm.

Hexokinase activity was assayed by coupling the reaction to the pyruvate kinase and lactate dehydrogenase systems and measuring NADH oxidation spectrophotometrically at 340 nm.

Pyruvate kinase activity was assayed by coupling the reaction to the lactate dehydrogenase system and measuring NADH oxidation spectrophotometrically at 340 nm.

Aldolase activity was measured according to a modification of the hydrazine method (19) in which 3-phosphoglyceraldehyde reacts with hydrazine to form a hydrazone which absorbs at 240 nm.

Phosphomonoesterase activity of alkaline phosphatase was determined by following the release of p-nitrophenol from p-nitrophenylphosphate spectrophotometrically at 405 nm.

RESULTS AND DISCUSSION

Table 1 summarizes some characteristics of the inhibition of mitochondrial ATPase (F_1) by BPh_3Fe^{2+}. As will be shown below, some of these characteristics are shared by a number of enzymes catalyzing the hydrolysis of pyrophosphate bonds.

Fig. 1 illustrates the inhibition of soluble F_1-ATPases from beef heart mitochondria, lettuce chloroplasts and R. rubrum chromatophores by BPh_3Fe^{2+}, and the relief of this inhibition by the uncoupler FCCP. A similar effect was observed with the membrane-bound mitochondrial ATPase (cf. ref. 2).

BPh_3Fe^{2+} inhibits the actomyosin ATPase and the Ca^{2+}-stimulated ATPase of sarcoplasmic reticulum as shown in Fig. 2 and Fig. 3, respectively. In both cases the activity is restored by uncouplers of oxidative phosphorylation (Figs. 2 and 3, Table 2). Uncouplers, when added in the absence of BPh_3Fe^{2+}, do not influence the activity of actomyosin ATPase or sarcoplasmic Ca^{2+}-ATPase. In Table 3 the inhibitory effects of various chelates on actomyosin ATPase and Ca^{2+}-ATPase are compared. In accordance with earlier results (2) obtained with mitochondrial ATPase, the Fe^{2+} trichelate of 4,4-diphenyl-2,2-bipyridine inhibits actomyosin ATPase and Ca^{2+}-ATPase, whereas the Fe^{2+} trichelates of orthophenanthroline and of bathophenanthroline sulfonate do not. The Fe^{2+} and Ni^{2+} trichelates, which are the most potent inhibitors of mitochondrial ATPase (2), also give the strongest inhibition of actomyosin ATPase and Ca^{2+}-ATPase.

As shown in Table 4, BPh_3Fe^{2+} inhibits the ouabain-sensitive Na^+K^+-ATPase isolated from rat kidney. Again, the inhibition is duplicated by the corresponding bipyridine derivative, but not by the Fe^{2+} trichelate of orthophenanthroline or bathophenanthroline sulfonate. It is relieved by uncouplers of oxidative phosphorylation.

TABLE 1. Some characteristics of the inhibition of mitochondrial ATPase (F_1) by BPh_3Fe^{2+}
(From refs. 1-3)

1. Inhibits both soluble and membrane-bound F_1.
2. Octahedral configuration and 4,7-diphenyl substitution essential.
3. Inhibition noncompetitive with respect to ATP.
4. BPh_3Fe^{2+} binds to β-subunit of F_1, probably to same site as aurovertin.
5. Stabilizes soluble F_1 against cold-inactivation.
6. All effects of BPh_3Fe^{2+} on both soluble and membrane-bound F_1 relieved by uncouplers of oxidative phosphorylation.
7. Uncouplers bind to BPh_3Fe^{2+} both in absence and presence of F_1.

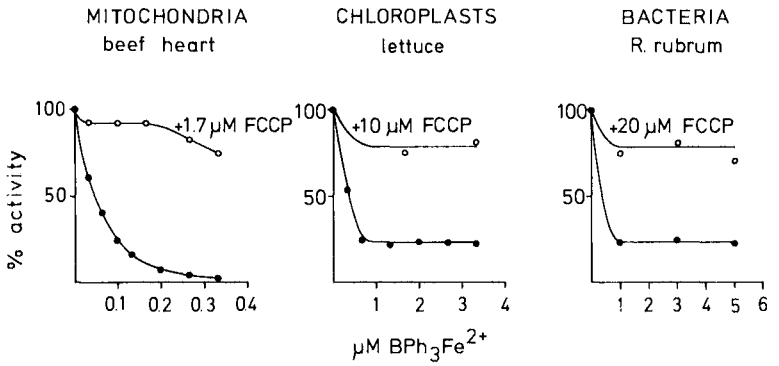

Fig. 1. Inhibition of F_1-ATPases from various sources by BPh_3Fe^{2+} and relief of the inhibition by FCCP. 100 % activities (µmoles/min/mg protein) were: mitochondrial F_1, 100; chloroplast F_1, 8; bacterial F_1, 13.

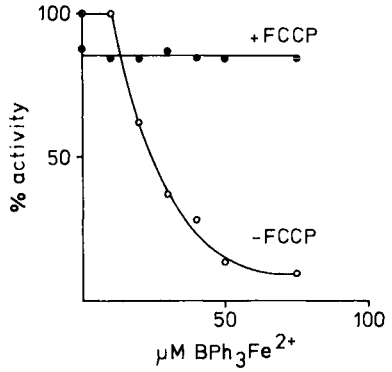

Fig. 2. Inhibition of actomyosin ATPase activity by BPh_3Fe^{2+} and relief of the inhibition by FCCP.

Activity was assayed at 30°C in 1 ml incubation mixture containing 25 mM Tris-Cl (pH 7.8), 40 mM KCl, 2 mM $MgCl_2$, 4 mM ATP, 0.08 mg actomyosin and varying concentrations of BPh_3Fe^{2+} in the absence and presence of 100 µM FCCP. Reaction was quenched after 15 minutes with 0.5 ml 10 % perchloric acid. 100 % activity equals 0.22 µmoles/min/mg protein

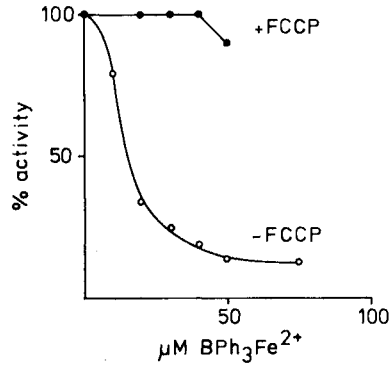

Fig. 3. Inhibition of Ca^{2+}-stimulated ATPase activity of sarcoplasmic reticulum by BPh_3Fe^{2+} and relief of the inhibition by FCCP.

Activity was assayed at 30°C in 1 ml incubation mixture containing 20 mM imidazole (pH 7.0), 40 mM KCl, 0.1 mM $CaCl_2$, 4 mM $MgCl_2$, 4 mM ATP, opened membrane vesicles corresponding to 0.1 mg protein and varying concentrations of BPh_3Fe^{2+} in the absence and presence of 100 µM FCCP. Reaction was quenched after 15 minutes with 0.5 ml 10 % perchloric acid. 100 % activity equals 0.65 µmoles/min/mg protein.

TABLE 2. Effects of various uncouplers on actomyosin ATPase activity and on Ca^{2+}-stimulated ATPase activity of sarcoplasmic reticulum inhibited by BPh_3Fe^{2+}
All samples contained 50 µM BPh_3Fe^{2+}, other conditions were the same as in Figs. 2 and 3.

Additions	Actomyosin ATPase	Sarcoplasmic ATPase
	(% activity)	
none	14	15
100 µM FCCP	84	100
100 µM S-13	61	63
100 µM TTFB	78	82
500 µM 1799	78	85

TABLE 3. Effects of various chelates in the absence and presence of uncouplers on actomyosin ATPase activity and on Ca^{2+}-stimulated ATPase activity of sarcoplasmic reticulum

Assay conditions were the same as in Figs. 2 and 3. When indicated, 100 µM FCCP was added.

Additions	Actomyosin ATPase (% activity)		Sarcoplasmic ATPase (% activity)	
	- FCCP	+ FCCP	- FCCP	+ FCCP
50 µM BPh_3Fe^{2+}	14	84	14	90
50 µM $DPBP_3Fe^{2+}$	19	81	4	100
50 µM OPh_3Fe^{2+}	105	95	100	100
50 µM BPh-sulf.$_3Fe^{2+}$	90	80	100	100
50 µM BPh_3Ni^{2+}	22	85	18	72
50 µM BPh_3Cu^{2+}	33	80	23	84
50 µM BPh_3Zn^{2+}	75	85	63	75
50 µM BPh_3Mn^{2+}	105	80	90	100
50 µM $DPBP_3Ni^{2+}$	47	83	20	75

Table 5 illustrates the effect of BPh_3Fe^{2+} in the absence and presence of the uncoupler TTFB on nucleoside di- and triphosphatase activities of rat liver microsomes. The ATPase, ITPase, UTPase, ADPase, IDPase and UDPase activities are all inhibited by BPh_3Fe^{2+} to about the same extent, and the inhibition is relieved by TTFB. IDPase activity released from the microsomal membrane by treatment with Ultra Turrax Blender, as described by Ernster and Jones (17), is likewise inhibited by BPh_3Fe^{2+} in an uncoupler-reversible manner (not shown).

In the experiment shown in Table 6, membrane-bound PP_iase activity of chromatophores from Rhodospirillum rubrum is totally inhibited by BPh_3Fe^{2+}. FCCP, which in itself activates the native enzyme, relieves the inhibition by BPh_3Fe^{2+}.

Soluble PP_iase from yeast is also inhibited by BPh_3Fe^{2+} (Fig. 4). In this case, however, the inhibition is not reversed by uncouplers (Fig. 4, Table 7). The soluble PP_iase shows a sensitivity to various chelates (Table 8) similar to that of mitochondrial ATPase, actomyosin ATPase, Ca^{2+}-ATPase of sarcoplasmic reticulum, and kidney Na^+K^+-ATPase.

TABLE 4. Effect of various chelates and uncouplers on Na^+K^+-ATPase activity from rat kidney

100 % activity equals 3.2 µmoles/min/mg protein. When indicated, 1 mM ouabain was added.

Additions	% activity	
	- ouabain	+ ouabain
none	100	8
5 µM BPh_3Fe^{2+}	42	12
10 µM BPh_3Fe^{2+}	4	4
10 µM $DPBP_3Fe^{2+}$	8	-
10 µM OPh_3Fe^{2+}	75	-
10 µM BPh-sulf.$_3Fe^{2+}$	92	-
20 µM FCCP	100	8
20 µM S-13	63	8
20 µM TTFB	96	8
100 µM 1799	92	8
5 µM BPh_3Fe^{2+} + 20 µM FCCP	104	-
10 µM BPh_3Fe^{2+} + 20 µM FCCP	96	8
10 µM BPh_3Fe^{2+} + 20 µM S-13	58	-
10 µM BPh_3Fe^{2+} + 20 µM TTFB	96	-
10 µM BPh_3Fe^{2+} + 100 µM 1799	96	-
10 µM $DPBP_3Fe^{2+}$ + 20 µM FCCP	96	-

TABLE 5. Inhibition of nucleoside di- and triphosphatases of liver microsomes by BPh_3Fe^{2+} and relief of the inhibition by TTFB

Activity was assayed at 30°C in 1 ml incubation mixture containing 50 mM Tris-Cl (pH 7.5), 4 mM $MgCl_2$, 5 mM nucleoside di- or triphosphate, 0.6 mg microsomal protein and BPh_3Fe^{2+} and/or TTFB as indicated. Reaction was quenched after 20 min with 0.5 ml 10 % perchloric acid. When indicated, 50 µM BPh_3Fe^{2+} and/or 100 µM TTFB was added.

Substrate	Activity	% inhibition by		
	(µmoles/min/g liver)	BPh_3Fe^{2+}	TTFB	BPh_3Fe^{2+} + TTFB
ATP	2.87	75	0	15
ITP	2.02	62	0	0
UTP	2.10	73	10	18
ADP	0.74	82	3	16
IDP	2.73	72	0	13
UDP	1.10	72	11	28

TABLE 6. Inhibition of PP_iase activity of chromatophores from Rhodospirillum rubrum by BPh_3Fe^{2+} and relief of the inhibition by TTFB

Activity was assayed at 30°C in 2 ml incubation mixture containing 50 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 5 mM PP_i, 0.85 mg protein and BPh_3Fe^{2+} and/or TTFB as indicated. Reaction was quenched with 1 ml 10 % trichloroacetic acid.

Additions	Activity (nmoles/min/mg protein)
none	3.4
50 µM BPh_3Fe^{2+}	0.0
100 µM TTFB	5.7
50 µM BPh_3Fe^{2+} + 100 µM TTFB	3.7

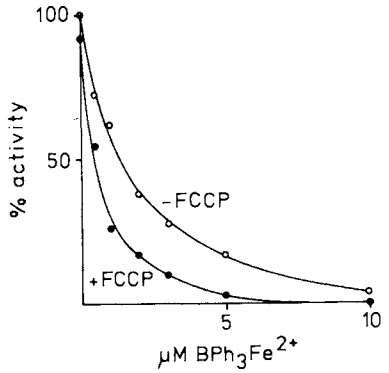

Fig. 4. Inhibition of soluble PP_iase from yeast by BPh_3Fe^{2+} in the absence and presence of FCCP.

Activity was assayed at 30°C in 1 ml incubation mixture containing 50 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 5 mM PP_i, 0.13 µg yeast PP_iase and varying concentrations of BPh_3Fe^{2+} in the absence and presence of 10 µM FCCP. Reaction was quenched after 15 minutes with 0.5 ml 10 % perchloric acid. 100 % activity equals 600 µmoles/min/mg protein.

TABLE 7. Effect of various uncouplers in the presence and absence of BPh_3Fe^{2+} on soluble PP_iase from yeast

Assay conditions were the same as in Fig. 4. When indicated, 5 µM BPh_3Fe^{2+} was added.

Addition	% activity	
	+ BPh_3Fe^{2+}	- BPh_3Fe^{2+}
none	17	100
10 µM FCCP	3	92
10 µM S-B	6	60
10 µM TTFB	14	100
50 µM 1799	4	89

TABLE 8. Effect of various chelates in the absence and presence of FCCP on soluble PP_iase from yeast

Assay conditions were the same as in Fig. 4. When indicated, 10 µM FCCP was added.

Addition	% activity	
	- FCCP	+ FCCP
5 µM BPh_3Fe^{2+}	18	3
5 µM $DPBP_3Fe^{2+}$	11	3
5 µM OPh_3Fe^{2+}	93	92
5 µM BPh-sulf.$_3Fe^{2+}$	91	91
5 µM BPh_3Ni^{2+}	7	2
5 µM BPh_3Cu^{2+}	45	12
5 µM BPh_3Zn^{2+}	14	9
5 µM BPh_3Mn^{2+}	59	11
5 µM $DPBP_3Ni^{2+}$	10	4

Table 9 summarizes the enzymes tested so far that are inhibited by BPh_3Fe^{2+}. Common to all of these enzymes is that they catalyze the hydrolysis of pyrophosphate bonds. Listed in Table 9 are also a number of enzymes that have been tested and found not to be inhibited by BPh_3Fe^{2+} at concentrations which were manifold above those causing inhibition of the first category of enzymes. These BPh_3Fe^{2+}-insensitive enzymes include partly catalysts which split pyrophosphate bonds but in a non-hydrolytic manner - i.e., transferases such as adenylate kinase, pyruvate kinase, or hexokinase - and partly hydrolytic enzymes which split non-pyrophosphate bonds such as phosphate esters - e.g. AMPase, glucose-6-phosphatase, alkaline phosphatase - or other types of bonds, e.g. aldolase. Interestingly, alkaline phosphatase was insensitive to BPh_3Fe^{2+} not only with p-nitrophenyl phosphate but also with inorganic pyrophosphate or ATP as substrate, indicating that only enzymes which hydrolyze pyrophosphate bonds in a specific manner are inhibited by BPh_3Fe^{2+}.

It should be noted, on the other hand, that the actual cleavage of a pyrophosphate bond may not be the step inhibited by BPh_3Fe^{2+}. This is indicated by the fact that the reactions catalyzed by two of the BPh_3Fe^{2+}-sensitive enzymes listed in Table 9, namely, the Na^+K^+-ATPase (20) and the Ca^{2+}-ATPase (21), are known to involve phosphoenzyme intermediates, where the phosphate is bound to a carboxyl group of the protein. It is unlikely - although experimentally not proven - that BPh_3Fe^{2+} inhibits the formation of these intermediates, in view of its lack of effect on various kinases. More probably it is the hydrolysis of the phosphoenzyme which is inhibited by BPh_3Fe^{2+}. It would thus appear that the criterion for a reaction to be sensitive to BPh_3Fe^{2+} is that it involves the hydrolytic cleavage of a phosphate bond of anhydride character, such as an acyl phosphate of or a pyrophosphate, in the active center of the enzyme. Another common feature of these enzymes seems to be that they catalyze a reversible hydrolytic cleavage of a P-O bond as revealed by a rapid oxygen exchange between P_i and water (22-30). This reaction may involve the transport of a proton, resulting from the hydrolytic cleavage of the P-O bond in the hydrophobic center of the enzyme, to the surrounding aqueous medium. This mechanism would be similar to that involved in the hydrolytic cleavage of a C-C bond in the reaction catalyzed by muscle aldolase (31). In that case the proton is transported through a number of special histidine residues located near the active center of the enzyme. In the present case, the proton transport may likewise involve some aromatic residues, which would serve as proton conductors between the hydrophobic pocket of the enzyme and the hydrophilic external environment. BPh_3Fe^{2+} may exert its inhibitory effect by blocking these aromatic residues through its phenyl groups, which are essential for inhibition (2).

The effect of uncouplers in relieving the BPh_3Fe^{2+} inhibition may be due to a mediation of protons between the hydrophobic center of the BPh_3Fe^{2+}-blocked enzyme and its aqueous environment (32). This proton conduction by uncouplers may be direct, similar to that assumed to occur through phospholipid bilayers (33). It may also be indirect, due to a removal of the phenyl groups of BPh_3Fe^{2+} from the aromatic residues of the enzyme. The latter alternative appears preferable in view of the earlier demonstrated nonenzymic interaction between BPh_3Fe^{2+} and various uncouplers (2) which requires the phenyl groups of BPh_3Fe^{2+}

TABLE 9. Selective inhibition by BPh_3Fe^{2+} of enzymes catalyzing the hydrolysis of pyrophosphate bonds

Enzymes inhibited	Enzymes not inhibited
Mitochondrial F_1-ATPase*	Adenylate kinase
Chloroplast F_1-ATPase*	Hexokinase
Bacterial F_1-ATPase*	Pyruvate kinase
Actomyosin ATPase*	AMPase
Ca^{2+}-ATPase*	Glucose-6-phosphatase
Na^+-K^+-ATPase*	Alkaline phosphatase
Microsomal NTPase*	Aldolase
Microsomal NDPase*	
Bacterial PP_iase*	
Yeast PP_iase	

*inhibition relieved by uncouplers

and which results in a spectral shift indicative of a conformational change of the interacting molecules. The somewhat puzzling finding that uncouplers do not relieve the BPh_3Fe^{2+} inhibition in the case yeast pyrophosphatase may be explained in terms of the proposed mechanism by assuming that the interaction between this enzyme and BPh_3Fe^{2+} is too strong to be overcome by uncouplers.

In conclusion, the results described in this paper suggest that various enzymes catalyzing the hydrolysis of pyrophosphate bonds may possess a basic similarity in their reaction mechanisms, in spite of their widely different gross chemical structures and biological functions. This common feature may concern the way in which these enzymes interact with protons during catalysis, and it may make BPh_3Fe^{2+} a particularly useful tool for elucidating the mechanism of pyrophosphate bond biosynthesis in oxidative and photosynthesic phosphorylation.

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HIGHLIGHTS IN THE STUDY OF ENZYMIC AMINO GROUP TRANSFER: METABOLIC ROLE, STRUCTURE AND CATALYTIC MECHANISM OF TRANSAMINASES

A. E. Braunstein

Institute of Molecular Biology, USSR Academy of Sciences, Moscow, USSR

Abstract - A survey is presented of key steps in the investigation of reactions and enzymes of amino group transfer. These include: discovery of biological transamination reactions;early studies of their occurrence, substrate range and presumable enzymic mechanism; studies revealing the principal biological functions of transaminations as essential steps in nitrogen assimilation, in transformations and catabolism of amino-acids, and in the chemical integration of intermediary nitrogen and energy metabolism; further, the detection and investigation of the cofactor role of pyridoxal phosphate in enzymic transamination and other transformations of amino--acids; essentials of the theory of biological vitamin B_6--dependent reactions; molecular-physical and bioorganic characterization of cytosolic aspartate transaminase (see Note a); elucidation of its peptide sequence, identification of some functionally important groups (and their presumable roles); hypothetic dynamic schemes of the molecular mechanism of enzymic transamination, and an outline of the first results obtained in X-ray structural investigations of cytosolic aspartate transaminase from chicken heart.

HISTORIC INTRODUCTION

In studies aiming primarily at identification of the metabolic precursor of amino groups for resynthesis of adenylate (AMP) from inosinate (IMP) in muscle tissue, Braunstein and coworkers discovered in 1937 a novel type of enzymic reactions, consisting in reversible transfer of the amino group, together with an electron pair and a proton,between an α-amino-acid (or other NH_2 donor) and an oxo-acid (or other acceptor molecule)(Refs. 1,2), or transamination. As indicated in the reaction scheme (Eqn.1), it was later demonstrated in experiments with isotopic labelling that the original amino-N is retained in the new amino-acid, whereas the α-hydrogen is exchanged for H atoms from the aqueous medium. In the following two decennia intensive research in the author's laboratories and elsewhere (see Refs. 2-6) demonstrated the ubiquitous occurrence of transaminating enzymes in the cells of all living beings, their broad substrate range with preference for dicarboxylic NH_2-donor/acceptor pairs, and rough outlines of the catalytic mechanism.

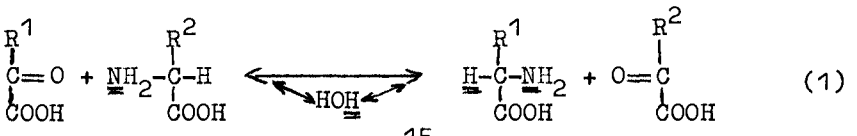
In our first experiments, in collaboration with Maria Kritzman (1) we explored the transformations, in minced muscle, of glutamic acid. It failed to act as a source of NH_2-N for the regeneration of AMP from IMP. The real precursor, identified 20 years later, is aspartic acid (see Fig.5 on p.7). Had we used aspartate rather than glutamate in our early attempts to reaminate IMP, this could possibly have been found from the outset.

Discovery and initial characterization of enzymic transamination. Actually the very first experiments (Refs.1,2) provided evidence that in

Note a. Abbreviations - Asp-transaminase, aspartate:oxoglutarate aminotransferase; PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; Pyr, pyruvate; Kgl, α-oxoglutarate, Lact, lactate.

muscle tissue there occurs under aerobic conditions equivalent disappearance of added glutamate and endogenous lactate, whereas in anaerobiosis a

TRANSAMINATION



(1)

($\underline{N}$, nitrogen labelled with ^{15}N; $\underline{H}$, hydrogen labelled with ^{2}H or ^{3}H)

supply of extraneous pyruvate is required (Table 1). The obvious inference that the product formed should be alanine was confirmed by analytic data and by the isolation, from scaled-up incubation samples, of optically active β-naphtalenesulfo-alanine (1939)(Ref.1).

TABLE 1. Balance of transformations of L-Glu in muscle pulp [(1937), from Ref.1]

Samples	Δ Pyr	Δ Lact	Δ Ala	Δ NH_2-Dicarbox
A. In O_2 atmosphere				
I. a. Muscle pulp (Control)	0	-39	0	0
b. same, + 70 μmol/g Glu	0	-34.5	+28.7	-32.6
II. a. Muscle pulp + + 160 μmol/g Pyr	-93	+48	0	0
b. same as a, + + 70 μmol/g Glu	-187	+120	+30.5	-29.0
B. In vacuo				
III. a. Muscle pulp + + 175 μmol/g Pyr	-71	+40	0	0
b. same as a + + 70 μmol/g Glu	-133	+70.5	+33.1	-32.6

In experiments with the primary end-products of NH_2 shift — L-alanine and α-ketoglutarate — added to muscle pulp, the back-reaction was observed to proceed rapidly to a certain equilibrium, close in rate and endpoint (approximately half-way) to that found in the forward reaction (Fig.1). These data, and the preparative isolation of L-glutamate (as the hydrochloride) produced in the back-reaction, completed the demonstration of occurrence in muscle (extended later to enzyme extracts from any type of cells) of reversible reactions of NH_2 transfer between donor amino-acids and acceptor oxo-acids. Close analogy[2] with well-known examples of non-enzymic (chemical) NH_2 transfer from amino-acids (or amines) to active carbonyl compounds (e.g. quinones, glyoxals, ninhydrin, oxo-acids) and the fate of isotopically tagged NH_2-N and α-H atoms (indicated in Eqn.1) suggested that the mechanism of enzymic transamination was based on formation and tautomeric rearrangement of Schiff-base intermediates. My conjecture that intermediary N and/or H acceptors might be involved in the enzymic reaction (Ref.2) was confirmed a few years later (see below).

From the outset it was apparent that many amino- and oxo-acids could participate in enzymic NH_2 transfer, and that the Glu/Kgl substrate couple was preferentially involved. With the very imperfect analytical methods and enzyme fractionation techniques then available, the substrate range and biological scope of the reaction could not be correctly assigned; but its rapid rates and ubiquity indicated the important physiological significance of transamination. Unfortunately, our main assay procedure, based on the separation of mono- and dicarboxylic amino-acids, precluded observation of the fastest and most cardinal transamination reaction — the NH_2 transfer between glutamate and aspartate.

Only in the late fourties the advent of partition chromatography and other refined fractionation methods allowed to demonstrate the occurrence in all living cells of a variety of transaminases which catalyse amino transfer between practically all amino- and oxo-acids, including transamination

reactions between monocarboxylic substrate pairs without dicarboxylic participants (Refs. 3,4,6,8).

PRINCIPAL METABOLIC FUNCTIONS OF NH_2 GROUP TRANSFER

Most active among NH_2 group transfer reactions in animals and higher plants are those involving three keto-acids arising in the glycolytic and respiratory cycles — viz., pyruvate, oxaloacetate and α-oxoglutarate or the corresponding α-amino-acids — as one or both substrate pairs.

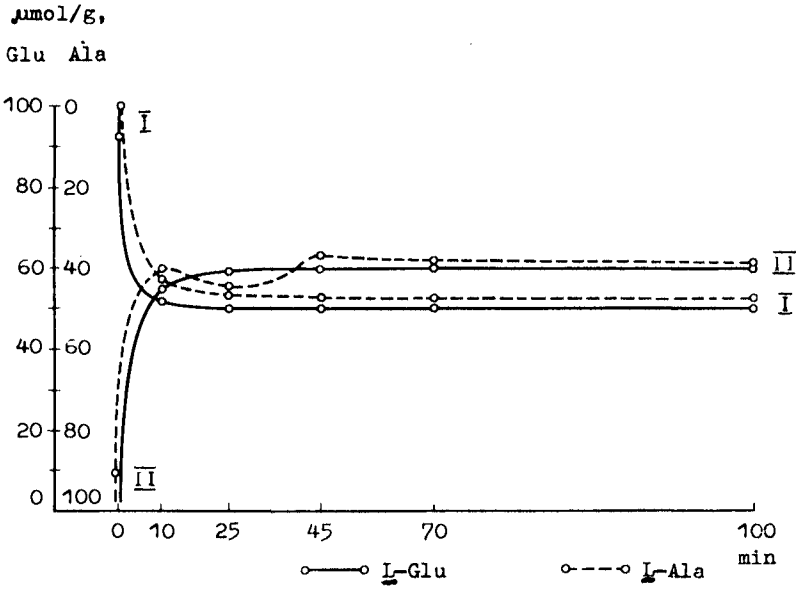


Fig.1. Reversibility and equilibrium of the transamination reaction (Braunstein and Kritzman,1937; see Ref.1).

Integration of intermediary nitrogen and energy metabolism. My suggestion (2,3) that one of the main physiological functions of transamination consists in the chemical integration of nitrogen metabolism and cellular oxidoreductions — especially in the regulation of glycolytic and respiratory cycles by the trapping or supply of intermediary oxo-acids — has found ample experimental support (see surveys cited in Refs.3-6).

Transamination-dependent pathways of N assimilation and dissimilation. In those years it was generally believed, mainly on the basis of indirect evidence, that amino acids were catabolized - at least in higher animals - chiefly by oxidative deamination, and the reverse process - synthesis of alanine from pyruvate and ammonia - had been demonstrated in vitro in liver and kidney slices (cf.Refs.3,5-8). Yet only one protein constituent - L-glutamic acid - is readily subject in all types of cells to oxidative deamination (and to synthesis by its reversal) by a specific glutamate dehydrogenase, requiring NAD(P).Ubiquitous occurrence,partial purification and properties of this enzyme were reported by H.v.Euler and associates in 1937-38, nearly coincident with our first publications on the role of glutamate in NH_2 transfer by transaminases. The inference was obvious that coupled action of glutamate dehydrogenase and transaminases could constitute a key stage in major indirect pathways of ammonia assimilation and amino acid degradation in animals (Fig.2). We designated these coupled processes as transreamination and transdeamination, respectively, and in a series of studies amply demonstrated their importance in the synthesis and degradation of most amino acids in mammals and in cell-free preparations from their tissues in vitro.

From semipurified alanine-glutamate transaminase and glutamate dehydrogenase we composed a model of "L-amino-acid oxidase" which actively deaminated

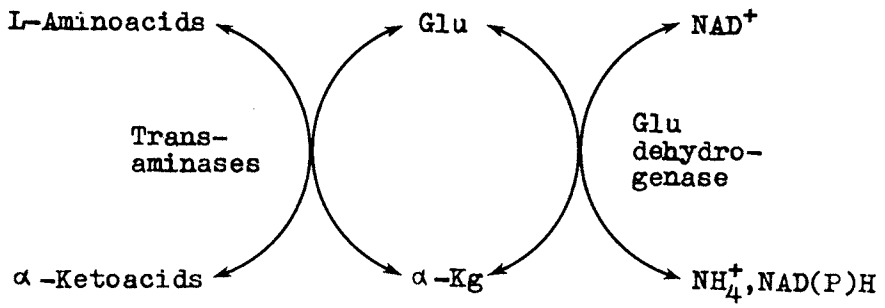


Fig.2. Transdeamination and transreamination via glutamate dehydrogenase; Braunstein (1947), see Ref.3.

L-alanine in presence of the required cofactors (Table 2), see Ref.7. The system should obviously function reversibly, though this was not actually tested. Our views implied that any agent interfering either with transamination or with generation of α-ketoglutarate in the citric acid cycle (like the inhibitory factors indicated in Fig.3) should interfere with oxidative deamination and with synthesis (from α-keto- or D-amino-acids) of L-amino-acids other than glutamic in liver or kidney; this was found to be the case (Ref.3). Figure 4 shows the impressive results obtained by me and R.Azarkh (see Refs.3,8) in experiments with rat liver homogenates where transamination was blocked *in vivo* by administration of isoniazid, and α-ketoglutarate formation was inhibited *in vitro* with fluorocitrate; the depressed amination of pyruvate (synthesis of alanine and aspartate) was restituted by addition to the incubation samples (a) of ketoglutarate, (b) of semi-purified alanine transaminase, and most efficiently - (c) of both. Evidence was obtained that the requirements for urea synthesis in liver tissue are similar, necessitating intermediary synthesis of glutamate and aspartate by transamination (in the case of uricotelic catabolism - also of glycine)(Ref.8). Glutamate dehydrogenase is not unique as the gateway for assimilation and release of NH_3; for example, in some microorganisms deficient in glutamate dehydrogenase activity (and in certain plants) this function may be taken over by other enzymes, such as alanine dehydrogenase, aspartase or (in the anabolic direction) by glutamine synthethase in conjunction with glutamate synthase (EC 1.4.1.13; 1.4.7.1).
A subsidiary transamination-mediated reaction sequence for oxidative NH_3 liberation (see Fig.5) is the process involving de- and reamination of AMP (3,8), designated by W.Lowenstein as "purine nucleotide cycle".

TABLE 2. Transdeamination of L-alanine in enzymic model system (Braunstein and Bychkov,1939; Ref.7)

Incubation samples	ΔNH_3, μmols
Complete model system	23.9
Without Glu-dehydrogenase	1.8
" " aminotransferase	5.6
" " codehydrogenase	0.1
" " ketoglutarate	0
" " pyocyanine	0.2
" " L-alanine	0.4

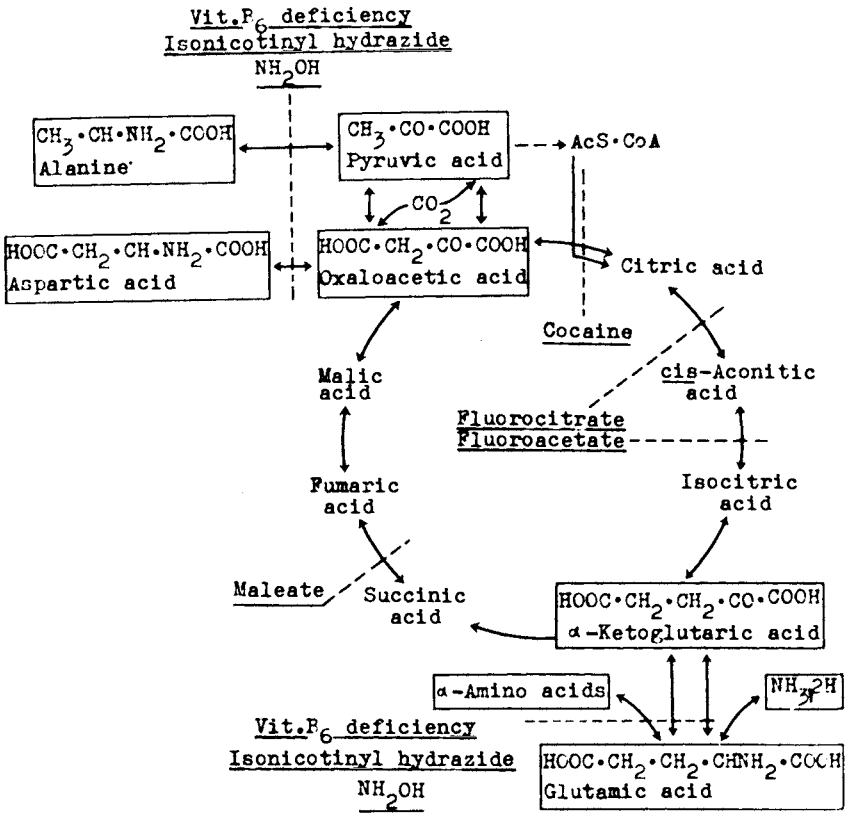


Fig. 3. Scheme of agents interfering with the respiratory cycle, and with nitrogen assimilation and dissimilation (Braunstein, 1957; Ref.3).

THE COENZYME FUNCTION OF PYRIDOXAL PHOSPHATE

In 1944-46, studies from several laboratories demonstrated that a vitamin B_6 derivative, recognized later as pyridoxal-5'-phosphate (PLP), was an essential cofactor of amino-acid decarboxylases and of the aminotransferases (or transaminases). In the latter case, studies by E.Snell and associates indicated that the cofactor performed a function I had originally suggested, viz. that of an intermediary NH_2 and hydrogen acceptor. Within the next few years many cofactor functions of PLP in a broad variety of metabolic reactions of amino-acids were discovered in different laboratories, including mine (Refs.9-13). Common features of the essence of pyridoxal--requiring enzymic reactions were briefly outlined, on the basis of analogies with well-known chemical reactions, in my paper (1949) on functions of vitamin B_6 in tryptophan metabolism (Ref.9). Closely similar interpretations of the general mechanisms of PLP-involving reactions of amino-acids were published in 1952-54 independently by me and M.Shemyakin (10) and D.Metzler, M.Ikawa and E.Snell (11); cf.Refs. (12-14). The common cause of such reactions was found in marked lowering of electron density of the aminated carbon atom in aldimines or Schiff bases, produced by condensation of the NH_2 group of amino-acids with the CO group of pyridoxal or similar active oxo compounds. The result is polarization and eventual disruption of single bonds between the α-C atom and any one of its substituents, followed by various secondary rearrangements (Fig.6). With certain amendments and supplements this general theory is still valid (Refs. 4a,14-16).
An aspect of special importance for the reaction specificity of PLP-enzymes, pointed out in 1966 by H.Dunathan (15), is the quantum-chemical postulation that in the E·S aldimines formed by PLP-enzymes the substituent group primarily released should be fixed in a plane perpendicular to that of the

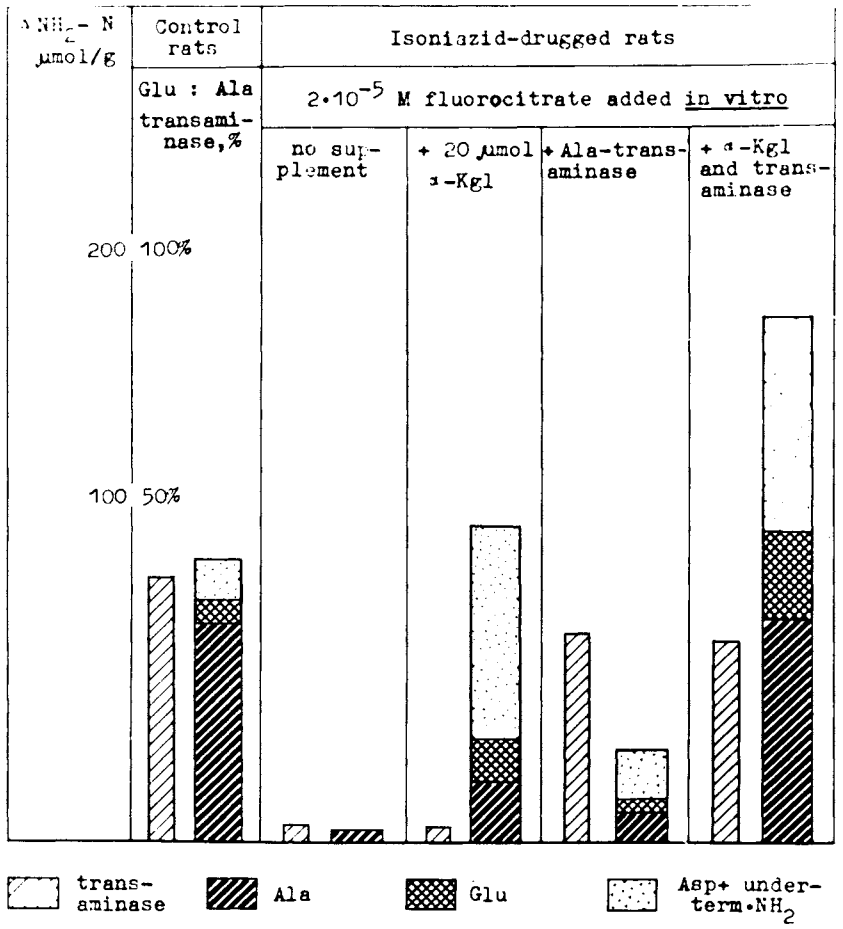


Fig. 4. Amination of pyruvate in liver homogenate of rats drugged with isoniazid (and fluorocitrate added in vitro). From Ref.8 (1960).

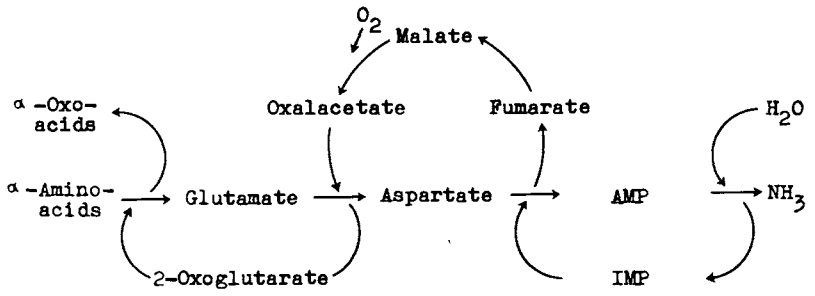


Fig.5. Indirect oxidative deamination of L-amino acids via glutamate, aspartate and AMP; Braunstein (1959), see Refs, (3,8).

planar conjugated $\sigma\pi$-system of the protein-bound coenzyme (Fig.7). In the case of transaminases and some other types of PLP-enzymes, the primary leaving group is the α-H atom, as in the upper scheme on this figure.

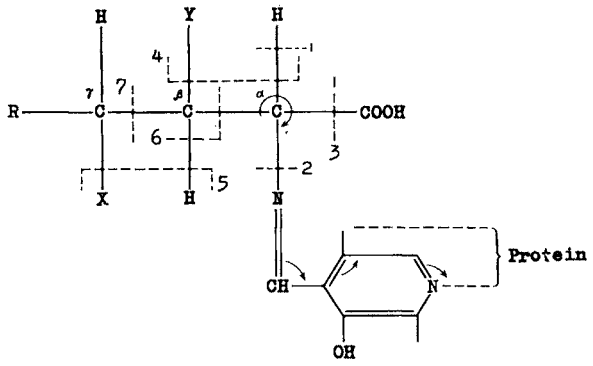


Fig.6. Scheme and list of main types of PLP-dependent transformations of amino acids; Braunstein (1947,1957), see Ref.12.

Type	Bonds disrupted	Reactions
1	αC—H	Racemization
1 + 2	αC—H, αC—N	Transamination
3	αC—COOH	α-Decarboxylation
4	αC—H, βC—Y	Elimination or replacement of αH and β-substituent
5	βC—H, γC—X	Elimination or replacement of βH and γ-substituent
6	αC—βC (and αH)	α,β-Cleavage (and condensation) of carbon chains
7	βC—γC	β,γ-Cleavage of carbon chain

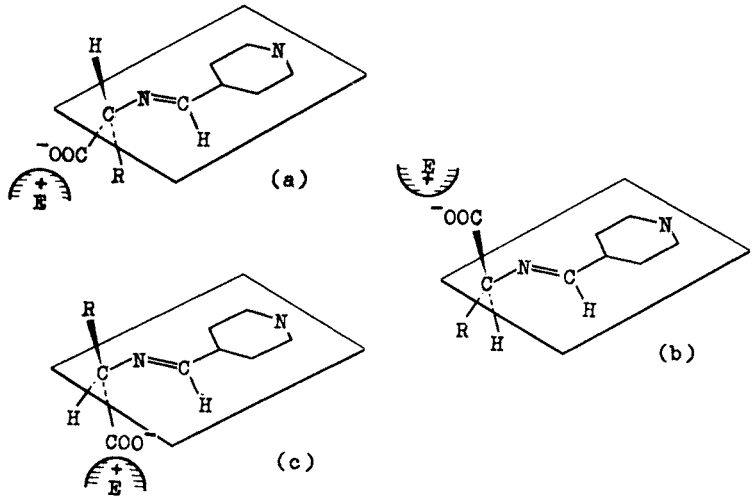


Fig. 7. Conformations of PLP-aminoacid aldimines in the active site, favouring disruption of: (a) C^{α}—H bond (transamination et al.);(b) C^{α}—C^{β} bond (threonine aldolase et al.); C^{α}—COOH bond (amino-acid α-decarboxylases). Scheme drawn by E.S.Severin after H.Dunathan (1966), cf.Refs.15,16).

PREPARATION AND CHARACTERIZATION OF PURE ASP-TRANSAMINASES

In 1958-60, high-purity preparations of aspartate transaminase from the myocard of several vertebrates became readily available; its isoenzymes from the cytosol and mitochondria were later separated and crystallized. In the following few years a vast body of evidence was accumulated, in our laboratory and elsewhere, about the physical parameters, chemical reactivity and catalytic properties of the enzyme; the topography of some functionally important groups; the sequence and stereochemistry of intermediary steps of the catalytic cycle. This information was obtained by means of a broad complex of elaborate (chiefly - indirect) chemical and physical approaches, including spectral, electro-, magneto- and hydrodynamic methods, chemical and isotopic labelling, kinetic and inhibitor analysis, etc. (Refs.4,4a). The sum of information provided the basis for alternative dynamic models of the molecular reaction mechanism of enzymic transamination, proposed in our Institute by colleagues of the author (Refs.17,18). Both hypothetical models implied the occurrence (and importance) of conformational changes (qualitatively different) in the enzyme molecule in the course of the catalytic cycle; these will be discussed later.

10 20
Ala-Pro-Pro-Ser-Val-Phe-Ala-Glu-Val-Pro-Gln-Ala-Gln-Pro-Val-Leu-Val-Phe-Lys-Leu-Ile-Ala-Asp-Phe-Arg-

30 40 50
Glu-Asp-Pro-Asp-Pro-Arg-Lys-Val-Asn-Leu-Gly-Val-Gly-Ala-Tyr-Arg-Thr-Asp-Asp-Cys-Glu-Pro-Trp-Val-Leu-

60 70
Pro-Val-Val-Arg-Lys-Val-Glu-Gln-Arg-Ile-Ala-Asn-Asp-Ser-Ser-Leu-Asn-His-Glu-Tyr-Leu-Pro-Ile-Leu-Gly-

80 90 100
Leu-Ala-Glu-Phe-Arg-Thr-Cys-Ala-Ser-Arg-Leu-Ala-Leu-Gly-Asp-Asp-Ser-Pro-Ala-Leu-Gln-Glu-Lys-Arg-Val-

110 120
Gly-Gly-Val-Gln-Ser-Leu-Gly-Gly-Thr-Gly-Ala-Leu-Arg-Ile-Gly-Ala-Glu-Phe-Leu-Ala-Arg-Trp-Tyr-Asn-Gly-

130 140 150
Thr-Asn-Asn-Lys-Asp-Thr-Pro-Val-Tyr-Val-Ser-Ser-Pro-Thr-Trp-Glu-Asn-His-Asp-Gly-Val-Phe-Thr-Thr-Ala-

160 170
Gly-Phe-Lys-Asp-Ile-Arg-Ser-Tyr-Arg-Tyr-Trp-Asp-Thr-Glu-Lys-Arg-Gly-Leu-Asp-Leu-Gln-Gly-Phe-Leu-Ser-

180 190 200
Asp-Leu-Glu-Asn-Ala-Pro-Glu-Phe-Ser-Ile-Phe-Val-Ile-His-Ala-Cys-Ala-His-Asn-Pro-Thr-Gly-Thr-Asp-Pro-

210 220
Thr-Pro-Glu-Glu-Trp-Lys-Gln-Ile-Ala-Ser-Val-Met-Lys-Arg-Arg-Phe-Leu-Phe-Pro-Phe-Phe-Asp-Ser-Ala-Tyr-

230 240 250
Gln-Gly-Phe-Ala-Ser-Gly-Asn-Leu-Glu-Lys-Asp-Ala-Trp-Ala-Ile-Arg-Tyr-Phe-Val-Ser-Glu-Gly-Phe-Glu-Leu-

260 270
Phe-Cys-Ala-Gln-Ser-Phe-Ser-Lys*-Asn-Phe-Gly-Leu-Tyr-Asn-Glu-Arg-Val-Gly-Asn-Leu-Thr-Val-Val-Ala-Lys-

280 290 300
Glu-Pro-Asp-Ser-Ile-Leu-Arg-Val-Leu-Ser-Gln-Met-Glu-Lys-Ile-Val-Arg-Val-Thr-Trp-Ser-Asn-Pro-Pro-Ala-

310 320
Gln-Gly-Ala-Arg-Ile-Val-Ala-Arg-Thr-Leu-Ser-Asp-Pro-Glu-Leu-Phe-His-Glu-Trp-Thr-Gly-Asn-Val-Lys-Thr-

330 340 350
Met-Ala-Asp-Arg-Ile-Leu-Ser-Met-Arg-Ser-Glu-Leu-Arg-Ala-Arg-Leu-Glu-Ala-Leu-Lys-Thr-Pro-Gly-Thr-Trp-

360 370
Asn-His-Ile-Thr-Asp-Glu-Ile-Gly-Met-Phe-Ser-Phe-Thr-Gly-Leu-Asn-Pro-Lys-Gln-Val-Glu-Tyr-Leu-Ile-Asn-

380 390 400
Glu-Lys-His-Ile-Tyr-Leu-Leu-Pro-Ser-Gly-Arg-Ile-Asn-Met-Cys-Gly-Leu-Thr-Thr-Lys-Asn-Leu-Asp-Tyr-Val-

410
Ala-Thr-Ser-Ile-His-Glu-Ala-Val-Thr-Lys-Ile-Gln

Fig. 8. Primary structure of Asp-transaminase$_{cyt}$ from pig heart (Ref.19).
*Lys, the lysine-258 residue forming the "internal" PLP-aldimine in the active centre of the holoenzyme.

The transaminating enzyme that ranks foremost in catalytic potency and biological significance is aspartate:oxoglutarate aminotransferase. Its cytosolic and mitochondrial isoenzymes from heart muscle are dimeric proteins of M.wt. about 93.000, containing two g/mols of PLP (the dissociated monomeric form is fully active). Each protomer consists of one long polypeptide chain; in the enzyme from pig heart cytosol it contains 412 residues. Its sequencing was completed in 1972 under Yu.A.Ovchinnikov's direction by a joint team of his coworkers and mine (Ref.19), see Fig.8.

Some residues, supposed to be functionally important, have been located in this sequence, including the PLP-binding Lys-258 in the active site, one of the five cysteines (Cys-390), one tyrosine (Tyr-40) (Refs.4a,20). The primary structure of the mitochondrial isozyme, reported in 1977 by Japanese authors (21; cf.4a), is strikingly similar: although the two isozymes are practically devoid of immunochemical cross-reactivity, their primary structures include more than 40% residues identically located in the peptide chain, and about 70% of the residues are closely related in chemical structure or genetic coding. This circumstance points to very ancient and evolutionarily conservative origin of the isozymic forms.

The molecular mechanism of enzymic transamination. It is well-established that transamination reactions proceed by a sequence of intermediary steps, constituting two half-reactions, as shown in the following equations (2a,2b) for the case of aspartate transaminase:

$$\text{L-Aspartate} + \text{PLP-Enzyme} \longleftrightarrow \text{Oxaloacetate} + \text{PMP-Enzyme} \quad (2\,a)$$
$$\text{PMP-Enzyme} + \text{2-Oxoglutarate} \longleftrightarrow \text{PLP-Enzyme} + \text{L-Glutamate} \quad (2\,b)$$

In the first step (a) the NH_2 group of amino-substrate is transferred to the enzyme-bound PLP to yield the oxo-acid product and the amino- or PMP-form of the enzyme; in step (b) the PLP-form is regenerated by transfer of the NH_2 group to another oxo-acid molecule, generating the new amino-acid (here, L-glutamate).

Using fast-reaction kinetics, G.Hammes and J.Haslam (22) demonstrated that each half-reaction includes no less than seven different intermediates. The scheme on figure 9 shows the sequence of major intermediates (cf.Refs. 4,4a,17). The first drawing (top left) represents the active non-protonated PLP-enzyme species, or "internal" aldimine, with the CO group of PLP linked to ε-NH_2 of the essential lysine residue in the active site by an imine double bond. This aldimine has a spectral absorption band at 362 nm. Interaction with the amino-substrate produces a non-covalent adsorption complex (Michaelis complex) with the same absorption band at 362 nm. Next, one proton of the substrate's NH_2^+ group is transferred either to the phenolic oxygen or the aldimino-N atom. The deprotonated, nucleophilic amino group now attacks the enzyme's C—4' atom and - via a tetrahedral transition complex (not shown) - displaces the ε-NH_2 group of Lys-258 from its PLP-aldimine. This "transaldimination" step results in formation of the PLP-substrate, or "external", aldimine displaying a characteristic absorption peak with λ_{max} 430 nm. Dissociation of its α-hydrogen atom leads to a carbanionic intermediate, and delocalization of the negative charge in the latter results in a set of resonance states, including a well-defined quinonoid species (featured on our scheme) with absorption peak at 492 nm. Its (chiral) protonation at the C—4' atom gives the PMP-ketimine intermediate whose absorption peak has λ_{max} ~340 nm. Hydrolysis of the latter imine finally releases the oxo-acid product and the PMP-form of the enzyme (λ_{max} 333 nm). Reversal of these steps with another oxo-acid molecule constitutes the second half-reaction, completing the transamination cycle. Within the scope of this article it is not possible to give a survey, ever so concise, of all the vast chemical and physical information relating to the catalytic function of aspartate transaminase, accumulated in our laboratory and elsewhere; see Refs.(4,4a,17). This paper deals in detail with one aspect only of the molecular mechanism of enzymic transamination, namely - the conformational changes in the enzyme molecule connected with the intermediary reaction steps just mentioned (Note a).

Conformational shifts in the acting transaminase molecule.

Evidence indicating that enzymic transamination is probably associated with conformational changes in the enzyme's active site was first obtained by Yu.Torchinsky et al. (21,22) in studies of the induced optical activity (i.o.a.) they had detected in absorption bands of the aspartate transaminase-linked coenzyme chromophore. This i.o.a., recorded either as anomalous

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Note a. Part of the available information was recently discussed by Dr.Yu.M. Torchinsky and me in a lecture given at the 12th FEBS Meeting in Dresden (July 1978).

rotatory dispersion (ORD) or as circular dichrois (CD), was found to display marked changes at sequential stages of the reaction: it is substantially reduced on formation of the Michaelis complex, disappears at the

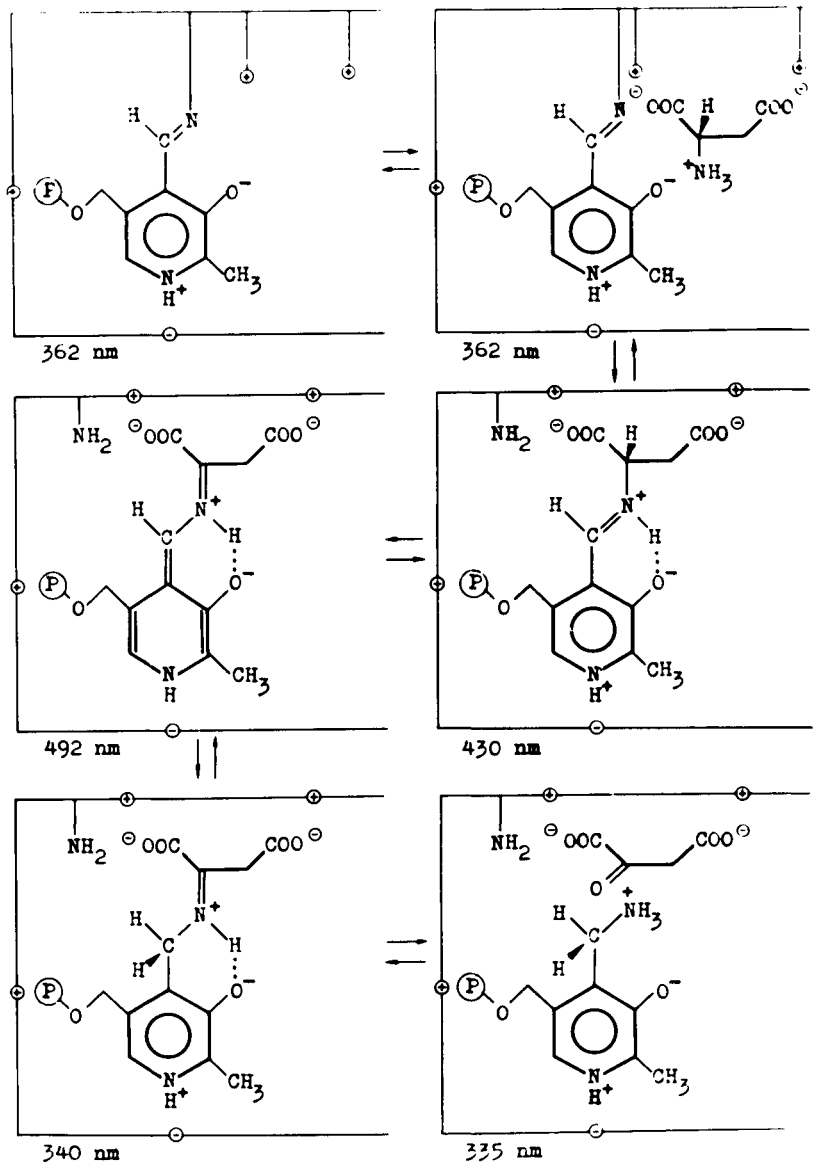


Fig. 9. Key intermediates of the enzymic transamination reaction. Yu.Torchinsky and A.Braunstein, 1978; condensed from Ref.17.

PLP-substrate aldimine stage, and acquires inverted sign (i.e. becomes negative) in the quinonoid intermediate. These phenomena, illustrated in Figs.10-11, were first observed in experiments with substrate analogues (allosubstrates); such analogues, e.g. α-methyl-aspartate (MeAsp) or erythro-β-hydroxyaspartate (HO-Asp), allow to stop artificially, or "freeze", intermediate steps of the enzymic reaction, corresponding to a PLP-substrate aldimine and the quinonoid intermediate, respectively. It appears justified to consider complexes of the enzyme with these allosubstrates as adequate models of intermediates formed in the course of the normal enzymic reaction. This is indicated, for instance, by the fact that quite similar (though less clearly pronounced) changes in absorption and CD spectra are

observed upon addition of the adequate substrate, L-aspartate (Ref.17). Figure 10 a shows the effect of maleate (an inhibitory substrate analogue) on the CD spectrum of the PMP-form of the enzyme. The complex with maleate is a model of the non-covalent Michaelis complex: although the absorption spectrum is unchanged, CD of the chromophore's peak at 333 nm is markedly lowered. Figure 10 b illustrates the changes in CD and absorption curves

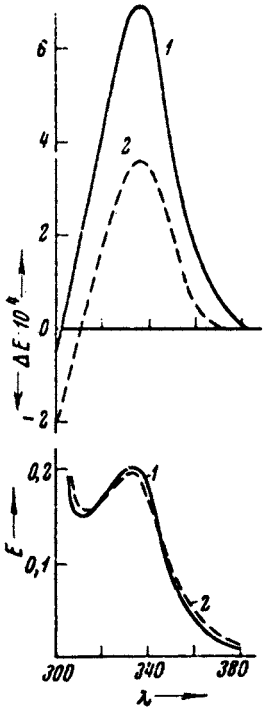


Fig. 10 a

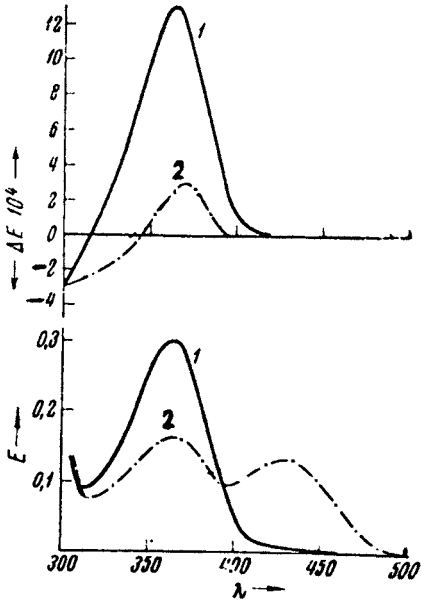


Fig. 10 b

Fig. 10 a. CD (above) and absorption spectra (below) of the PMP-form of cytosolic pig heart Asp-transaminase, before (curve 1), and after (curve 2) addition of 0.05 M maleate; pH 8.1; protein concentration 1.15 mg/ml (Yu.Torchinsky and A.Braunstein, 1977).

Fig. 10 b. CD (above) and absorption spectra (below) of the form of the enzyme before (curve 1) and after (curve 2) addition of 0.2 M DL-MeAsp; pH 8.3; protein concentration 1.74 mg/ml (Yu.Torchinsky and A.Braunstein, 1977).

of the PLP-form in the presence of MeAsp. This allosubstrate produces at least two intermediates: - a Michaelis complex with unchanged position of the absorption peak (λ_{max} 362 nm) but greatly reduced CD maximum at this wavelength, and an allosubstrate aldimine with an absorption maximum at 430 nm, devoid of induced optical activity. Reaction with MeAsp does not go beyond the aldimine stage, because of the absence of an α-H atom (replaced in this analogue by a methyl group).
Figure 11 represents the CD curve of the enzyme complex with another allosubstrate, HO-Asp. This complex imitates the next step of the catalytic reaction, viz., formation of the quinonoid intermediate with a characteristic absorption band near 495 nm, which exhibits negative CD. The marked alterations in i.o.a. described above have been interpreted as manifestations of changes in the mutual positions of the coenzyme chromophore and its dissymmetric protein surroundings at sequential steps of enzyme-catalysed transamination (Ivanov and Karpeisky; Ref.17). These findings and general stereochemical considerations provided the basis for an hypothetic dynamic model of enzymic transamination (Ref.17). According to the model, transition from the noncovalent Michaelis complex to the substrate aldimine, i.e.

the transaldimination step, is associated with rotation of the coenzyme ring around an axis presumably passing through the 2-methyl and 5'-phosphate groups of the coenzyme (as represented schematically in Fig.12).

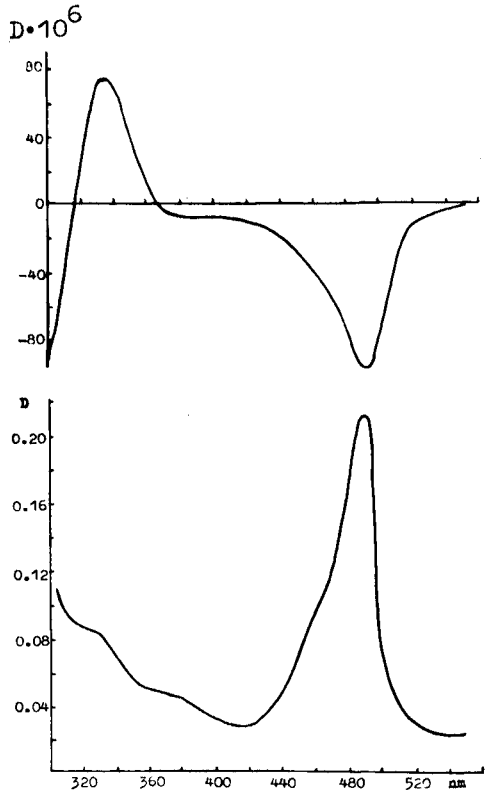


Fig. 11. CD curve of the complex of cytosolic pig-heart Asp--transaminase with 0.01 M erythro-β-hydroxy DL-aspartate; pH 8.6; 0.5 mg protein per ml (Yu.Torchinsky and A.Braunstein, 1978).

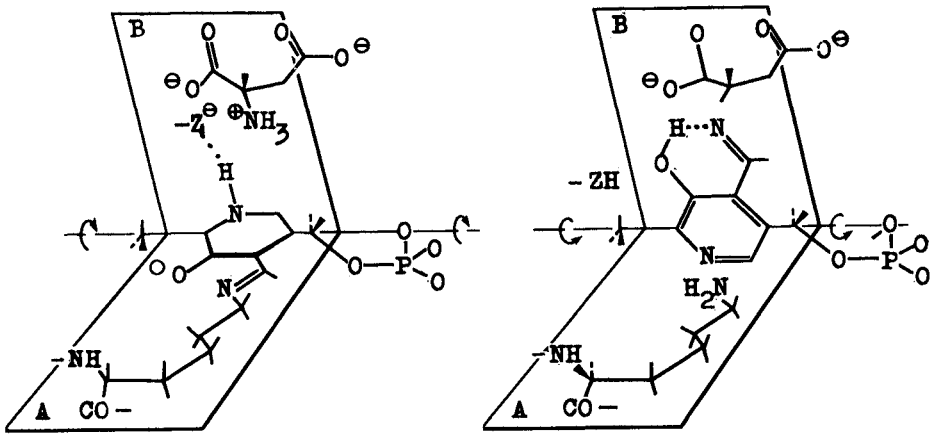
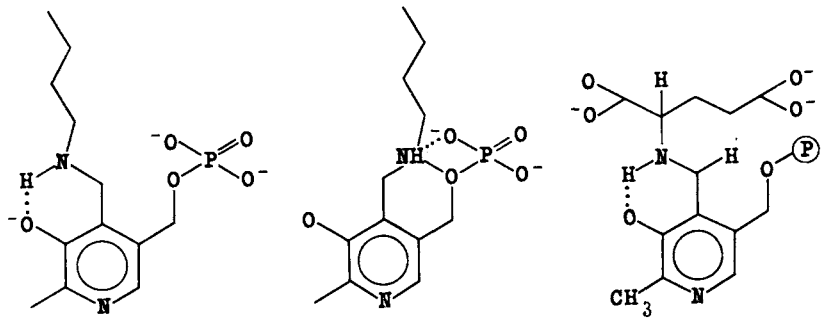


Fig.12. Reorientation of the plane of the coenzyme's pyridine ring in the course of the transaldimination step, according to the model of Ivanov and Karpeisky(Ref.17), as represented schem tically by D.Arigoni (1972); cf.Ref.4a.

Reversal, in the first half-reaction, to original orientation of the plane of the pyridine ring should occur at the stage of transition from PMP-ketimine to free PMP-enzyme and oxo-acid product (see Fig.9, bottom).
Important experimental findings demonstrating the occurrence of spatial translocations of the coenzyme in the functional catalytic site, were made by Besmer and Arigoni (1971); cf.Refs.(4a,16). They studied the stereochemistry of ^3H addition to carbon atom C-4' of the coenzyme, on reduction with sodium borotritide of PLP-aldimines in the active site of Asp-transaminase. According to their data, tritium addition occurs from the re-side in the case of reduction of internal aldimine (free enzyme), whereas the substrate aldimine accepts tritium from the si-side. It thus appears that the faces of the imine double bond accessible for reduction are opposite in the internal and in the external (PLP-substrate) aldimine. This observation could be explained on the basis of Ivanov and Karpeisky's hypothesis of reversible rotatory dislocation of the cofactor, as shown in the diagrammatic representation of such reorientation on figure 12. As already mentioned, there is also a possible alternative explanation of the observed steric inversion of the coenzyme-substrate aldimine bond. As mentioned earlier, this has been done in our Institute by E.S.Severin, R.M.Khomutov and their associates (Ref.18). From extensive studies of the interactions of Asp-transaminase with sterically rigid substrate analogues (stereoisomeric aminoisoxazolidones) and other active-site-directed inhibitors, they have drawn the conclusion that in the internal PLP-lysine aldimine the imino-N of the pro-R-oriented azomethine group faces the 5'-phosphate group rather than the phenolic 3-O-atom as generally assumed (Ref.4,4a,17). Hence they inferred that steric inversion of the azomethine link to pro-S orientation may occur by way of isolated formal rotation of the imino group through an angle of approx. 120° around the $C^4—C^{4'}$ σ-bond (via the tetrahedral transitional complex); this interpretation is shown schematically in Fig.13.



A B C

Fig. 13. Conformation of "internal" [A - according to the usual point of view (Refs.4,4a,17), B - after E.S.Severin et al. (Ref.18)] and C - "external" PLP-substrate aldimines.

Evidence strongly supporting the view that the transamination reaction is associated with substantial conformational alterations in the enzyme protein is provided by observations, first reported by P.Christen et al. (Ref.23), which revealed that chemical reactivities of some functional groups in the transaminase molecule are strikingly and specifically enhanced upon addition to the enzyme solution of the substrate couple, glutamate + oxoglutarate; this phenomenon was designated as "syncatalytic reactivity" (23). In Asp-transaminase from pig-heart cytosol the semiburied systeine-390 residue displays such reactivity, in the chicken-heart enzyme the phenomenon is displayed by a tyrosine residue not yet identified (Ref.24). Elucidation of the underlying conformational changes must await precise description of the three-dimensional structure of the transaminase molecule and its complexes with specific ligands.
New evidence indicating changes in orientation of the coenzyme in the active site in the course of the catalytic reaction was recently obtained in studies of the absorption spectra of plane-polarized light transmitted

through a single crystal of transaminase. Such studies were initiated by Metzler et al. (25) who used crystals of Asp-transaminase from pig-heart cytosol, and similar results were obtained by Torchinsky and Yu.Morosov (1978) with a crystallographically dissimilar form (see Ref.27) of the chicken heart enzyme. In solution the MeAsp-enzyme complexes exhibit two absorption bands - at 362 and 430 nm, and the same bands are seen in spectra of the crystalline complex (see Fig.10 b). In the crystals, the two bands proved to be polarized in opposite directions, i.e. rotation of the polarization plane of the incident light beam induced opposite alterations in the intensities of the absorption peaks: one peak is increased while the other one is drastically lowered. These observations are readily explained if we assume a difference in orientation of the coenzyme ring in the internal aldimine (362 nm band) and in the PLP-substrate aldimine (430 nm band). At first sight, these data are concordant with the dynamic model of Ivanov and Karpeisky (Ref.17). But in reality the situation is not so straightforward. Quantum-chemical calculations carried out by P.S.Song (1978, personal communication from D.E.Metzler) and by F.Savin (cf.26) revealed that for most derivatives of vitamin B_6 the transition dipole moment (TDM) is very close to the C-2—C-5 axis across the coenzyme ring (Fig.14). Arrow 1 in this figure shows, approximately, the direction of transition dipole moment for the structure as drawn, and arrow 2 - for the

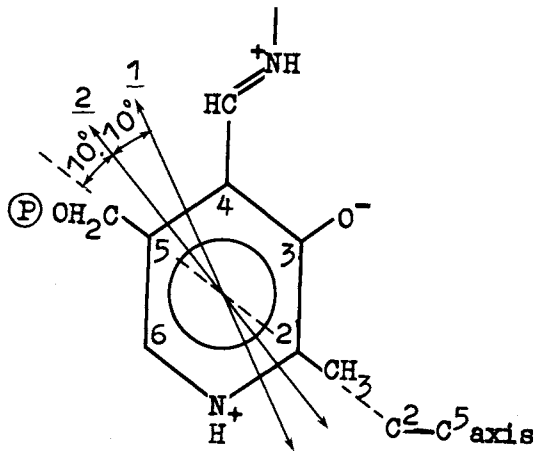


Fig. 14. Direction of the transition dipole moment, TDM, in PLP-aldimines. Computed by Savin (1978) by the all--valence-electrons CNDO/S method.
Arrow 1 shows approximate direction of TDM for aldimine with imine double bond facing the phenolic O-atom, and arrow 2 — with the double bond facing 5'-phosphate.

corresponding structure with imine double bond facing the 5'-phosphate group (cf.Ref.18).
Two conclusions can be drawn from these data. First, it is improbable that reorientation of the imine double bond alone (Ref.18), involving an approx. 10° change in direction of TDM, might account for the fact that the absorption bands of internal and external (substrate) aldimines of PLP in the active site are polarized in opposite directions. Second, rotation of the coenzyme ring around the C-2—C-5 axis (Ref.17) would likewise hardly account for that fact, since polarized light is maximally absorbed roughly along this axis.
The sum of spectroscopic and chemical data discussed above constitutes a large body of evidence supporting the occurrence of coenzyme translocations in the enzyme's catalytic site in the course of the transamination reaction. However, the question as to the actual nature of these translocations — whether rotational, pendulum-like, or other — cannot as yet be answered. Solution of this and other questions regarding the catalytic mechanism of transaminase will become possible on the basis of determination of the three-dimensional structure of the enzyme and its complexes with specific ligands (substrates and inhibitors) in crystalline state by means of X-ray studies.

During several years workers in a number of laboratories failed in assiduous attempts to prepare transaminase crystals satisfactory for this purpose, and only in 1975 Yu.Torchinsky et al. in our laboratory succeeded in growing sufficiently large and perfectly ordered crystals of Asp-transaminase from chicken-heart cytosol, as well as crystals of its complexes with specific ligands (see, for example, Fig.15) and several heavy-atom substituted isomorphous forms which proved adequate for X-ray diffractometric studies. As reported in Prof.B.K.Vainstein's paper, he and his associates at the Institute of Crystallography of the USSR Academy of Sciences started such studies of these crystal forms, in close cooperation with our laboratory. To-date, the results of three-dimensional synthesis of the structure at 5Å resolution have been reported (Ref.27), and the study at 3.5Å resolution is nearing completion.

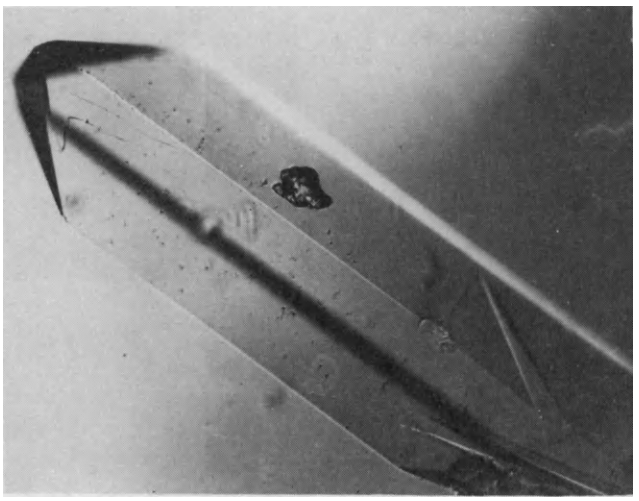


Fig.15. Micrograph of an orthorombic crystal of Asp-transaminase from chicken-heart cytosol (complex with MeAsp). The crystal belongs to space group $P2_12_12_1$ (Torchinsky and Volkova, 1975; from Ref.27).

Figure 16, representing a three-dimensional model of the transaminase molecule based on the electron density maps at 5Å resolution (Ref.27), clearly revealed two similar subunits related by non-crystallographic diad. As stated already by Prof.B.Vainstein (see Fig.5 in Ref.28), it was possible to discern in the 5 Å Fourier synthesis the position and sizes of nine α-helical segments of the peptide chain in each subunit, accounting for approx. 40% of its total length, in agreement with earlier CD spectrometric estimates.

The enzyme was shown to be catalytically active in the crystals. Removal of coenzyme from the crystals allowed to locate, using difference Fourier maps of holo- versus apo-enzyme (Ref.27), the position of the active sites in the dimeric enzyme molecule (indicated by arrows in Fig.16). The active site is situated in a fairly shallow niche in proximity of the intersubunit contact area; it seems that elements of the adjoining subunit may take part in formation of one of the surfaces bordering on the niche. In the other subunit the active site is symmetrically situated in an identical way at a distance of 30 Å. Crystalline complexes were prepared of Asp-transaminase with MeAsp, HO-Asp, 2-oxoglutarate, and with the carbonyl reagent, isonicotinoyl hydrazine (INH), as well as of the free PLP- and amino- or PMP--forms of the enzyme, and difference electron density maps were analyzed at 5 Å resolution.

The maps of the PLP- and PMP-forms of the enzyme proved very similar, thus indicating close resemblance of the protein conformations in these species. On the other hand, formation of complexes of the PLP-form with 0.03 M

MeAsp or 0.05 M INH is apparently associated with diffuse conformational changes, as indicated by reproducible emergence of a number of positive and negative peaks in the difference electron density maps (Ref.27 b). Some of these peaks are rather remote from the active site. It should be

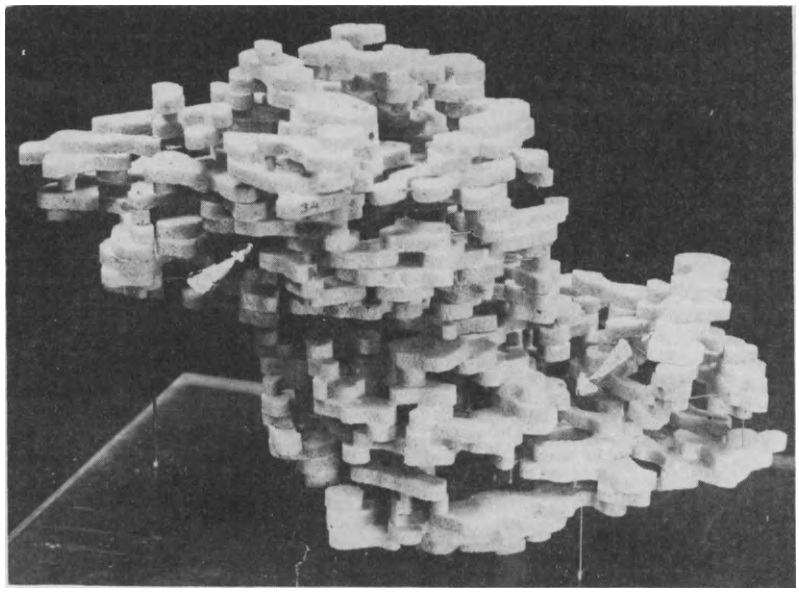


Fig. 16. Model of the dimeric molecule of Asp-transaminase from chicken-heart cytosol (at 5 Å resolution); from Ref. 27.

noted that conformational changes associated with formation of enzyme-substrate (or -allosubstrate) complexes do not affect the ORD and CD spectra of Asp-transaminase in solution in the far UV range (250-200 nm); see Refs.(29,30). This means that in the course of the catalytic cycle there occur no significant changes in the enzyme's content of α-helical and β-structures; it is rather their orientation, i.e., the tertiary structure of the globular protein, that undergoes alterations.

An important implication of the available X-ray diffraction data is that conformational changes associated with the catalytic cycle are not confined to the active-site region, but appear to involve a considerable part of the enzyme's protein molecule. Similar observations were reported in recent years with regard to complexes of several other enzymes (and other biologically active proteins) with their specific ligands, and plausible conjectures are being made about the probable role of such reversible diffuse transconfomations in the protein molecule (along with continuous rapid micro-fluctuations in the peptide chains) as factors contributing to unique biological functions — extremely high selective affinities, specific catalytic activity, and the like.

Detailed description and evaluation of the nature and role of the dynamic changes in three-dimensional structure of aspartate transaminase associated with its catalytic function will be possible on completion of X-ray investigations of the enzyme at higher resolution. Such studies are currently in active progress on three alloenzymic forms of aspartate transaminase — in Moscow, in Basel (on the mitochondrial isozyme from chicken heart) and in Ames, Iowa (on the pig-heart cytosolic enzyme).

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Note a - Language of cited paper is indicated as follows: (R), Russian; (E), English; (G), German; (F), French.

THE ROLE OF SECONDARY SPECIFICITY IN CATALYSIS BY PROTEOLYTIC ENZYMES

V. K. Antonov, L. D. Rumsh and A. A. Zinchenko

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow 117988, GSP-1, USSR

Abstract - The secondary specificity of proteolytic enzymes is discussed in terms of the stepwise formation of an enzyme-substrate complex. It is concluded that the secondary enzyme-substrate interactions induce the transformation of the initially formed complex into a productive form in which the maximal complementarity of the enzyme and the substrate is realized. This explains increase of k_{cat} and constancy of $K_{M(app)}$ for a series of substrates differing in the secondary interaction sites.

Substrate specificity of many enzymes is determined not only by substrate groups directly adjacent to the sensitive bond (primary specificity) but also by those regions of the molecule which are located for off this group (secondary specificity). The secondary substrate specificity is distinctly displayed by acid proteases. For example, an increase in the length of a polypeptide chain of the substrates for pepsin from two to four amino acid residues changes the hydrolysis rate constant more than 10^5 times (Refs. 1, 2). The secondary specificity of other acid proteases, such as chymosin, cathepsin D, and kidney renin is even higher (Refs. 3,4).

The secondary specificity can be manifested in the value of the catalytic rate constant (k_{cat}), the Michaelis constant $K_{M(app)}$, or these two kinetic parameters simultaneously. Pepsin is an example of enzymes for which the secondary specificity influences almost exclusively the catalytic constant, the effect depending not only on the length of a substrate polypeptide chain, but also on the nature of side chains remoted from the sessile bond.

In order to evaluate the contribution of each residue of a substrate polypeptide chain into the specificity of pepsin, we have analysed statistically digestion of over 500 amino acid sequences in different proteins by pepsin (Ref. 5). The so-called specificity indices (S_{ij}) were calculated for each amino acid (i) at each of 20 positions (j) around the bond being hydrolyzed. These indices reflect the probability for a given residue in a given position to be included in the sequence being split. The mean value of the specificity index for a given position (S_j) characterizes the rigidity of requirements imposed by the appropriate locus of the active site on the structure of amino acid side chains. These values indicate that pepsin is most specific towards a residue in the position P_1 and that the binding site of the enzyme, which specifically interacts with amino acid side chains in the substrate, comprises 5-7 residues (Ref. 5).

Furthermore, a linear correlation has been established between the total specificity indices for a large number of synthetic peptides, on the one hand, and the kinetic parameters (k_{cat} and k_{cat}/K_M) of their enzymatic hydrolysis, on the other (Ref. 2) (Fig. 1). Correlation equations make it possible to calculate *a priori* the rate of hydrolysis by pepsin of any bond in a given amino acid sequence.

The secondary specificity of pepsin was revealed also while studying the activation of cleavage of di- and tripeptides in the presence of peptides-activators that cannot be hydrolyzed by pepsin. The observed value of k_{cat} was shown to increase several dozen times in the presence of these activators, the increase correlating with the specificity of the activator (Ref. 6). The mechanism of activation seems to involve synthesis of

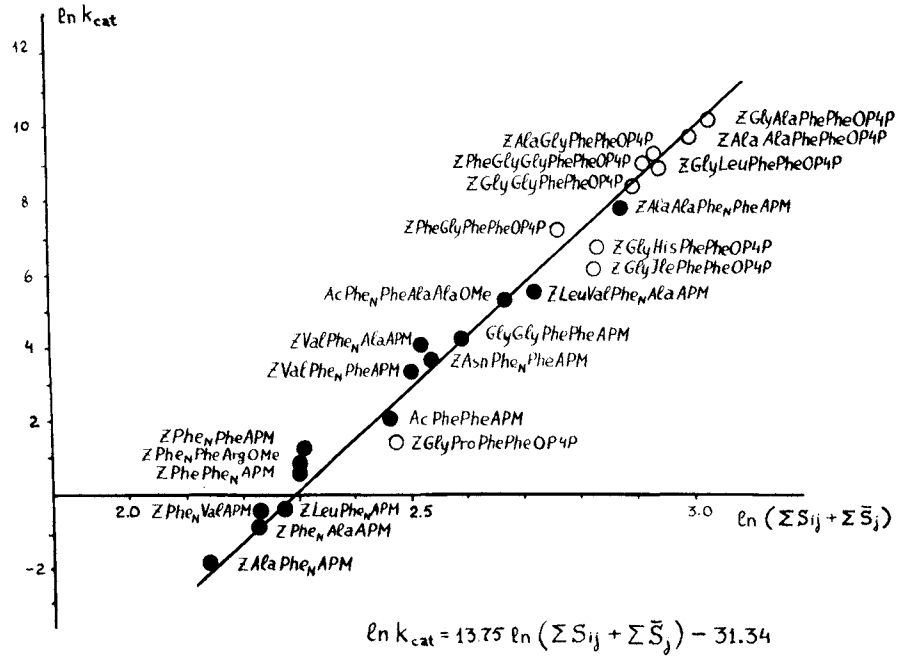

Fig. 1. Dependence of k_{cat} for synthetic substrates on the sum of specificity indices. Open circles - data from Ref. 1 and filled circles, from Ref. 2.

a peptide from the activator and the substrate with the following cleavage of this peptide across the bond which is split in the substrate in the absence of the activator.

How do the secondary interactions of a substrate with the enzyme affect the rate constant for enzymatic hydrolysis? In order to answer this question, we have determined the thermodynamic parameters of binding and catalysis for a number of peptides which differ in the catalytic constant by more than two orders of magnitude (Ref. 2). First, the binding of substrates was found to be due to a significant positive change in the entropy (Table 1). The enthalpy of complex formation was also positive and slightly increased when passing from a dipeptide to a tetrapeptide. In this case, a compensation change in the entropy and the enthalpy of binding with the isoergonic temperature of 278°C was observed. These data suggest that when substrates are bound to the enzyme the solvation state of the reagents considerably changes. Second, the free energy of activation decreased while going from a dipeptide to a tetrapeptide at the account of a decrease in the enthalpy of activation. Therefore, the secondary interactions contribute to stabilizing the transition state, chiefly by decreasing its energy, but display only a minor effect on the probability of its formation.

TABLE 1. Thermodynamic parameters of binding and hydrolysis

Substrate	ΔG^o	ΔH^o	ΔS^o	ΔG^*	ΔH^*	ΔS^*
	kcal/mole		e.u.	kcal/mole		e.u.
ZPhe-PheAPM* (Phe: NO_2)	-4.25	0.6	16.3	19.5	7.8	-39.3
ZVal-Phe-AlaAPM (Phe: NO_2)	-4.15	1.2	18.0	18.0	6.1	-40.0
ZLeu-Val-Phe-AlaAPM (Phe: NO_2)	-4.35	1.9	21.0	16.7	3.4	-44.6

*APM - γ-aminopropylmorpholine

It has been accepted so far that the transition state is stabilized either due to realization in it of new interactions between the enzyme and a substrate which are not observed in the ground state, or because a strain arising in the ground state when the substrate is bound are eliminated in the transition state (Ref. 7). Either of these interpretations means that the enzyme is complementary to the transition state of the substrate. Then, a decrease in the enthalpy of activation caused by the secondary interactions would signify that the affinity of the enzyme for the substrate groups located far off the bond being broken increases in the transition state. A change in the affinity must be accompanied with the formation of new non-covalent bonds and the rupture of the existing bonds between the enzyme and the substrate, as well as with conformational changes of the reagents. We think, however, that it is highly improbable that considerable rearrangements may occur in the reacting system within the fluctuation time, when the transition state is attained.

We propose a different interpretation of these facts. This interpretation is based on the conception of stepwise formation of an enzyme-substrate complex. At the first step, the substrate is "anchored" by the enzyme at the most specific binding residue. In the case of pepsin substrates, this residue is (as was mentioned above) in the position P_1. Then, the secondary interactions are realized, which convert the first enzyme-substrate complex (ES_o) into a second one which will be referred to as the productive complex (ES_p). In the second complex, all possible interactions between the enzyme and the substrate take place. To put it differently, the enzyme must be complementary to the substrate not in the transition state but in the ground state of the productive complex. The formation of the transition state (ES_p) may be accompanied also with new interactions, but only between the atoms of the substrate reacting group and the enzyme. These interactions have to be prepared in the ground state; they do not require any rearrangements in the reacting system and, for a series of substrates of the same type, make identical contribution in the energy of the transition state.

$$E + S \underset{k_{-I}}{\overset{k_I}{\rightleftharpoons}} ES_o \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_p \xrightarrow{k_3} E + P \qquad (I)$$

when $k_3 \ll k_{-I}$

$$v = d\,P/dt = k_3 |E|_o |S|_o / K_s K_p + (I + K_p)|S|_o \qquad (2)$$

where $K_s = (k_{-I} + k_2)/k_I$ and $K_p = (k_{-2} + k_3)/k_2$

$$k_{cat} = k_3 / I + K_p \qquad (3)$$

$$K_{M(app)} = K_s K_p / I + K_p \qquad (4)$$

The simplest kinetic scheme (1) based on these conceptions can be described by Equation (2) for the reaction rate. Analysis of this equation shows that the value of $K_{M(app)}$ (Eq.4) may remain constant whereas the value of k_{cat} (Eq.3) increases ($K_p \gg 1$) in a series of pepsin substrates belonging to the same type, particularly of those which contain the same amino acid in the position P_1. Indeed, such a situation has been found for the majority of synthetic substrates of pepsin studied so far. The step limiting the rate of hydrolysis can be either cleavage of the bond ($k_2 \gg k_3 \ll k_{-2}$ or $K_p \ll 1$), or formation of the productive complex ($k_2 \ll k_3 \gg k_{-2}$). Depending on this, a number of characteristics of enzymatic catalysis (pH dependence, the value of kinetic isotopic effect, etc.) can undergo changes. In fact, these characteristics for the so-called "fast" substrates of pepsin differ from

those for "slow" substrates (Refs.8,9).

Therefore, as follows from the above, the secondary interactions between the enzyme and the substrate change the level of free energy for the ground state at the step of the productive complex, thus altering the observed values of k_{cat} and $k_{cat}/K_{M(app)}$ (Fig. 2).

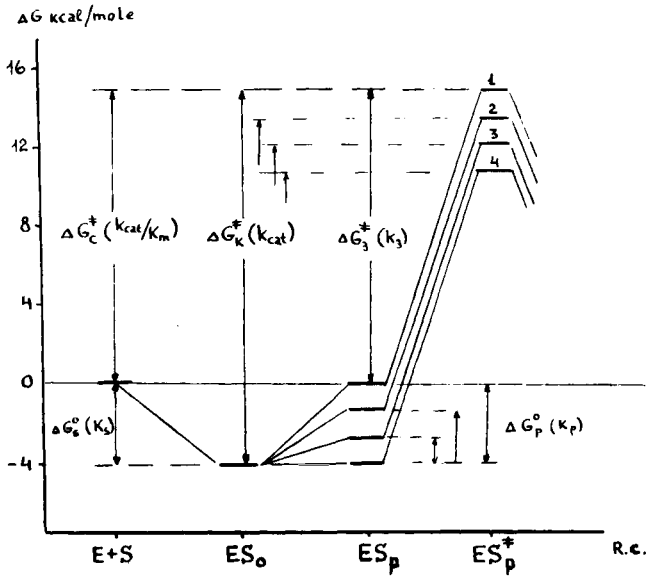

Fig. 2. Diagram of changes in free energy along the reaction coordinate for an arbitrary series of substrates, having $K_{M(app)} = 1 \times 10^{-3}$M, $k_3 = 1000$ sec^{-1} and k_{cat}=1(1), 10(2), 100(3) and 1000(4) sec^{-1}.

For a number of proteolytic enzymes, e.g. chymotrypsin and trypsin, the values of k_{cat} increase while those of $K_{M(app)}$ decrease, i.e. the well-known rule "better binding - better catalysis" is obeyed (Ref.10). There is a linear dependence of the kinetic parameters on hydrophobicity of the side chain in a series of simple substrates, derivatives of acylamino acids (Ref. 11). Apparently, for such substrates, all energy involved in the interaction of the substrate side chain with the enzyme is expressed by the dissociation constant for the enzyme-substrate complex. However, the interaction of the acylamino group with the enzyme hardly makes any contribution into binding, but considerably increases the value of k_{cat}. One may assume that it is this interaction which favours the formation of the productive complex. Here, the energy of interaction can be evaluated by comparing the rate constants for acylation of chymotrypsin by esters of N-acetyl-L-phenylalanine and hydrocinnamic acid (Ref. 12). This energy is about 6.5 kcal/mole for acetylamino groups, which is close to the energy of the hydrogen bond in a non-polar medium. This value is likely to vary for different substrates, provided that the extent of shielding of the hydrogen bond formed between the acylamino group and the carbonyl group of Ser-214 from the environment is not the same in complexes with different substrates.

Chymotrypsin is known to cleave the esters of acylamino acids more than 10^3 times as fast as amides (Ref. 12). This is entirely determined by a difference in free energy of the starting compounds, whereas the level of free energy in the transition state is almost the same for all "semispecific" substrates as well as for the dipeptide $AcPheGlyNH_2$(Ref. 13) (Fig. 3). However, the free energy of activation decreases by 3 kcal/mole when passing to $AcPheAlaNH_2$, i.e. upon introduction of a methyl group. This example illustrates how the favourable effect of the secondary specificity is structurally realized. As follows from the data of x-ray analysis of the complex between chymotrypsin and a pancreatic inhibitor (Ref. 14), the methyl group of an alanine residue in the position P_1 forms favourable for catalysis contacts with Cys-42 and His-57 of the enzyme, but an unfavourable

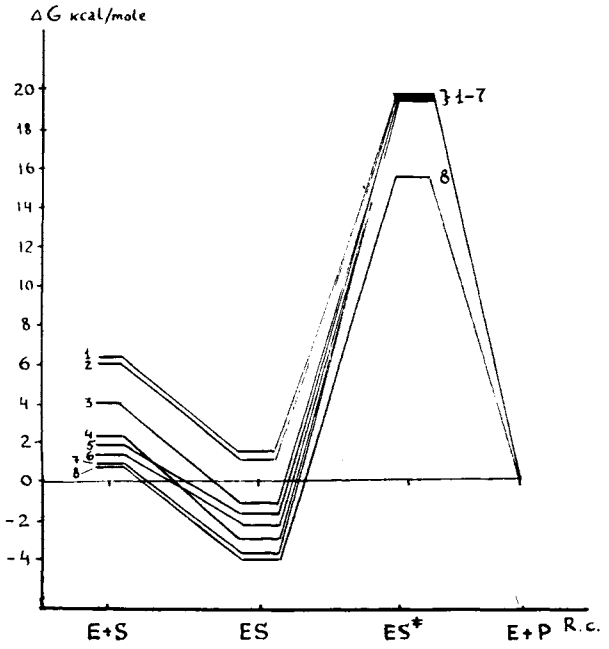

Fig. 3. Diagram of changes in free energy along the reaction coordinate for semispecific substrates of chymotrypsin (Ref. 13) - AcPhe(NO_2-p)-R, where R = 1 -OEt, 2-OMe, 3 - $NHC_6H_4NO_2$-p, 4 - NHC_6H_5, 5 - NH_2, 6 - $NHNH_2$, 7 - $GlyNH_2$ and and 8 - $AlaNH_2$.

contact with the hydroxyl of Ser-195. Apparently, this produces strain which is eliminated in the transition state because of the rotation of the Ser-195 side chain to a position typical of the acyl enzyme. As was demonstrated by conformation analysis (Ref. 15), this rotation is realized very easily, upon introducing the substrate, due to abstraction of a water molecule from the enzyme active site that is, the "acyl-enzyme" position of Ser-195 in the enzyme-substrate complex is more favoured by energy considerations than the "native" one. Consequently, there is no need to assume that the "stress" is removed in the transition state. We believe that "anchoring" of the substrate (complex ES_o), by eliminating a molecule of water, provides for favourable interaction of the Ala methyl group with Cys-42 and His-57 in the productive complex (ES_p).

Therefore, we believe that the role of the secondary interactions in catalysis by proteolytic enzymes is as follows: these interactions induce the transformation of a system into a productive enzyme-substrate complex in which the maximal complementarity of the enzyme and the substrate is realized. Thus, in a series of substrates of the same type, the enzyme specificity is manifested at the stage of the productive complex rather than at the stage of the transition state.

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ENZYME HAVING COVALENTLY BOUND FLAVINS

K. Yagi

Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya 466, Japan

Abstract — To screen the enzymes having covalently bound flavins in animal tissues, cells, or cell organelles, a novel method was devised. The flavin linked covalently to protein in rat liver was labelled by injecting ^{14}C-labelled riboflavin into a rat and the covalently bound flavin was detected by its radioactivity after electrophoresis on sodium dodecyl sulfate-acrylamide gel. By this procedure, the numbers of proteins having covalently bound flavins in rat liver mitochondria and microsomes were found to be four and one, respectively.

INTRODUCTION

Following to the discovery of "unextractable flavin" in succinate dehydrogenase complex (1) and that of "tightly bound riboflavin" in vegetables (2), Kearney and Singer (3) obtained this kind of flavin from succinate dehydrogenase by tryptic digestion and found that the flavin is bound covalently to the protein moiety. Since then, more than ten enzymes having covalently bound flavins are disclosed. In animal body, four enzymes having covalently bound flavins are known. They are succinate dehydrogenase [succinate : (acceptor) oxidoreductase, EC 1.3.99.1] (3,4), monoamine oxidase [amine : oxygen oxidoreductase (deaminating), EC 1.4.3.4] (5), sarcosine dehydrogenase [sarcosine : (acceptor) oxidoreductase (demethylating), EC 1.5.99.1] (6), and L-gulonolactone oxidase [L-gulono-γ-lactone : oxygen 2-oxidoreductase, EC 1.1.3.8] (7,8). In microorganisms, nine enzymes containing covalently bound flavins were found. They are D-6-hydroxynicotine oxidase [6-hydroxy-D-nicotine : oxygen oxidoreductase, EC 1.5.3.6] (9), thiamine dehydrogenase (10), β-cyclopiazonate oxidocyclase (11), trimethylamine dehydrogenase [trimethylamine : (acceptor) oxidoreductase (demethylating), EC 1.5.99.7] (12), p-cresol oxidase (13), L-galactonolactone oxidase (14,15), cholesterol oxidase [cholesterol : oxygen oxidoreductase, EC 1.1.3.6] (16), flavocytochromes c (cytochrome c-552 and cytochrome c-553) (17) and choline oxidase (18).
The number of these enzymes, however, is still small as compared with that of another category of flavoproteins, viz., dissociable flavoproteins. Accordingly, it seemed worthwhile to develop a method to demonstrate how many enzymes having covalently bound flavins remain to be elucidated. For this purpose, we devised a method to screen all such enzymes in animal tissues, cells, or cell organelles. The principle of this method is due to the fact that radioactive riboflavin administered to a rat is incorporated, even slowly, into the covalently bound flavins in the liver (19) and behaves as an indicator of the protein having covalently bound flavins. The present paper deals briefly with the method and examples of its application to rat liver mitochondria and microsomes.

MATERIALS AND METHODS

Male Wistar rats weighing approximately 50 g were used. Liver mitochondria and microsomes were prepared according to Jhonson and Lardy (20) and Hogeboom (21), respectively. [2-^{14}C] Riboflavin (specific radioactivity, 32 mCi/mmol) was purchased from the Radiochemical Centre, Amersham. For slab gel electrophoresis, the discontinuous buffer system described by Laemmli (22) was used with a slight modification. The separation gel was prepared with 1% acrylamide in 0.375 M Tris-HCl (pH 8.8), and the stacking gel with 1% sodium dodecyl sulfate (SDS) and 3% acrylamide in 0.125 M Tris-HCl (pH 6.8). The sample for electrophoresis was prepared as follows : mitochondrial or microsomal suspension was mixed with 0.02 M sodium phosphate buffer (pH 7.2) containing 8 M urea, 2% SDS and 1% 2-mercaptoethanol, and the mixture was heated in boiling water for 3 minutes. After gel electrophoresis, 5 cm portion of the gel was cut into approximately 2 mm slices. Each slice was incubated with 0.4 ml of 15% H_2O_2 in a glass scintillation vial at 70°C for 16 hours. After the gel slice was dissolved, protosol and 0.4% omnifluor-toluene solution were added. The radioactivity was estimated in a Beckman LSC-335 scintillation counter. Mitochondrial and microsomal protein was determined by the biuret method (23).

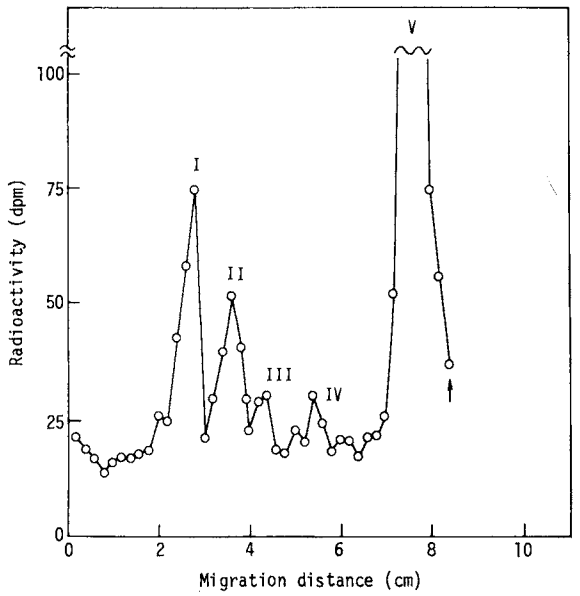

Fig. 1. Pattern of radioactivity after slab gel electrophoresis of rat liver mitochondria. Mitochondrial suspension (8.8 mg protein) was heated for 3 minutes in 0.02 M sodium phosphate buffer (pH 7.2) containing 8 M urea, 2% SDS and 1% 2-mercaptoethanol and subjected to electrophoresis.

RESULTS AND DISCUSSION

A rat was fed on the riboflavin-deficient diet (24) for two weeks, and was injected daily with 0.2 ml of radioactive riboflavin solution (0.2 mg of [2-^{14}C]riboflavin in 1.0 ml of physiological saline) through tail vein for one week. During this period, the rat was fed on the riboflavin-deficient diet. One day after the last injection, the rat was sacrificed and the liver was excised. Mitochondrial and microsomal fractions of the liver were subjected to the analysis by slab gel electrophoresis.

Figure 1 shows the pattern of the radioactivity of the mitochondrial sample, and figure 2 that of the microsomes. In mitochondria, five peaks are observed in the radioactivity pattern. The highest peak (V) observed near the tracking dye, is due to free flavin. The molecular weights of the protein subunits corresponding to peaks I to IV were calculated to be 91,000, 72,000, 60,000, and 44,000, respectively, from the calibration curve obtained with the marker proteins (25). It is known that in mitochondrial flavoproteins, succinate dehydrogenase, monoamine oxidase and sarcosine dehydrogenase contain covalently bound flavin as coenzyme. The subunit molecular weight of succinate dehydrogenase was estimated to be 70,000 (26,27). This value is in good accord with that of peak II in the present pattern. As to the subunit of monoamine oxidase, the experimental data using radioactive inhibitors were reported. Collins and Youdim (28) reported that ^{14}C-phenethylhydrazine-binding monoamine oxidase has a molecular weight of 60,500. It was also shown by using a specific inhibitor, ^{14}C-pargylin, that the radioactivity was associated with the protein of 60,000 in outer membrane of rat liver mitochondria (29). More recently, Minamiura and Yasunobu (30) and Salach (31) reported that the subunit molecular weight of the highly purified monoamine oxidase from bovine liver mitochondria was about 52,000 and 62,000. Taking these results into consideration, the protein of peak III is assigned to monoamine oxidase. As to peak I, our recent work revealed that it corresponds to sarcosine dehydrogenase (32). Accordingly, peak IV remained to be elucidated. This protein seems to be a new enzyme having covalently bound flavin.

In the pattern shown in figure 2, two peaks are observed. Since peak II is due to free flavin, it is clear that rat liver microsomes contain only one protein containing covalently bound flavin (33). As to the entity of this peak, it is recalled that L-gulonolactone oxidase was isolated from rat liver microsomes (7). Accordingly, this peak is considered to correspond to L-gulonolactone oxidase. This was confirmed by identifying it with purified L-gulonolactone oxidase on SDS polyacrylamide gel by observing their intrinsic fluorescence (34). It should be emphasized from the present study that rat liver microsomes do not contain any new protein having covalently bound flavin.

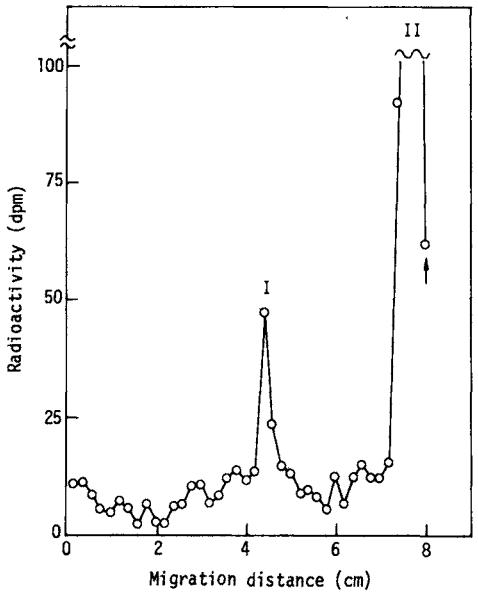

Fig. 2. Pattern of radioactivity after slab gel electrophoresis of rat liver microsomes. Microsomal suspension (12.5 mg protein) was heated for 3 minutes in 0.02 M sodium phosphate buffer (pH 7.2) containing 8 M urea, 2% SDS and 1% 2-mercaptoethanol and subjected to electrophoresis.

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STRUCTURE AND EVOLUTION OF GLUTAMATE DEHYDROGENASES

E. L. Smith

Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, California 90024, USA

Abstract - Prokaryotes usually possess only one type of glutamate dehydrogenase which is specific for NADP. The liver enzyme of vertebrates functions with either NADP or NAD and its activity is modulated allosterically -- ADP and GDP acting as positive effectors and ATP and GTP as negative effectors. In Neurospora crassa there is a coordinate induction-repression system that regulates the biosynthesis of two individual glutamate dehydrogenases -- one, specific for NADP, is maximally produced in media lacking a source of organic nitrogen, and the other, specific for NAD, is maximally produced in the presence of glutamate or certain other amino acids. The vertebrate enzymes are hexameric with approximately 500 residues per subunit polypeptide chain. The hexameric NADP-specific enzyme of Neurospora manifests considerable homology to the vertebrate enzymes, particularly in the NH_2-terminal portion of the molecule, whereas the NAD-specific enzyme of Neurospora is tetrameric with each subunit containing more than 1000 residues. The homology of the NAD enzyme to the others is limited to the neighborhood of certain functional residues. Comparison of the glutamate dehydrogenases shows in the sequences large gaps or additions involving many residues. The origin of the large gaps or additions, which also occur in other homologous proteins, appears to reside in the nature of eukaryotic DNA which contains long non-coding regions between the coding regions. The RNA transcript of eukaryotic DNA is processed to join the coding regions producing a messenger RNA which is translated into the polypeptide sequence. Addition of interior amino acid sequences of a protein would appear to involve incorporation of non-coding DNA into the mRNA and deletion to involve addition of part of a coding region to a non-coding segment. This appears to be the simplest way of explaining the great diversity of sequences which appeared during the evolution of many homologous, eukaryotic proteins, such as the glutamate dehydrogenases.

INTRODUCTION

Essentially all living organisms contain a glutamate dehydrogenase (see (1)). In most prokaryotic species, there is usually only one type of enzyme which is generally specific for one of the two pyridine nucleotide coenzymes, NADP or NAD. The glutamate dehydrogenases of the vertebrates can function with either coenzyme and their activity is modulated by the levels of the purine nucleoside di- and triphosphates -- ADP and GDP acting as positive effectors and ATP and GTP as negative effectors. Thus, the vertebrate enzymes respond to the energy requirements of the cell by using dehydrogenation of glutamate as a source both of reduced coenzyme for energy, and of α-ketoglutarate for degradation by the tricarboxylic acid cycle.

$$\text{Glutamate} + \text{NAD(P)} + H_2O \rightleftharpoons \alpha\text{-ketoglutarate} + \text{NAD(P)H} + NH_4^+$$

In the Ascomycete, Neurospora crassa, instead of allosteric regulation of activity, there is a coordinate induction-repression system (2,3) that regulates the biosynthesis of two distinct glutamate dehydrogenases -- one, specific for NADP, is maximally produced in media lacking a significant source of organic nitrogen; the other, specific for NAD, is maximally produced in the presence of glutamate and certain other amino acids. Presumably, the role of the NADP enzyme is in biosynthesis, making available glutamate for the synthesis of other amino acids and, therefore, also purine and pyrimidine nucleotides, as precursors for synthesis of proteins, nucleic acids and other nitrogenous materials required for growth. In contrast, the NAD enzyme serves to provide reduced coenzyme and α-ketoglutarate for the energy requirements of the cell.

STRUCTURES OF GLUTAMATE DEHYDROGENASES

For some years work in our laboratory has been devoted to investigations of the amino acid sequences and other properties of the glutamate dehydrogenases of eukaryotes. In this presentation we shall be concerned primarily with the primary structure and subunit character

of these enzymes as well as the evolution of their sequences and regulatory behavior.

TABLE I. Comparison of various kinds of glutamate dehydrogenases

Type	Coenzyme	Subunit size	Number of subunits
Vertebrate liver*	NAD: NADP	~56,000	6
Neurospora crassa	NADP	48,400	6
Neurospora crassa	NAD	116,000	4
Escherichia coli	NADP	46,000	6

*These include bovine, chicken, human, rat, tuna and others.

In Table I, there is summarized the general structures of various types of glutamate dehydrogenases. It is apparent that most of these enzymes are hexameric with identical subunits of approximately 45,000 to 56,000 daltons. The exception is the NAD-specific enzyme of Neurospora which is tetrameric and has a subunit which is more than twice the size of those of the other enzymes.

At the present time, the sequences of the glutamate dehydrogenases from bovine (4,5) and chicken (4,6) liver have been completely determined. The sequence of the enzyme is almost completely known from a single human liver (7) and partial sequences have been obtained from rat liver (1). Fig. 1 presents the sequence of the bovine enzyme and, for comparison, the known differences in the sequences of the chicken and human enzymes.

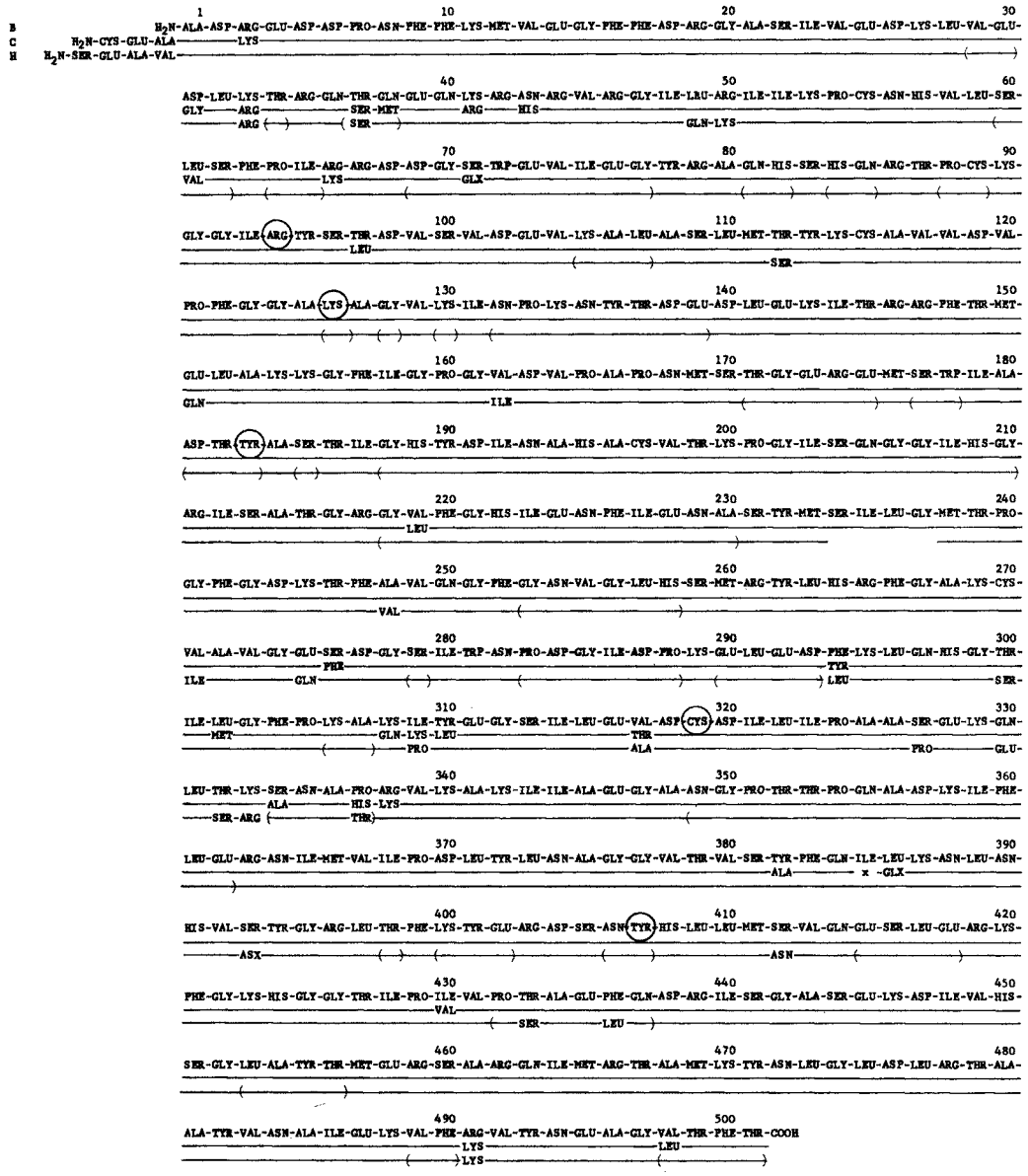

Fig. 1. The amino acid sequence of the bovine (B) liver glutamate dehydrogenase and for comparison, those residues that differ in the chicken (C) enzyme and that are known to differ in the still incomplete sequence of the human (H) enzyme. The sequences have been corrected by inclusion of isoleucine residue-385 which was previously overlooked. The five circled residues have been identified with specific functions that are presented below.

Inspection of these sequences indicates that both the chicken and the human enzymes possess extra residues at the amino terminal ends, as compared to the bovine enzyme. The number of other differences in the sequences of the four vertebrate enzymes is relatively small, approximately 5 percent. Both this fact and the nature of the amino acid replacements indicates a high degree of conservatism in the evolution of this enzyme in the higher vertebrates.

Fig. 2 shows a comparison of the amino acid sequences of the Neurospora NADP-specific enzyme (8) and the bovine enzyme. These sequences reveal that although there is considerable homology between them, particularly in the amino-terminal halves of the sequences there are a large number of differences. The Neurospora enzyme is much smaller lacking a significant number of residues both at the amino- and carboxyl-terminal ends. In addition, in order to obtain good homology between these two sequences, it is evident that a number of large gaps had to be introduced. The problem of these gaps is considered later in this presentation.

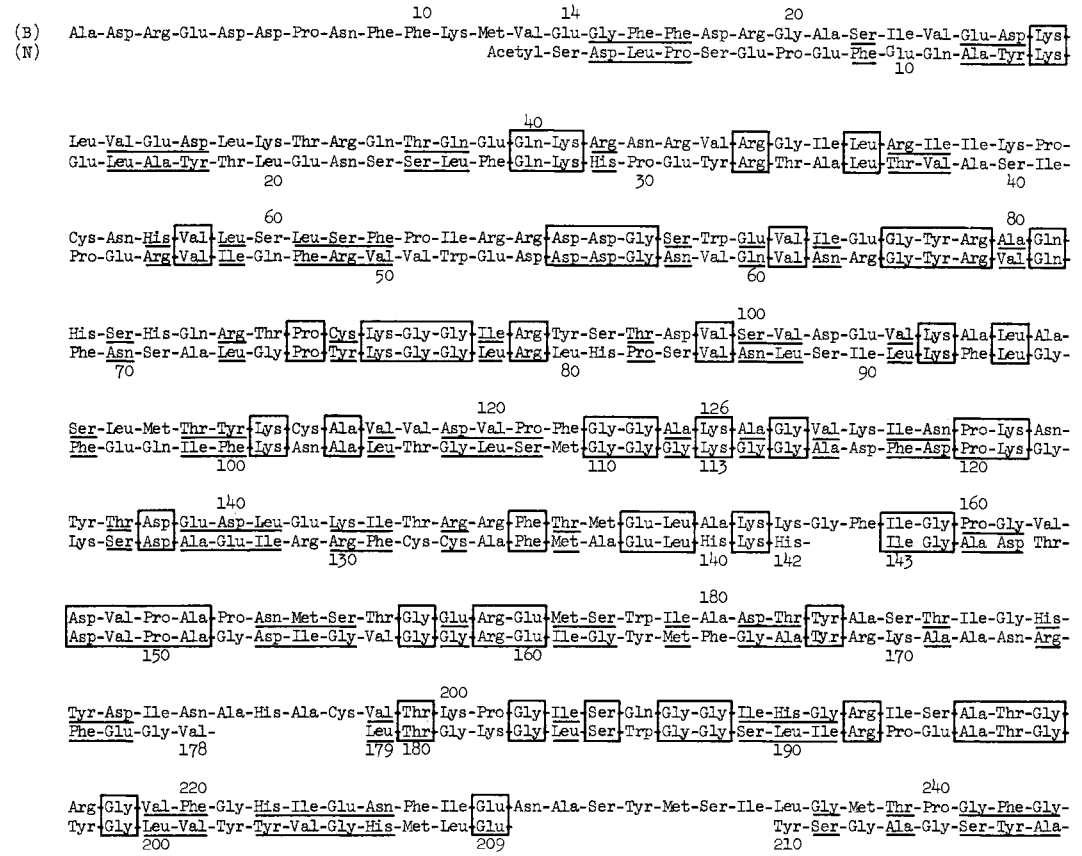

Fig. 2A. Comparison of the amino acid sequences of the bovine (B) enzyme and the NADP-specific glutamate dehydrogenase of Neurospora (N). Boxed residues are identical; underlined residues differ by a single base change in the codons.

Comparison of the presently known sequence of the NAD-specific glutamate dehydrogenase of Neurospora with the others reveals little overall sequence homology. Presently the sequence of the carboxyl-terminal 669 residues has been established (9) (Fig. 3) as well as large portions of the amino-terminal part of the enzyme (10). Only around certain types of reactive residues does there appear to be significant similarity in the sequences when compared to the other types of glutamate dehydrogenases. Each of the presently identified reactive residues and the sequences around them will be considered in turn.

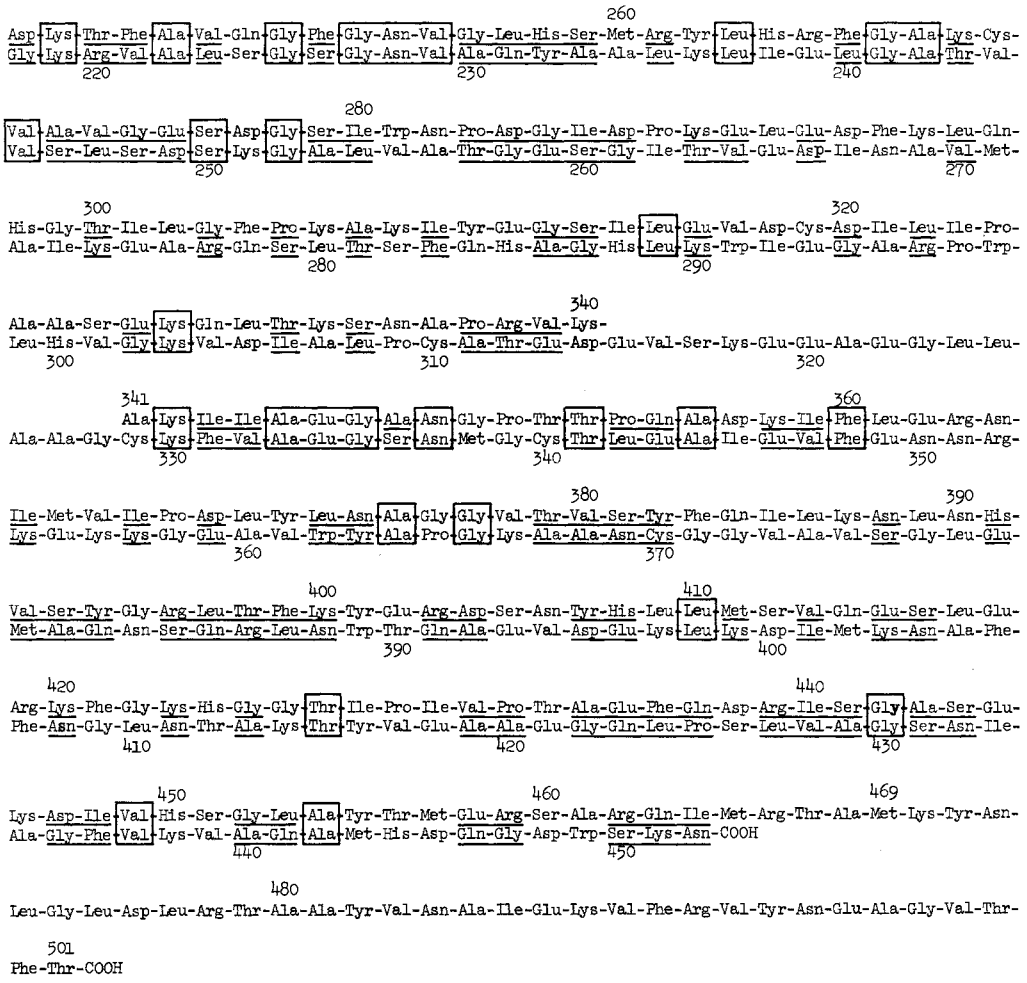

Fig. 2B. Continuation of Fig. 2A.

Reactive lysine

All of these enzymes are strongly inhibited by reaction with pyridoxal phosphate, pyridoxal, cyanate, or other reagents that react with ϵ-amino groups. Peptides containing the labeled essential lysine residue have been isolated from several of the vertebrate glutamate dehydrogenases (1). This has usually been accomplished by reaction with pyridoxal phosphate followed by reduction of the Schiff base with borohydride. In all cases a lysine residue in the identical sequence has been identified. In kinetic and equilibrium studies with pyridoxal phosphate (11), pyridoxal (11) and carbamyl phosphate (12) it has been demonstrated that pK' is 7.8 ± 0.2 at 30° for the reaction of this amino group in the bovine enzyme. A similar study has also been performed with the NADP enzyme of Neurospora by using N-ethylmaleimide as the inhibitor (13). At 34° , pK' = 7.6 and at 2°, pK' = 8.7. The apparent ΔH is 12.6 ± 2.0 Cal per mole, which is the heat of ionization characteristic of an ϵ-amino group. The amino acid sequence around the reactive lysine residue is shown for the three types of glutamate dehydrogenases in Fig. 4.

With the NAD enzyme from Neurospora, the reaction of pyridoxal phosphate at neutral pH occurred not with one but with six lysine residues of the enzyme. Although the inhibition was similar to the other glutamate dehydrogenases, it was impossible to identify directly the essential lysine residue (13,14). However, inspection revealed that the sequence of the region around one of the reactive lysine residues was homologous to the sequences of the other enzymes (Fig. 4) (9,15).

Reactive tyrosine residues

The initial reaction of tetranitromethane with the bovine enzyme modifies a single tyrosine residue without loss of enzyme activity, but abolishes the allosteric inhibition by guanosine triphosphate (GTP) (16). The peptide containing nitrotyrosine was isolated from both the bovine and the chicken enzymes (1) and it was thereby demonstrated that tyrosine-407* was the modified residue. (This is in accord with the revised sequence for these enzymes; see legend to Fig. 1.). Prolongation of the reaction of tetranitromethane led to a gradual loss of enzyme activity but several different tyrosine residues were partially modified.

Leu-Gly-Thr-Glu-Tyr-Thr-Ser-Leu-Ile-Ala-Ala-Leu-Asp-Pro-Lys-Asn-Asn-Ser-His-Val-Glu-Ile-Leu-Ser-Lys-Met-Lys-
669 660 650

Lys-Arg-Leu-Arg-Thr-Glu-Thr-Phe-Thr-Pro-Asp-Tyr-Ile-Leu-Glu-Ile-Ile-Ser-Ser-His-Pro-Gln-Leu-Val-Arg-Ala-Leu-
640 630 620

Tyr-Ala-Ser-Phe-Ala-Ser-Val-His-Leu-Arg-Val-Gly-Ser-Asp-Tyr-Asp-Arg-His-Leu-Ile-Ala-Pro-Thr-Pro-Val-Met-Ser-
610 600 590

Glu-Val-Leu-Asp-Ala-Arg-Leu-Lys-Glu-Lys-Ile-Thr-Lys-Asp-Val-Ser-Asn-Glu-His-Glu-Glu-Met-Val-Met-Thr-Ala-Phe-
580 570

Arg-Val-Phe-Asn-Asn-Ala-Val-Leu-Lys-Thr-Asn-Phe-Phe-Thr-Pro-Thr-Lys-Val-Ala-Leu-Ser-Phe-Arg-Leu-Asn-Pro-Ser-
560 550 540

Phe-Leu-Pro-Glu-Val-Glu-Tyr-Pro-Lys-Pro-Leu-Tyr-Gly-Met-Phe-Leu-Val-Ile-Thr-Ser-Glu-Ser-Arg-Gly-Phe-His-Leu-
530 520 510

Arg-Phe-Lys-Asp-Ile-Ala-Arg-Gly-Gly-Ile-Arg-Ile-Val-Lys-Ser-Arg-Ser-Lys-Glu-Ala-Tyr-Gln-Ile-Asn-Ala-Arg-Asn-
500 490

Leu-Phe-Asp-Glu-Asn-Tyr-Gly-Leu-Ala-Ser-Thr-Gln-Gln-Arg-Lys-Asn-Lys-Asp-Ile-Pro-Glu-Gly-Gly-Ser-Lys-Gly-Val-
480 470 460

Ile-Leu-Leu-Asp-Pro-Lys-Gln-Gln-Asp-Arg-His-Arg-Glu-Ala-Phe-Glu-Lys-Tyr-Ile-Asp-Ser-Ile-Leu-Asp-Leu-Leu-Leu-
450 440 430

Lys-Ala-Glu-Thr-Pro-Gly-Ile-Lys-Asn-Pro-Ile-Val-Asp-Leu-Tyr-Gly-Lys-Glu-Glu-Ile-Leu-Phe-Met-Gly-Pro-Asp-Glu-
420 410 400

Asn-Thr-Ala-Asp-Leu-Val-Asp-Trp-Ala-Thr-Glu-His-Ala-Arg-Ala-Arg-Gly-Ala-Pro-Trp-Trp-Lys-Ser-Phe-Phe-Thr-Gly-
390 380

Lys-Ser-Pro-Arg-Leu-Gly-Gly-Ile-Pro-His-Asp-Ser-Tyr-Gly-Met-Thr-Thr-Leu-Ser-Val-Arg-Glu-Tyr-Val-Lys-Gly-Ile-
370 360 350

Tyr-Arg-Lys-Leu-Glu-Leu-Asp-Pro-Ser-Lys-Ile-Arg-Lys-Met-Gln-Thr-Gly-Gly-Pro-Asp-Gly-Asp-Leu Gly Ser Asn Glu-
345 330 320

Ile-Leu-Leu-Ser-Asn-Glu-Thr-Tyr-Thr-Ala-Ile-Val-Asp-Gly-Ser-Gly-Val-Leu-Cys-Asp-Pro-Asp-Gly-Ile-Asp-Lys-Asp-
310 300

Glu-Leu-Arg-Arg-Leu-Ala-Lys-Ala-Arg-Ala-Met-Ile-Ser-Asn-Phe-Asp-Ile-Ala-Lys-Leu-Ser-Lys-Asp-Gly-Tyr-Arg-Val-
290 280 270

Leu-Cys-Asp-Asp-Thr-Asn-Val-Thr-Leu-Pro-Asn-Gly-Glu-His-Val-Asn-Gly-Thr-Ala-Phe-Arg-Asn-Thr-Tyr-His-Leu-Arg-
260 250 240

Fig. 3A. The sequence of the NAD-specific enzyme of Neurospora from residues 669 through 238 from the COOH-terminus.

Asp-Asn-Gly-Ile-Thr-Asp-Met-Phe-Val-Pro-Cys-Gly-Gly-Arg-Pro-Glu-Ser-Ile-Asp-Leu-Ser-Ser-Val-Asn-Lys-Leu-Ile-
230 220

Lys Asp-Gly-Lys-Thr-Ser-Ile-Pro-Tyr-Ile-Val-Glu-Gly-Ala-Asn-Leu-Phe-Ile-Thr-Gln-Asp-Ala-Lys-Leu-Arg-Leu-Glu-
210 200 190

Glu-Ala-Gly-Cys-Ile-Val-Tyr-Lys-Asp-Ala-Ser-Ala-Asn-Lys-Gly-Gly-Val-Thr-Ser-Ser-Ser-Leu-Glu-Val-Leu-Ala-Ser-
180 170 160

Leu-Ser-Phe-Asp-Asp-Lys-Gly-Phe-Val-Thr-His-Met-Cys-His-Asn-Ser-Arg-Gly-Asn-Ala-Pro-Glu-Phe-Tyr-Gln-Ala-Tyr-
150 140 130

Val-Lys-Glu-Val-Gln-Asn-Lys-Ile-Gln-Asp-Asn-Ala-Arg-Leu-Glu-Phe-Glu-Ala-Ile-Trp-Arg-Glu-His-Glu-Gln-Thr-Gly-
120 110

Leu-Pro-Arg-Ser-Val-Leu-Ser-Asp-Lys-Leu-Ser-Leu-Ala-Ile-Thr-Ser-Leu-Asp-Glu-Asp-Leu-Gln-Arg-Ser-Glu-Leu-Trp-
100 90 80

Asp-Asn-Glu-Lys-Ile-Arg-Arg-Ser-Val-Leu-Ala-Asp-Ala-Leu-Pro-Asn-Leu-Leu-Ile-Asn-Lys-Ile-Gly-Leu-Asp-Thr-Ile-
70 60 50

Ile-Glu-Arg-Val-Pro-Asp-Ser-Tyr-Leu-Arg-Ala-Ile-Phe-Gly-Ser-Tyr-Leu-Ala-Ser-Arg-Phe-Val-Glu-Tyr-Glu-Phe-Gly-
40 30

Ser-Ser-Pro-Ser-Gln-Phe-(Phe,Tyr)-Ala-Asp-Met-Ser-Lys-Arg-Met-Gly-Asn-Ile-Asn-Lys-Glu-COOH
20 10

Fig. 3B. The sequence shown in Fig. 3A continued from residue 237 to the COOH-terminus.

It was, therefore, impossible with this enzyme or the chicken enzyme to determine which tyrosine residue was responsible for the loss of activity.

The sequences in Fig. 1 show that tyrosine-407 modified by tetranitromethane in the bovine and the chicken enzymes is also present in the identical sequence in the human enzyme; however, no corresponding tyrosine residue is present in the sequences of either the NADP-(Fig.2) or the NAD-dependent (Fig. 3) enzymes of Neurospora. This is not unexpected since neither of these two enzymes is allosterically inhibited by GTP.

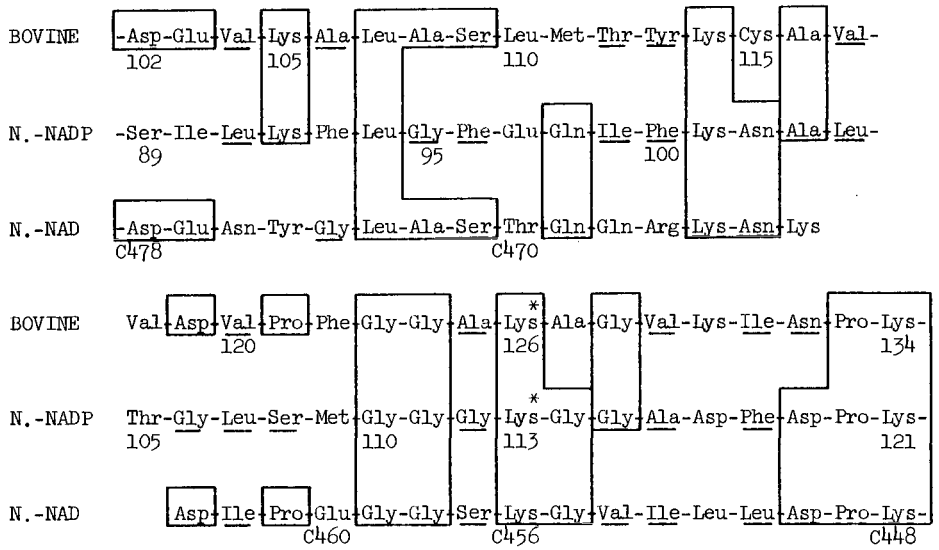

Fig. 4. The sequence around the single reactive lysine residue (*) of the bovine enzyme (which is identical in the human and chicken enzymes) and for the NADP-specific enzyme of Neurospora. For the NAD-enzyme of Neurospora, only the sequence which is shown resembles those of the other enzymes. Boxed residues are identical; underlined residues represent single base changes in the codons. Residues in the NAD-enzyme are identified by the letter C to indicate numbering from the carboxyl end; this is also done in Fig. 6,7,8 and 9.

Treatment of the NADP enzyme of Neurospora with tetranitromethane produces a rapid loss of enzyme activity (Fig. 5) (17). The enzyme could be protected against inactivation by tetranitromethane by the coenzyme NADP, but not by substrates or by NAD (Fig. 5). Interestingly enough, although the enzyme is competitively inhibited by nicotinamide mononucleotide and by 2'-phosphoadenosine-5'-diphosphoribose, both of which show K_i values of the order of 4.5×10^{-4}, only nicotinamide mononucleotide protected the enzyme against inactivation by tetranitromethane (Fig. 5). This indicates that the nitrotyrosine residue formed in the enzyme is presumably present in the area of coenzyme binding, and more specifically, in the region binding the nicotinamide portion of the co-enzyme.

A peptide containing this modified tyrosine, residue-168, was isolated from the NADP enzyme (17); a portion of the sequence around this residue is shown in Fig. 6 in comparison with the previously aligned sequence of the bovine enzyme (8), which was made before the specific inactivation of the NADP enzyme by tetranitromethane was known.

Reactive arginine residues

In recent years various types of diketones or other 1,2-dicarbonyl compounds have been found to inhibit a large variety of enzymes. Presumably all of these reagents react with the guanidine groups of arginine residues. In our laboratory we have studied the conditions for using 1,2-cyclohexanedione to bind reversibly to arginine residues and have determined the nature of the product produced under the conditions where the reaction is reversible, namely, at slightly alkaline pH values (18).

Cyclohexanedione produces complete or partial inhibition of all three types of glutamate dehydrogenases (19,20). With the NADP enzyme of Neurospora, the reaction is reversible and by the time essentially complete inhibition has been produced only two arginine residues have reacted (19). Kinetic analysis showed that one of these arginine residues reacts very much more rapidly than the other. Under conditions where mainly the most reactive arginine residue has been modified by 1,2-cyclo- [1-^{14}C] hexanedione, the labeled peptide was isolated and the tagged residue was identified as arginine-81 (21). Fig. 7 shows the amino acid sequence around this arginine residue and for comparison the homologous regions of the other glutamate dehydrogenases.

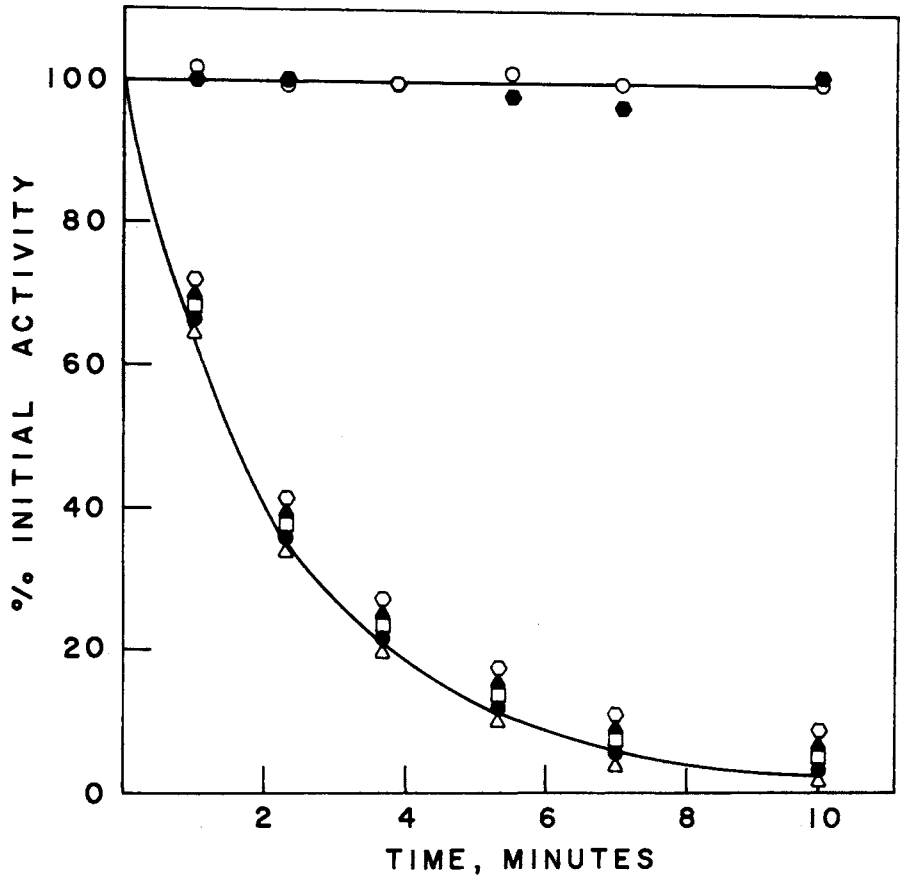

Fig. 5. Effect of various substances on the rate of inactivation of the NADP-specific glutamate dehydrogenase of Neurospora by tetranitromethane. ● no addition, △ α-ketoglutarate, □ NAD(H), ⬡ 2'-phosphoadenosine-5'-diphosphoribose, ▲ N-Methylnicotinamide, ○ NADP(H), ⬢ nicotinamide mononucleotide.

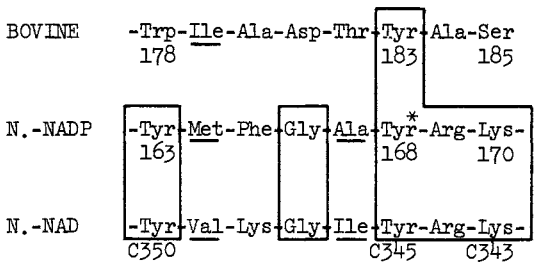

Fig. 6. The sequence around tyrosine-168 (*) of the NADP enzyme of Neurospora. Tyrosine-183 of the bovine enzyme had previously been aligned based on the general homology of the two enzymes. Tyrosine-C345 of the NAD enzyme of Neurospora is in a sequence similar to that of tyrosine-168 of the NADP enzyme. Boxes indicate identical residues; underlined residues indicate single base changes in the codons.

Partial reaction of the NADP-enzyme with cyclohexanedione results in an increase in K_m for coenzyme and only a slight change in the K_m for glutamate (19). Moreover, the inhibitory effect of nicotinamide mononucleotide is lost but not that of 2'-phosphoadenosine-5'-diphosphoribose. Presumably, a possible role of this arginine residue is to bind with one of the phosphates in the pyrophosphate bridge of the coenzyme (19).

Role of cysteine residues

The role of sulfhydryl groups in the different types of glutamate dehydrogenases differs considerably. In the case of the NADP-specific enzyme, all of the sulfhydryl groups are

available for reaction in the native enzyme with either iodoacetate or iodoacetamide (13). Blocking these sulfhydryl groups has no effect on the activity of the enzyme although the stability of the enzyme is decreased. From this finding,it is evident that sulfhydryl groups play no catalytic role in this enzyme.

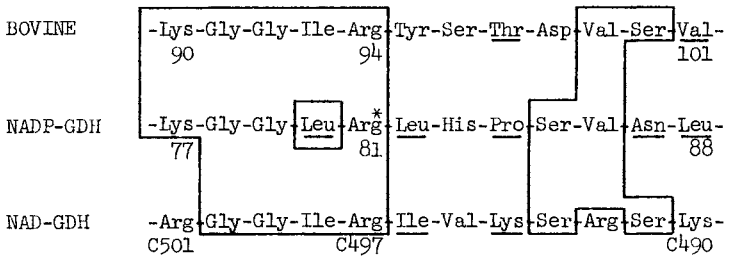

Fig. 7. The sequence around arginine-81 (*) reactive with 1,2-cyclohexanedione in the NADP enzyme, and for comparison, the similar sequences in the other two types of glutamate dehydrogenases.

For the NAD-specific enzyme of Neurospora, the situation is strikingly different (22). With iodoacetate or iodoacetamide there is no reaction whatsoever; however, one sulfhydryl group reacts with a variety of other reagents. Hydroxymercuribenzoate produces almost complete inactivation whereas formation of the cyano derivative has only a slight effect on the activity. Complete reaction with N-ethylmaleimide does not alter V_{max} but the K_m values for both glutamate and NAD are increased more than five fold. These results suggest that modification of the sulfhydryl group by a bulky or a charged group distorts the conformation of the enzyme so that binding with both the coenzyme and the substrate is modified. The sequence around the reactive sulfhydryl, residue-C263, of the NAD enzyme (9,23) is shown in Fig. 8. There is no similar sequence around any of the thiol groups in the NADP enzyme.

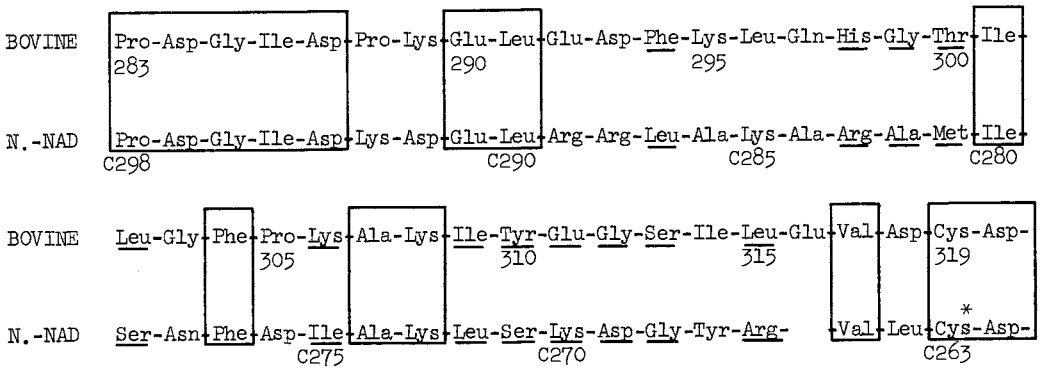

Fig. 8. Comparison of the sequences NH_2-terminal to cysteine-C263 of the NAD enzyme of Neurospora and cysteine-319 of the bovine enzyme.

Comparison of the sequence shown in Fig. 8 with the sequences around the cysteine residues of the vertebrate enzymes indicated that cysteine-319 is in a part of the molecule with some resemblance to the 35-residue sequence NH_2-terminal to cysteine-C263 of the NAD-enzyme of Neurospora (9). Included is the pentapeptide sequence, Pro-Asp-Gly-Ile-Asp, the longest known identical sequence in the two types of enzymes. Reaction of the bovine enzyme with mercurials has been reported to modify selectively cysteine-319 (24) and to affect the binding of the regulatory activator, ADP (25).

RELATIONSHIP OF REACTIVE RESIDUES

It is interesting to compare the relationships of the above-described four types of reactive residues in the various glutamate dehydrogenases, namely, the lysine, tyrosine, arginine and cysteine residues already described. In Fig. 9, there is shown for comparison the relative positions of these residues in the three types of glutamate dehydrogenases (9).

First, it is apparent that the order in which these four residues occurs in the sequences is the same in the three types of enzymes, despite the fact that there is so little overall homology between the NAD enzyme and the other types (9). This finding suggests a distant evolutionary relationship of the NAD enzyme to the others. The NAD enzyme is also distinctive in possessing a tetrameric subunit structure instead of the hexameric structure found for other glutamate dehydrogenases. Second, it is noteworthy that the large polypeptide

chain of the NAD enzyme possesses all of the apparently homologous functional residues in the carboxyl-terminal half of the molecule. Third, inspection of Fig. 9 shows that the numbers of intervening residues between the similar reactive ones are very different among the three types of enzymes. The problem of explaining the origin of such differences, which can be regarded as either large gaps or insertions of many residues, is considered below.

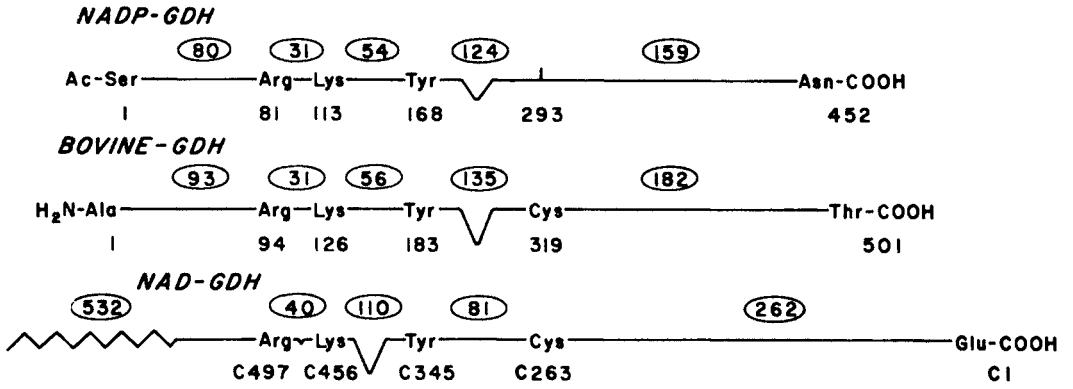

Fig. 9. Schematic representation of the peptide chains of the three types of glutamate dehydrogenases. The presumptively similar functional residues are indicated. Circled numbers above the lines indicate the numbers of residues between each of the identified residues. For the NAD enzyme of Neurospora, residues are numbered from the carboxyl end.

Despite the various differences among the three types of glutamate dehydrogenases, it must be assumed that all are evolutionarily related. In addition to the similarities already mentioned, i.e., the homologies around the reactive residues and the clear homology between the NADP-enzyme of the Neurospora and the vertebrate enzymes, there are two additional points to be noted. First, all of the competitive inhibitors for substrate inhibit all three types, e.g., isophthalate (1); this indicates that the binding site for substrate is similar or identical in all three types of enzymes. Second, present evidence clearly indicates that the coenzyme binding sites in various dehydrogenases possess a similar conformation, as determined by X-ray diffraction analysis (26). Further, it is known that the NAD binding domains for various dehydrogenases may be present in the middle or at either end of the polypeptide chains (26). Although this has not yet been shown for the glutamate dehydrogenases, the known sequences of these enzymes are compatible with this type of conformation.

ORIGIN OF GAPS OR ADDITIONS IN HOMOLOGOUS PROTEINS

Among homologous proteins of closely related species the most common differences are amino acid substitutions generally involving a mutational change in a single base of the triplet nucleotide codon. Such substitutions have now been reported for the amino acid sequences of many homologous proteins, e.g., cytochromes c, hemoglobins, ribonucleases, etc. In general, for a given protein the greater the evolutionary distance of the species from one another, the larger is the number of amino acid substitutions.

Among smaller proteins and even many larger ones, the next most common type of difference is in the addition or deletion of a variable number of residues at either the amino-terminal or the carboxyl-terminal end of the homologous protein. For example among the cytochromes c of vertebrates there are generally 104 residues. However, the cytochromes of both invertebrates and higher plants have additional residues at the amino-terminal ends.

Whether the changes have occurred by addition or deletion of residues, it is relatively easy to visualize that a mutation to form a new chain initiation codon from one specifying an internal residue would decrease the number of residues at the amino-terminal end, whereas a mutation from an initiation codon to a codon specifying an amino acid would result in the synthesis of the polypeptide chain beginning at the next earlier initiation codon. Similarly, one can visualize the opposite situation at the carboxyl terminal end. A change from a chain termination codon to a codon prescribing an amino acid will result in an extension of the peptide chain to the next termination codon. Shortening of the chain at the carboxyl end can result from a mutation in an amino acid codon to a termination codon. Both of these situations have obviously occurred many times, since many homologous proteins are now known that differ in the numbers of residues at the amino- and carboxyl-terminal ends. The known glutamate dehydrogenases of the vertebrates manifest such differences at the NH_2-terminus (Fig. 1). As already noted (Fig. 2), the Neurospora NADP-specific glutamate dehydrogenase is much shorter at both ends than the vertebrate enzymes.

Addition or deletion of residues within a peptide chain poses a different problem. This cannot be accomplished simply by point mutations because elimination of one or more amino

acid residues requires the deletion of three nucleotides or some multiple of three, and addition of one or more residues requires the addition of three nucleotides or some multiple of three. This is obviously essential to conserve the amino acid sequence carboxyl-terminal from the eliminated or added residues in order to produce a viable protein. Many examples are known of homologous proteins that show the elimination or addition of one or a few residues. A few examples may be mentioned. The known sequences of three subtilisins indicate a difference of a single deleted internal residue (27,28). For the glyceraldehyde 3-phosphate dehydrogenases of pig and lobster muscles there is a difference in the chains of only a single residue which has been either added or deleted (29). The penicillinases of Staphylococcus aureus and Bacillus licheniformis show a deletion (or addition) of two residues (30,31). The sequences of chicken egg white lysozyme (129 residues) and bovine α-lactalbumin (123 residues), homologous proteins with rather different functions, can be aligned only by assuming the addition or deletion of one or two residues in various parts of the sequences (32).

Comparison of the sequences of other types of homologous but more distantly related proteins, shows that the number of gaps or additions is greater. Furthermore, the numbers of residues in these gaps or additions are rather substantial in relation to the total sequence, as in the instance of the glutamate dehydrogenases (Fig. 9). This has posed a somewhat embarrassing problem since it has been difficult to understand how such large numbers of nucleotides could be added or deleted in the interior of structural genes without disturbing the remainder of the sequences of the resulting proteins. Studies with prokaryotic organisms and bacteriophages have demonstrated that there is strict colinearity for the sequences of the DNA, the messenger RNA and the protein. Deletion has been explained by a process of pinching off in a loop of DNA three or a multiple of three nucleotides. Deletion or addition has also been assumed to be due to unequal crossing-over giving two DNA segments of unequal lengths. These explanations may indeed be valid in some cases, particularly for prokaryotes, but there is now a simpler and better explanation for such changes in eukaryotic genes.

In 1977 it was reported from several laboratories that the situation for several eukaryotic genes is different. For ovalbumin (33), globin (34), and immunoglobulins (35) as well as for certain viruses of eukaryotes, it has been reported that in both the DNA and, what had been termed heterogeneous nuclear RNA or hnRNA, the coding regions are interrupted by non-coding regions, as shown schematically in Fig. 10. Indeed, it has been reported that the ovalbumin gene contains seven intervening sequences (36). In addition, the coding regions are generally preceded and followed by non-coding regions. It now appears that the DNA is transcribed to an RNA that retains the non-coding regions and, in some manner, this nuclear or nRNA is subsequently processed to remove the non-coding regions; coding regions are then joined to produce a messenger RNA that is translated into the colinear protein sequence (Fig. 10A). We have termed the "intervening sequences" as non-coding, simply for convenience; there is no implication that such sequences may not have other specific functions.

With this information it is now possible to propose a simple mechanism for deletion or addition of a large number of residues within a gene and thus in a protein sequence, by assuming that such changes are due only to point mutations. Such means have been independently proposed by Gilbert (37) and by Tonegawa et al. (35). As yet, the nature of the signals is unknown by which non-coding regions are eliminated to form messenger RNA. Nevertheless, the signals must be in the hnRNA sequences but the number of ribonucleotides or the kind of nucleotide sequence required for processing has yet to be determined. The simplest assumption is that a point mutation can change a signal so that a portion of a coding region can be eliminated in the transcribed RNA (Fig. 10B) or, conversely, that a portion of a non-coding region can be included within the coding region of the protein (Fig. 10C). This assumption furnishes a mechanism which is not only relatively simple but it also assures that all of the RNA sequence which is carboxyl-terminal to the insertion or deletion will be read correctly and in phase without alteration, since the nucleotides in the coding regions will be read in a normal phase relationship. This view offers a relatively simple explanation as to how in the genes of eukaryotes large numbers of residues can be added or deleted in the interior of polypeptide sequences.

If we now examine the types of evolutionary changes that must be explained among the three types of glutamate dehydrogenases, the problems of homology and the relative lengths of the chains do not loom as large as at first glance. The evidence that we have presented suggests that the sequences around the known functional residues have been usually conserved whereas in the intervening and terminal sequences there have been many changes which have altered the properties of the enzymes without destroying the catalytic mechanism, the binding sites for the substrates, glutamate and α-ketoglutarate, or for the coenzyme. We must recall, however, that those enzymes specific for NADP are not inhibited by and do not function with NAD and vice-versa, yet the vertebrate enzymes can function with either coenzyme. Thus, evolutionary changes have occurred in the coenzyme binding site. Moreover, the evolution of allosteric regulation among the vertebrate enzymes has involved the incorporation into the polypeptide sequences the information for two additional specific binding sites: one for the inhibitory effectors, GTP and ATP, and the other for the positive effectors, ADP and GDP; neither of these sites exists in the enzymes of prokaryotes or the eukaryote, Neurospora.

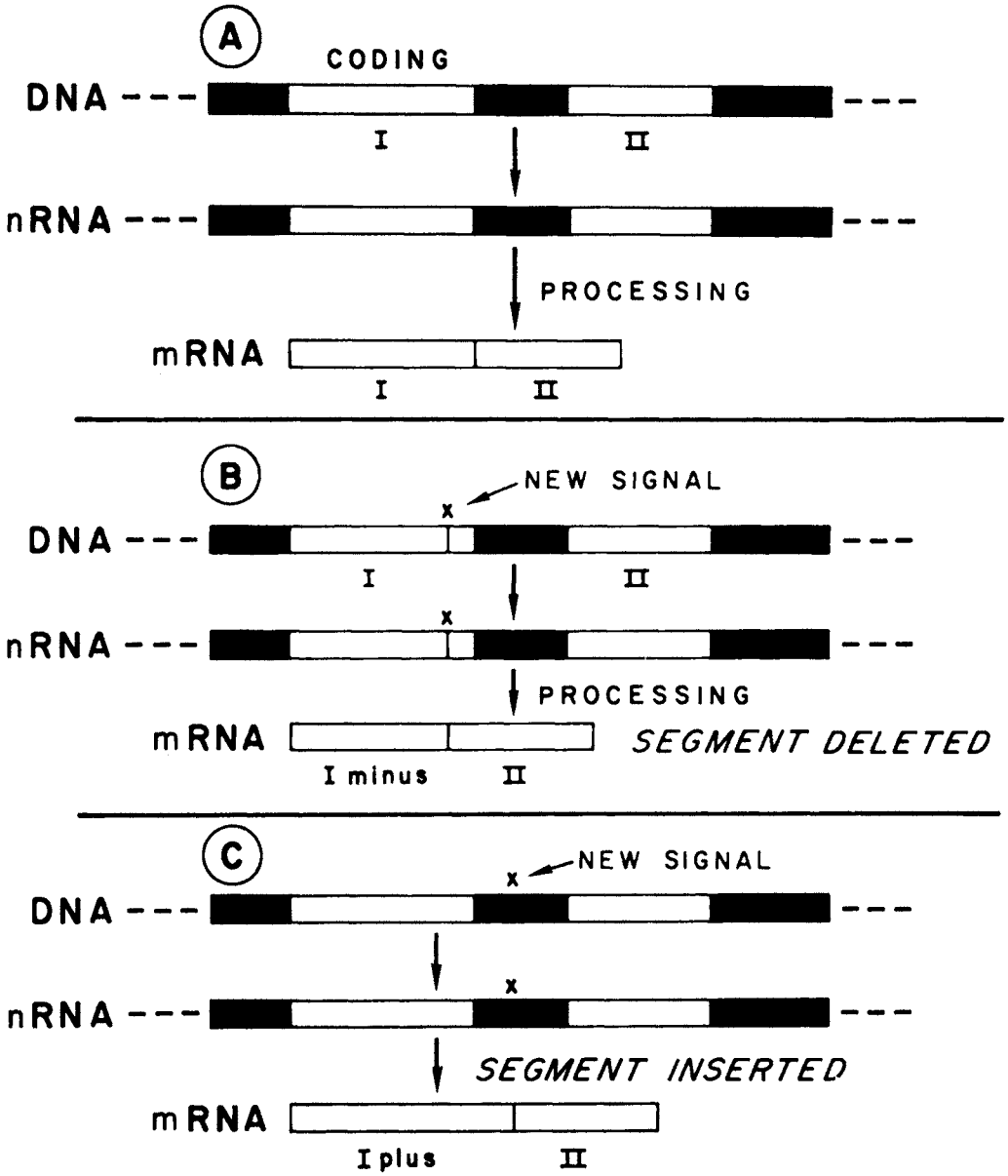

Fig. 10. A. Schematic representation of coding and non-coding regions in a eukaryotic gene, the copy in nRNA and the processing to yield messenger RNA. B. A point mutation (shown as x) to give a new signal that would decrease the size of coding sequence I. C. A point mutation (x) to give a new signal to increase the size of coding region I by incorporating a portion of the adjacent non-coding region.

At the present time, the evolution of the glutamate dehydrogenases is assumed to have proceeded along the following lines. It appears likely that the presently known oldest type is that of the prokaryotes and the NADP-specific enzyme of Neurospora with a six subunit structure and a chain length of approximately 450 residues. This type was presumably the ancestor of the vertebrate enzymes which possess a similar subunit structure but with a longer sequence and the ability to utilize either NADP or NAD. In addition, the capacity for allosteric regulation was developed in the ancestors of contemporary vertebrates. The four subunit NAD enzyme of Neurospora presumably represents a remote gene duplication from a more primitive type since it seems that an additional long segment of approximately 400 residues has been added to the amino-terminal end and a shorter segment to the carboxyl end of each peptide chain, as well as numerous changes elsewhere in the molecule. Furthermore, although induction-repression systems for single enzymes or metabolic pathways are fairly common in prokaryotes, such as E. coli, the development of a coordinate induction-repression system involves a novel type of control mechanism. Presently, this has been found for the glutamate dehydrogenases only in the Ascomycetes -- Neurospora and yeast. A tentative scheme for these evolutionary relationships is shown in Fig. 11.

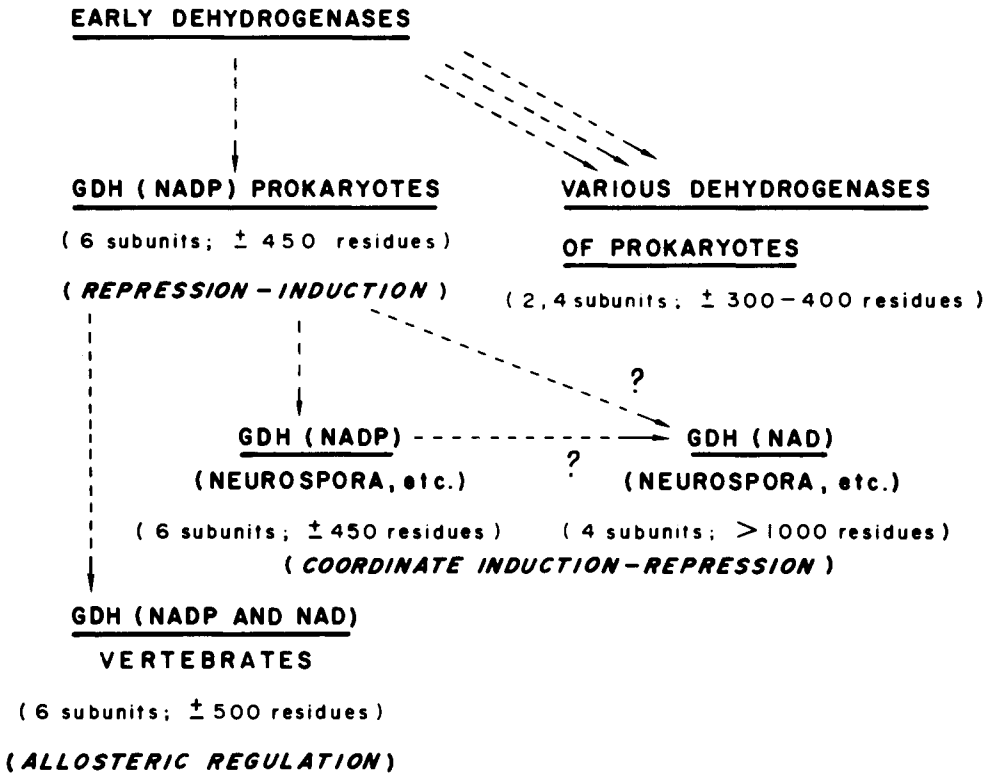

Fig. 11. A schematic representation of the possible course of the evolution of the three known types of glutamate dehydrogenases. Most of the presently well-studied dehydrogenases possess 2 to 4 small subunits of 300 to 400 residues. The similar conformation of the coenzyme binding sites in the known structures of various dehydrogenases suggests a common origin for all of these enzymes (26). It is assumed that the NAD-specific glutamate dehydrogenases with large subunits, more than 1000 residues, have been derived from the genes for the smaller subunit enzymes of similar catalytic properties.

Although the evidence is still fragmentary, it is assumed that interrupted coding regions occur in many, but not necessarily all, eukaryotic genes. (Eukaryotic genes coding for such small proteins as cytochrome c and histone H4 do not manifest insertion or deletion of residues in the sequence.) However, it is assumed that non-coding regions are present in all eukaryotes. If this be so, one can visualize that the presence of non-coding regions, however these may have arisen during the evolution of the eukaryotes, provided two new factors during the evolution of higher organisms. First, as already discussed, a way is provided for incorporating or deleting large segments of protein sequence without altering the essential function of the protein, but also a means for sequence modifications that could assume new functions. Second, non-coding regions, either within the genes or outside of the genes, provide a large reservoir of DNA that can be used not only for the fabrication of considerably modified proteins but also for the synthesis of novel types of new proteins. This aids in explaining the fact that the NAD-binding domains of dehydrogenases of different specificity occur in different positions in the peptide chains. These views may aid in explaining the explosive evolution of the eukaryotes which led to their enormous diversity.

In the past many investigators have assumed, tacitly or explicitly, that the evolution of proteins has occurred mainly by amino acid substitution as a result of point mutations and, of course, by gene duplication followed by additional point mutations. There is no doubt that these processes have contributed to the evolution of new proteins and of new functions. However, it is now recognized that substitution of single amino acid residues is unlikely to have produced the great diversity of new proteins and of new functions that are characteristic of higher organisms. As yet there is insufficient information to visualize completely the potentialities for evolutionary change that are represented by the reservoirs of non-coding regions of DNA. It is evident, however, that we can now begin to reevaluate the processes involved in the molecular evolution of eukaryotes.

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STRUCTURAL AND FUNCTIONAL PROPERTIES OF CYTOCHROME C OXIDASE

A. Azzi, R. Bisson, R. P. Casey,
J. B. Chappell, H. Gutweniger, C. Montecucco
and K. Sigrist-Nelson

Medizinisch-chemisches Institut, Universität Bern, Switzerland and
Center for the Study of Mitochondrial Physiology, University of Padova, Italy

Abstract - Photolabeling techniques have been employed to elucidate some structural characteristics of cytochrome c oxidase. Azido-phospholipids have been useful to investigate which and at what depth of the oxidase subunits interact with lipid fatty acid. It appears that all subunits, except V and VI interact to a certain extent with phospholipid fatty acid chains. Azido cytochrome c derivatives have ascertained the binding site of cytochrome c to be on subunit II of the oxidase. Finally, single and multiple turn-over studies have confirmed that cytochrome oxidase behaves as a proton pump.

INTRODUCTION

Cytochrome c oxidase is a multipeptide enzyme complex which contains heme and copper, and requires lipid for functioning (1). It catalyses the reduction of dioxygen to water and concomitantly it may function as a proton pump, which is able to conserve the free energy liberated in the redox process in the form of an electrochemical proton gradient (2).

The fundamental biological role of cytochrome c oxidase, as terminal component of the mitochondrial respiratory chain and as an energy transducing component, has stimulated intense and widespread efforts directed to the classification of its structural features and its enzymatic mechanisms.

The early studies of Schneider et al. (3) and Eytan et al. (4) have indicated that cytochrome c oxidase is asymmetrically inserted into the membrane, with one subunit (IV, Note a) facing the inside of the mitochondrial compartment, two subunits (I and V) deeply inserted in the lipid phase, and the remaining subunits facing the cytoplasmic side. More recently the studies of Henderson et al. (5) have elegantly confirmed the asymmetric insertion of cytochrome c oxidase in membranes and its gross structural features.

At the same time Ludwig and Capaldi (6) have confirmed that subunits II and III are facing the cytoplasmic side of mitochondria, although because they span the membrane they protrude to a certain extent from the matrix side as well. From the work of Schneider et al. (3) of Eytan et al. (4) Henderson et al. (5) Ludwig and Capaldi (6) and the recent study of Chan and Tracy (7) it is possible to construct a picture of the subunits of cytochrome oxidase, or their portions, deeply inserted in the membrane, from what is not labelled by water soluble reagents or does not react with membrane impermeable antibodies.

The present study was undertaken for direct identification of hydrophobic cytochrome oxidase subunits (or part of a subunit) using lipid soluble labels. The choice was for azidophospholipid derivatives, as the less pertubing non-group-specific hydrophobic labels (8).

Another goal of the present research was to establish with which of the seven subunits of cytochrome oxidase cytochrome c interacts. In this case a cytochrome c-azido derivative was employed as a photoaffinity label for cytochrome oxidase (9).

Note a. The numbering of subunits is made in accordance with Ludwig and Capaldi (6).

The last part of this communication deals with the evidence obtained in favour of cytochrome c oxidase being a proton pump. According to the chemiosmotic hypothesis of Mitchell (10), cytochrome c oxidase forms part of the electron carrying arm of the third loop of his system of alternating hydrogen and electron carriers. Thus, while cytochrome c oxidase may carry out vectorial electron transport, it cannot achieve proton translocation. Wikström, however, has proposed that cytochrome c oxidase is itself a proton pump, i.e. concomitantly with electron transport the enzyme undergoes conformational changes resulting in the extrusion of protons from the mitochondrial interior (2). There is much controversy at present concerning which is the true mode of action of the enzyme.

Most observations of proton translocation by cytochrome c oxidase have been made using mitochondria (11,12) or sub-mitochondrial particles (13) in the presence of various inhibitors, to allow visualisation of proton efflux. This work has received strong criticism (14) and clearly the complexities of these mitochondrial systems make them unsuitable for providing unequivocal evidence for cytochrome c oxidase being a proton pump. A more reliable system for functional studies of cytochrome c oxidase is provided by lipid vesicles prepared in the presence of the purified enzyme. Krab and Wikström (15) have shown recently that ferrocytochrome c and ferrocyanide induced proton extrusion is observable in such reconstituted cytochrome c oxidase vesicles and we have employed this system in our studies.

MATERIALS AND METHODS

Preparation of cytochrome c oxidase

Cytochrome c oxidase was prepared from beef heart according to the method of Yu et al. (16), suspended in 0.25 M sucrose, 0.05 M phosphate buffer, pH 7.4, and stored in small aliquots at -80°. The enzyme had a heme content of 8 to 11 nmol/mg of proton, and a low lipid content determined, respectively, according to Erecinska et al. (17) and Rouser et al. (18). The enzymatic activity, measured spectrophotometrically at 550 nm (19), was 95 nmol of cytochrome c s^{-1} $nmol^{-1}$ heme a.

The detailed preparation of phospholipid I and phospholipid II (Fig. 1), will be described in detail elsewhere (Bisson et al., in preparation). Labeling with ^{14}C of all the three methyl groups of phospholipid I was performed as previously reported by Smith et al. (20). The specific radioactivity was 177 µCi/µmol. The three methyl groups of phospholipid II choline were tritiated and their specific activity was 3.9 Ci/mmol.

$[^{14}C]$methyliodide and $[^{3}H]$methyliodide were purchased from the Radiochemical Center Amersham, U.K. All operations were performed under red light. Vesicles of egg lecithin (0.4 % w/w in 10 mM phosphate buffer, pH 7.4) and mixed vesicles of egg lecithin 80 % and diphosphatidylglycerol (DPG) 20 % (0.4 % w/w in the same buffer) were prepared by ultrasonic dispersion under nitrogen. The visicles contained 1 % of the radioactive azido lecithin. Cytochrome c oxidase (290 µg) was incubated with 150 µl of vesicles at 0° for 1 hour and then irradiated in glass cuvettes at 0° C for thirty minutes with a 100 W ultraviolet lamp. The suspensions were layered into a 10 % sucrose, 10 mM phosphate buffer, pH 7.4 and centrifuged for three hours at 39000 rpm in a SW-39 Beckman rotor. The pellets were analyzed by SDS polyacrylamide gel electrophoresis as previously reported (9). After illumination, the enzymatic activity of cytochrome oxidase remained within acceptable limits (more than 85 % of the control).

After staining with amidoblack and recording of the densitometric trace, the gels were sliced in 1.8 mm thick slices. The slices were finally dispersed in a mixture of Lumasolve (1 ml), Lipololuma (9 ml) and H_2O (0.1 ml), Lumec System AG, Basel) and after overnight incubation at 40° C counted in a Packard Tricarb Liquid Scintillation Spectrometer. Alternatively autoradiographic images were recorded (21) and their optical density scanned.

Interaction of cytochrome c with cytochrome c oxidase

Samples of 5 µM cytochrome c oxidase were dissolved in 0.5 mM EDTA/Tris, pH 6.5, containing 0.1 % Tween 80 in the presence of 12 µM cytochrome c or its derivatives. In a 4-mm thick layer, they were illuminated by a

100-W ultraviolet lamp shielded with a glass filter for 45 min at 0°, at a distance of 10 mm while being stirred. After illumination, the samples (2 ml) were passed rapidly through a Pasteur pipette (1 x 80 mm) filled with Amberlite CG-50 equilibrated with 50 mM ammonium acetate, pH 7.2. The eluate was lyophilized, dissolved in a small volume of the incubation buffer, and used for spectral analysis and gel electrophoresis.

Electrophoresis
The protein content of the samples was 7 mg/ml and fractions of 20 µl were applied to the gel. Dissociation of the protein complex was obtained by treatment with 25 mM Tris/acetate, pH 8.2, containing 3 % sodium dodecyl sulfate, 1.5 % β-mercaptoethanol, and 2 mM ethylenediaminetetraacetate.

Electrophoresis was carried out in a 1-mm thick gel (100 x 140 mm) made from 12 % acrylamide and 0.32 % bisacrylamide, 0.15 % N,N,N', N'-tetramethylethylenediamine (v/v), 0.7 % sodium dodecyl sulfate, and 10 mM Tris/acetate, pH 8.2, polymerized by addition of 0.03 % persulfate. 40 mA were applied for 5 h in a medium containing 0.05 M Tris/acetate, pH 8.2, and 0.5 % sodium dodecyl sulfate. The gels were fixed and stained in a solution containing 45 % methanol, 10 % acetic acid, 1 % Amido black 10B, and destained electrically. Under these conditions, a complete separation of Bands II and III of the oxidase was achieved, as tested by two-dimensional electrophoresis using the method of Downer et al. (22).

Cytochrome c, myoglobin, chymotrypsin, and aldolase were used as protein standards. Unstained gels were fixed in a solution of 45 % methanol, 10 % acetic acid. Stained and unstained gels were analyzed in a Hitachi-Perkin-Elmer MPF-2 spectrophotofluorimeter with a scanning attachment.

Preparation and utilization of reconstituted cytochrome c oxidase vesicles
Asolectin was added to a solution containing 100 mM Hepes and 10 mg/ml cholic acid adjusted to pH 7.0 with 1 M-KOH, to give a concentration of 40 mg/ml. This suspension was sonicated and cytochrome c oxidase added to a concentration of 15 µM on a heme basis. The resulting mixture was dialysed for 4 hours versus 100 volumes of 0.1 M Hepes/30 mM K^+, then for a further 4 hours versus 200 volumes of 10 mM Hepes/3 mM K^+, 27 mM KCl, 73 mM sucrose than for 12 hours versus 200 volumes of 1 mM Hepes/0.3 mM K^+, 30 mM KCl, 79 mM sucrose. The procedure was carried out at 4° C.

Charge separation occurring on reduction of O_2 via cytochrome c oxidase in these vesicles would produce a large transmembrane electrical potential. This would rapidly force any extruded protons back into the vesicles. Thus, in order that proton expulsion could be visualised, valinomycin and external K^+ were always present in our experiments, to allow collapse of this charge differential. Changes in the pH of the extravesicular medium were detected by monitoring the absorbance of phenol red. Whilst similar results were obtained with a pH electrode, its response was slower than that of the indicator dye. Consequently, the latter was used to provide a more accurate indication of the kinetics of proton extrusion.

RESULTS AND DISCUSSION

Labeling of cytochrome c oxidase with azidophospholipids
The structural formula of the azidophospholipid employed in this study are shown in Fig. 1. The two molecules were synthesized with the azidoreactive group at two different distances from the polar head group of the phospholipid, 19 and 5 Å respectively. Such molecules, similarly to nitroxide phospholipids, should have the azido group penetrating to different depths when inserted in a membrane bilayer. They should be able, after their activation to nitrene, under illumination, to crosslink with proteins enbedded at different depths in the membrane. It is clear that the values of 19 and 5 Å are maximum depths, due to the inherent flexibility of phospholipid fatty acid residues.

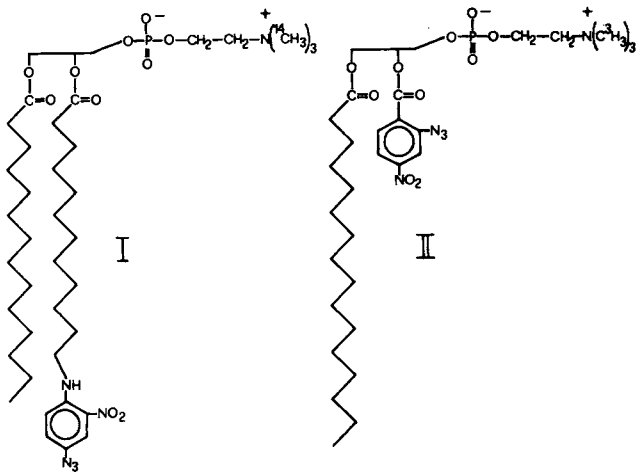

Fig. 1. Structure formulae of azido phospholipid derivatives.

The illumination of cytochrome c oxidase in the presence of lecithin containing 1 % radioactive azidophospholipid I or II results in a covalent association of radioactivity to the protein, as indicated by the fact that neither ammonium sulfate precipitation or acetone washing could extract all the radioactivity. The polyacrylamide - SDS gel electrophoretic pattern (Fig. 2) of the enzyme after illumination is characteristic of cytochrome c oxidase with seven distinct components of different molecular weights. The gel can be cut and analyzed for radioactivity by

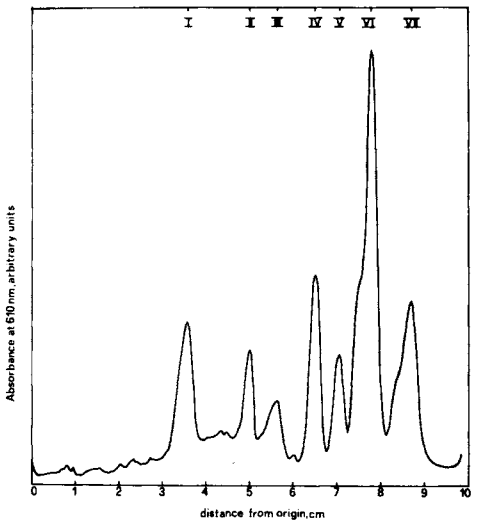

Fig. 2. Electrophoretic analysis of cytochrome c oxidase in polyacrylamide-sodium dodecylsulfate gels. Experimental details are given in Materials and Methods.

scintillation counting or by autoradiography. In the latter case the autoradiogram is scanned and its profile compared with that of the protein distribution pattern (Fig. 3). The most evident and immediate conclusion is that all subunits, except V are labeled by azido phospholipid derivatives, and, therefore, are inserted at least with some segment into the phospholipid bilayer. Subunit V is most probably not inserted in the bilayer.

Subunit VI is only apparently highly labeled. Control experiments using highly cross-linked-urea gels (22) have shown that the label is not precisely associated with subunit VI, but only with a minor component (component a), normally present, but not essential for activity in cytochrome c oxidase. There is general agreement between the data from the labeling of the oxidase with hydrophilic probes and the present ones obtained directly with hydrophobic probes.

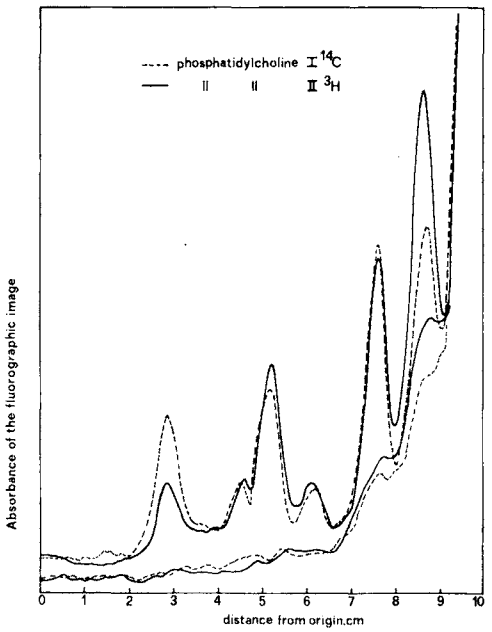

Fig. 3. Distribution of the radioactivity of phospholipids I and II in cytochrome oxidase subunits. Gel electrophoresis and autoradiographies were obtained as described in Materials and Methods and text.

Although it is not possible to obtain quantitative data on the depth which different proteins reach in the membrane, the differences in labeling with the two probes suggest that subunit I is inserted more into the membrane than at its surface and the opposite being true for subunit VII. The other subunits are probably traversing the membrane (Fig. 4).

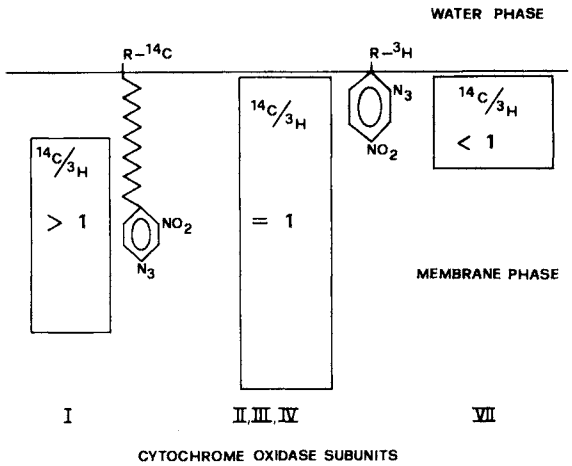

Fig. 4. Scheme of the penetration of the different subunits of cytochrome oxidase in the membrane.

Labeling of cytochrome c oxidase with azido-cytochrome c derivatives

The easy reaction of cytochrome c ε-amino groups with fluoronitrophenyl azide allows the preparation of mono-nitrophenyl derivatives of cytochrome c with a large number of lysine residues. Two of these, lysine 22- and 13-cytochrome c derivatives have been employed to label cytochrome c oxidase. In fact lysine 13 is located in the surface portion of cytochrome c which interacts with the oxidase, while lysine 22 is not involved in such an interaction. When a lysine 22 cytochrome c derivative (22-NAP-cytochrome c) or a lysine 13 (13-NAP-cytochrome c) is incubated with cytochrome c oxidase and then illuminated, the latter derivative but not the former one establishes a covalent interaction with the oxidase (Fig. 5). Most of the cytochrome c can in fact be removed as a free component after amberlite CG-50 chromatography of the 22-NAP-cytochrome c-cytochrome oxidase illumination product, but a large amount remains bound in the case of the 13-NAP-cytochrome c-cytochrome oxidase illumination product.

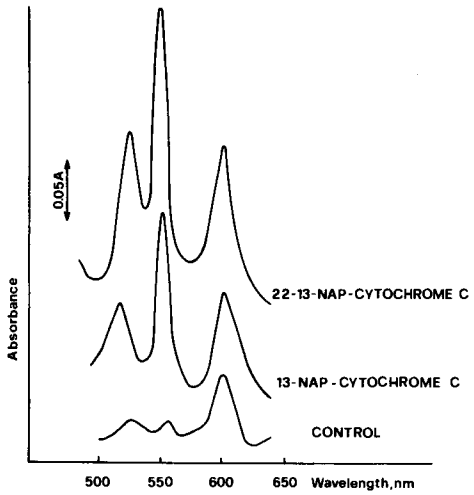

Fig. 5. Binding of azidocytochrome c derivatives to cytochrome c oxidase. 13-NAP and 22-13-NAP-cytochrome c are the nitro-azylazido derivatives of cytochrome c at the indicated lysyl residue. Binding of cytochrome c derivatives and assays are described in the text.

In Fig. 6, the electrophoretic analysis of samples of oxidase illuminated in the presence of 22-NAP-cytochrome c or 13-NAP-cytochrome c is shown. The pattern of cytochrome oxidase polypeptides for the 22-NAP-derivative is identical to that of native cytochrome c oxidase. The pattern of the 13-NAP-derivative-treated oxidase lacks of one subunit, namely subunit II and has a more pronounced subunit I. This pattern is consistent with the crosslinking of 13-NAP-cytochrome c with subunit II of cytochrome oxidase.

Such a polypeptide of molecular weight 24,000 daltons after crosslinking with the 12,000 dalton cytochrome c disappears as band II and migrates as band I of molecular weight 36,000 daltons. The supposition is confirmed by the heme c absorbance in coincidence with the 36,000 molecular weight peptide (Fig. 6, dotted traces).

Thus subunit II of cytochrome c oxidase (both of yeast and beef) is most probably the site of binding (or part of it) for cytochrome c. It may be that the first steps of the electron transfer reaction to cytochrome c oxidase occur at this level.

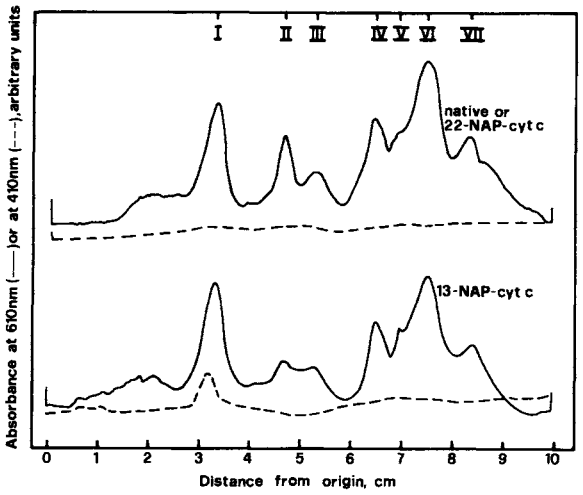

Fig. 6. Electrophoretic analysis of cytochrome c oxidase after reaction with azido cytochrome c derivatives. Details of the experiment are given in the text.

Determination of the H^+/e stoichiometry of cytochrome c oxidase associated pump

In Fig. 7 (upper traces) is shown the effect of adding ferrocytochrome c to a suspension of cytochrome c oxidase vesicles. There is an acidification of the medium and parallel experiments showed that this is concomitant with oxidation of ferrocytochrome c (data not shown). The broken trace shows that when sufficient ferrocytochrome c is added for one turnover of the oxidase, a fairly stable acidification occurs with a stoichiometry of 0.9 H^+ extruded

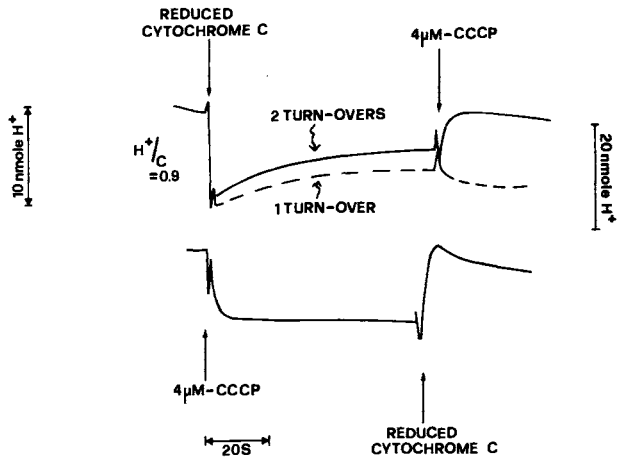

Fig. 7. Changes in pH associated with reduction of cytochrome oxidase by ferrocytochrome c, in coupled and uncoupled vesicles.

per ferrocytochrome c oxidised. When sufficient ferrocytochrome c was added for 2 turnovers of the enzyme, however (solid trace), a slightly lower stoichiometry is obtained and there is a more rapid decay of the acidification. This behaviour is explained as follows. The contribution of the potential difference to the electrochemical proton gradient is low and thus the main force driving extruded protons back into the vesicles is the pH

difference. The consumption of protons in the reduction of O_2 occurs inside the vesicles. The volume of the vesicle interior is however very small. Thus the internal pH change provides the main contribution to the pH difference. With one turnover of the oxidase, the relatively high internal buffering capacity keeps the internal pH change sufficiently low so that there is only a slow back-flux of extruded protons. With two turnovers, however, the internal buffering capacity is exceeded and an accelerated back-flux of protons results.

When the uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) is present in a suspension of cytochrome c oxidase vesicles, ferrocytochrome c does not induce an acidification of the medium (Fig. 7, lower trace). Instead an alkalinisation occurs with a stoichiometry of 1 H^+ consumed per ferrocytochrome c oxidase according to the equation.

$$4 \text{ ferrocytochrome c} + 4\ H^+ + O_2 \rightarrow 2\ H_2O + 4 \text{ ferricytochrome c}$$

This behaviour is to be expected if a proton pump were operating. The high proton permeability of the membrane in the presence of CCCP allows proton reentry to occur so rapidly that the extruded protons are not detected and proton consumption is seen immediately.

In the experiments of Fig. 7 appearance of protons in the external medium and consumptions of protons in the reduction of O_2 are detected separately. If cytochrome c oxidase is indeed a proton pump then these processes should occur concomitantly. Provided that a very low concentration of CCCP ($\sim$ 20 nM) is present, then it is indeed possible in a single experiment to detect both the ferrocytochrome c induced proton extrusion and the subsequent alkalinisation of the medium. Using this system the effect of varying the amount of added ferrocytochrome c on the above pH changes was investigated (Table). The number of protons consumed per ferrocytochrome c

H^+- AND OH^--CHANGES ON ADDITION OF REDUCED CYTOCHROME C TO CYTOCHROME OXIDASE VESICLES

(CONDITIONS: 1.46 NMOLE OF CYTOCHROME OXIDASE INCORPORATED IN 8 MG OF PHOSPHOLIPID AND SUSPENDED IN 2.5 ML OF A SOLUTION CONTAINING 0.066M-CHOLINE CL, 0.027M-KCL AND 50µM-PHENOL RED. THE PH WAS ADJUSTED TO 7.4 WITH NAOH. TEMPERATURE, 15° C. IMMEDIATELY PRIOR TO THE ADDITION OF VARYING AMOUNTS OF REDUCED CYTOCHROME C, 20NM-CCCP AND 1.6 µM-VALINOMYCIN WAS ADDED TO THE SUSPENSION).

REDOX EQUIVALENT OF CYTOCHROME OXIDASE WAS 1.46 x 4 = 5.84 NMOLE

REDUCED CYTOCHROME C ADDED (NMOLE)	NUMBER OF TURNOVERS	H^+-EJECTED (NMOLE)	H^+/REDUCED CYTOCHROME	OH^--PRODUCED (NMOLE)	OH^-/REDUCED CYTOCHROME C
5.85	1	4.8	0.82	5.8	0.99
11.7	2	8.8	0.75	11.5	0.98
17.6	3	11.1	0.63	17.7	1.01
23.4	4	14.5	0.61	23.2	0.99

oxidised is approximately 1 regardless of the amount of ferrocytochrome c added. The ratio of H^+ extruded to ferrocytochrome c oxidised, however, decreases with increasing number of turnovers of the oxidase. This can again be explained by the larger proton gradient induced on increasing turnovers of the cytochrome oxidase accelerating the back-flux of extruded protons such that less are detected. This would lead to an underestimation of the true stoichiometry and it is noteworthy that extrapolation of H^+ ejected/cytochrome c to zero turnovers (and thus zero electrochemical H^+ gradient) indicates a stoichiometry of 0.9. It is possible that there is a further slight underestimation of the true stoichiometry owing to the presence of unincorporated cytochrome c oxidase in the suspension. It is thus, reasonable to conclude that 1 proton is expelled per ferrocytochrome c oxidised. The possibility of errors in the measured stoichiometries due to misorientation of the oxidase are excluded by appropriate control experiments.

The above data provide a strong indication that cytochrome c oxidase is indeed a proton pump. The oxidation of 4 molecules of ferrocytochrome c in the reduction of O_2 results in the consumption of 4 H^+ from the vesicular interior and the translocation of a further 4 H^+ which appear in the external medium.

Conclusions

The present study on some structural and functional characteristics of cytochrome c oxidase has given information on the features of its interaction with cytochrome c. Subunit II appears to be cytochrome c binding site for mammalian and yeast cytochrome oxidase. It has also been shown that almost all the subunits of cytochrome oxidase have interactions with the hydrophobic phase of the membrane. Subunits I and VII are located closer to the center and the water interface, respectively.

Finally cytochrome c oxidase has been shown to be a proton pump. The stoichiometry of the protons translocated per electron equivalent which reduces oxygen is 1.

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GLYCOSYL TRANSFERASES IN OLIGOSACCHARIDE BIOSYNTHESIS AND THEIR USE IN STRUCTURE - FUNCTION ANALYSIS OF GLYCOPROTEINS

R. L. Hill, T. A. Beyer, J. C. Paulson,
J-P. Prieels, J. I. Rearick and J. Evan Sadler

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710, USA

Abstract - Eleven different glycosyl transferases have been highly purified, or purified to homogeneity, and each has been found to have a very strict acceptor substrate specificity, in accord with the hypothesis that one enzyme is required for the synthesis of each type of disaccharide sequence found in the oligosaccharides of glycoconjugates. Several of the transferases act reciprocally, that is, each have the same acceptor substrate, but the product of one transferase may be a poor acceptor for another transferase, or not an acceptor at all. The combined actions of the transferases not only indicate the types of oligosaccharide structures permitted in animal oligosaccharides, but also give some insight into the basis of the structural heterogeneity observed in many oligosaccharides. Moreover, it is also possible to predict the preferred pathways of biosynthesis of certain oligosaccharides.

The pure glycosyl transferases have been shown to be very useful reagents for the structural analysis of oligosaccharides and for the assessment of the structure-function relationships of glycoconjugates. Examples of their use for this purpose include studies on the rabbit hepatic galactose binding protein, α_1-acid glycoprotein, human blood coagulation factor VIII and a heretofore unrecognized oligosaccharide binding activity of hepatocytes that binds specifically certain fucose containing ligands. The structure-function relationships of cell surface oligosaccharides can also be assessed with the aid of the transferases, as illustrated by studies on the role of sialic acid in expression of erythrocyte MN antigens and in viral adherence to erythrocytes.

INTRODUCTION

The increasing interest over the past ten years in the structures, functions and biosynthetic pathways of animal glycoproteins and glycolipids is the result of the growing body of evidence suggesting that these glycoconjugates are involved in a variety of cellular processes (1). The oligosaccharides of cell surface glycoconjugates have been suggested to be involved in such diverse phenomena as cellular adhesion, fusion, differentiation, and transformation, viral and bacterial adherence to host cells, fertilization, localization of glycoproteins on subcellular organelles, the clearance from blood of plasma glycoproteins, and a variety of reactions of the immune system. The molecules that control these complex processes must have the potential for much informational content. Such potential is available in animal cell oligosaccharides, since the 10 monosaccharides known with certainty as components of glycoconjugates can, in theory, give rise to 700 different disaccharides, each differing only in the position and the anomeric configuration of the glycosidic linkage (2). Thus, the potential for structural variability of yet larger oligosaccharides is enormous, and well within that required for directing the many cellular processes in which they have been implicated. But in spite of the compelling evidence that oligosaccharides are involved in these cellular phenomena, molecular explanations for their actions are, at present, totally lacking.

Of the several different experimental approaches currently used to study the structure-function relationships of the oligosaccharides at a molecular level, at least three different, although related approaches, seem minimally required. First, and of primary importance, is the isolation and characterization of different glycoconjugates, including the determination of their complete structures. The almost unlimited number of structures that the ten monosaccharides in glycoconjugates can form is obviously not realized in nature, and many different glycoconjugates are observed to contain identical or closely related structures. Yet new types of structures are reported each year, and it is only from a precise knowledge of these structures that a full understanding of their biological functions will be obtained. Second, is the detailed characterization of the enzymes involved in the biosynthesis and the degradation of oligosaccharides. Understanding how glycosyl transferases act in concert to

synthesize specific oligosaccharides, and how glycosidases degrade these structures, provides useful insight into the origins of different oligosaccharide structures as well as the structural heterogeneity often observed in glycoconjugates. Perhaps equally important is that well-characterized transferases and glycosidases will prove to be valuable additions to the presently meager armamentarium of reagents that can be used to specifically modify oligosaccharides when attempting to assess their biological functions. Third, is the isolation and characterization of the specific receptors on animal cell surfaces, or on the surfaces of subcellular organelles, whose binding of a specific ligand is carbohydrate mediated. Only in the past decade have improvements in biochemical methodologies allowed a molecular description of cell surface receptors, including several which bind specifically to glycoconjugates through a monosaccharide at the non-reducing terminus of their oligosaccharide chains. These receptors have proven to be membrane glycoproteins themselves, and are possibly the animal counterparts of plant lectins, if not in function at least in their specificity for binding sugars. Their unique binding specificities certainly suggest that they may well be involved in any number of cellular processes. With detailed knowledge of the structures of cell surface oligosaccharides and the ability to specifically modify these structures on intact cells, a more precise description of the biological functions of glycoconjugates in a variety of cellular phenomena can be ascertained. There is a vast literature concerning the effects of glycosidases or plant lectins on the behavior of cells, but until it is understood how specific cell surface glycoconjugates are affected by such treatments, it is unlikely that detailed molecular explanations of the roles of oligosaccharides in various cellular processes will be forthcoming.

We wish to summarize here recent studies from our laboratory on the enzymic properties of several glycosyl transferases that have been obtained in homogeneous, or near homogeneous form. The remarkably strict substrate acceptor specificities of these transferases reflect the high degree of specificity in the carbohydrate-protein interactions in animal cells, and are similar to, and perhaps more specific, than those observed by currently known animal oligosaccharide binding proteins (lectins). Studies are also summarized illustrating the use of these transferases to modify glycoproteins and human erythrocytes. From these studies it has been possible to identify a heretofore unrecognized lectin activity in liver and to understand more clearly the roles of sialic acids in erythrocyte membranes in the expression of the MN blood group system, and in the adsorption of certain myxoviruses to erythrocytes.

PURIFICATION AND SOME ENZYMATIC PROPERTIES OF HIGHLY PURIFIED GLYCOSYL TRANSFERASES

A large number of glycosyl transferase activities have been reported since the first enzyme of this type from animal cells, glycogen synthase, was described in 1957 (3). Each catalyzes a reaction of the following general form,

$$\text{Glycosyl}_A\text{-nucleotide} + \text{Glycose}_B \rightarrow \text{Glycosyl}_A\text{-glycose}_B + \text{Nucleotide} \quad (1)$$

Most of the donor substrates are nucleoside diphospho sugars; the exception is cytidine monophosphosialic acid for the sialyl transferases. Considerable insight into the substrate requirements of the transferases was first obtained by examination of preparations purified only a few fold from tissue homogenates (4). From these studies it was concluded that a different transferase is required for synthesis of each type of glycosyl-glycose linkage (2), a concept called the "one linkage-one enzyme" hypothesis.

In the past seven years, eleven different glycosyl transferases involved in the synthesis of the oligosaccharides in glycoconjugates have been highly purified or purified to homogeneity (Tables 1 and 2). Purification of these enzymes required the use of specific affinity chromatographic methods, since each of the enzymes is present in extremely small amounts in tissues, and except for the enzymes from serum, colostrum and milk, requires solubilization from membranes with detergents. Each enzyme transfers a single sugar from its donor substrate to a preformed oligosaccharide acceptor in a glycoprotein, except for the chondroitin sulfate core-protein xylosyl transferase, which transfers xylose to the hydroxyl group of serine. The substrate acceptor specificities of each pure transferase are also listed in Tables 1 and 2. In general, the observed specificities support the one enzyme-one linkage hypothesis, first proposed on the basis of studies with very impure enzymes. Although other acceptors may be used by these transferases, they are usually < 1% as effective as those listed. The glycose$_B$ residue (reaction 1) must often be in a specific glycosidic linkage with another specific sugar residue. For example, two sialyl transferases transfer sialic acid to a galactose residue, but for one, the β1,3 galactoside α2,3 sialyl transferase, the galactose must be linked β1,3 to N-acetylgalactosamine whereas for the other, the β1,4 galactoside α2,6 sialyl transferase, sialic acid is transferred only to galactosyl β1,4 N-acetylglucosamine containing structures. Moreover, the best acceptor for each of the sialyl transferases is very ineffective as an acceptor for the other transferase. Often, the best acceptors for a transferase require additional structures, for example, the β1,4 galactoside α2,6 sialyl transferase appears to transfer optimally to acceptors with the sequence Galβ1,4-

TABLE 1. THE SUBSTRATE ACCEPTOR SPECIFICITIES FOR SOME GLYCOSYLTRANSFERASES

GLYCOSYLTRANSFERASE	SUBSTRATES		PRODUCT
	DONOR	ACCEPTOR	
N-ACETYLGALACTOSAMINIDE α2,6 SIALYL (PURE FROM PORCINE SUBMAXILLARY GLANDS)	CMP-SIA	GALNACαSER/THR	SIAα2,6GALNACαSER/THR
β1,3 GALACTOSIDE α2,3 SIALYL (PURE FROM PORCINE SUBMAXILLARY GLANDS, MW 50,000)	CMP-SIA	GALβ1,3GALNACαSER/THR	SIAα2,3GALβ1,3GALNACαSER/THR
β1,4 GALACTOSIDE α2,6 SIALYL (PURE FROM BOVINE COLOSTRUM, MW 43,000 AND 50,000)	CMP-SIA	GALβ1,4GLCNACβ1,2MAN	SIAα2,6GALβ1,4GLCNACβ1,2MAN
β-GALACTOSIDE α1,2 FUCOSYL (PURE FROM PORCINE SUBMAXILLARY GLANDS, MW 55,000 AND 59,000, Mn^{2+} ACTIVATED)	GDP-FUC	GALβ-	FUCα1,2GALβ-
N-ACETYLGLUCOSAMINIDE α1,3 FUCOSYL (135,000 FOLD PURIFIED FROM HUMAN MILK, Mn^{2+} ACTIVATED)	GDP-FUC	GALβ1,4GLCNACβ-	GALβ1,4 / FUCα1,3 > GLCNACβ-

TABLE 2. THE SUBSTRATE ACCEPTOR SPECIFICITIES FOR SOME GLYCOSYLTRANSFERASES

GLYCOSYLTRANSFERASE	SUBSTRATES		PRODUCT
	DONOR	ACCEPTOR	
α1,2 FUCOSYL β-GALACTOSIDE α1,4 N-ACETYLGALACTOSAMINIDE (PURE FROM PORCINE SUBMAXILLARY GLANDS, MW 100,000, Mn^{2+} DEPENDENT)	UDP-GALNAC	FUCα1,2GALβ-	FUCα1,2 / GALNACα1,3 > GALβ-
α1,2 FUCOSYL β-GALACTOSIDE α1,3 GALACTOSYL TRANSFERASE (PURE FORM HUMAN SERA)	UDP-GAL	FUCα1,2GALβ-	FUCα1,2 / GALα1,3 > GALβ-
N-ACETYLGLUCOSAMINIDE β1,4 GALACTOSYL (PURE FROM BOVINE MILK, MW 50,000 - 60,000, Mn^{2+} DEPENDENT)	UDP-GAL	GLCNACβ1,2MANβ1,3 / GLCNACβ1,2MANβ1,6 > MAN-	GALβ1,4GLCNACβ1,2MANβ1,3 / GLCNACβ1,2MANβ1,6 > MAN-
α-MANNOSIDE β1,2 N-ACETYL-GLUCOSAMINYL (400 FOLD PURIFIED FROM RABBIT LIVER, Mn^{2+} DEPENDENT)	UDP-GLCNAC	MAN / MAN > MANα1,6 / MANα1,3 > MANβ1,4-	MAN / MAN > MANα1,6 / GLCNACβ1,2MANα1,3 > MANβ1,4-
CHONDROITIN SULFATE CORE PROTEIN XYLOSYL	UDP-XYL	HO-SER	XYL-O-SER
β-GALACTOSYL COLLAGEN α1,2 GLUCOSYL (PURE FROM CHICK EMBRYOS, MW 72,000 - 78,000)	UDP-GLC	GAL-O-β-HYL	GLCα1,2GAL-O-β-HYL

GlcNAcβ1,2Man, rather than to similar structures in the sequence Galβ1,4GlcNAc, β1,4 or β1,6Man (12). A similar specificity may be shown by the N-acetylglucosaminide β1,4 galactosyl transferase, which readily incorporates galactose into β1,4 linkage with one of the two N-acetylglucosaminyl groups in a biantennary acceptor, such as found in transferrin (Table 2), but the rate of addition of the second galactose residue is much slower (20). Kinetic studies indicate that the K_m of the galactosyl transferase for the monogalactosylated transferrin acceptor is about 10 times higher than that for the acceptor without any galactose.

Although each of the transferases shown in Tables 1 and 2 is involved in a single step during the biosynthesis of either O- or N-linked oligosaccharides in glycoproteins, the β1,3 galactoside α2,3 sialyl transferase, and the α1,2 fucosyl β-galactoside α1,3 N-acetylgalactosaminyl transferase, also effectively use glycolipid acceptors containing oligosaccharide structures that are identical to those found in specific glycoprotein acceptors. Thus it appears that some transferases may act with the same specificity on oligosaccharides of both glycoproteins and glycolipids. To date, none of the transferases that utilize only glycolipid acceptors has been purified to homogeneity, including those involved in the synthesis of the dolichol phospho-oligosaccharides, whose oligosaccharide groups are transferred en bloc to asparaginyl groups in glycoproteins (23).

Some purified glycosyl transferases have been useful in elucidating the biosynthetic relationships that determine the oligosaccharide structures of glycoconjugates. For example, an α-mannoside β1,2 N-acetylglucosaminyl transferase, which has been purified over 400 fold from rabbit liver, appears to be involved in the processing of the oligosaccharides in N-linked glycoproteins (24). In Chinese hamster ovary cells in culture, this transferase catalyzes the specific incorporation of N-acetylglucosamine into a pentamannoside intermediate as shown in Table 2. Only then can a mannosidase in ovary cells hydrolyze the two terminal mannose residues and the terminal Siaα2,6Galβ1,4GlcNAcβ1,2 be added to complete the structure of the N-linked oligosaccharides (see Note a).

THE RECIPROCAL ACTIONS OF GLYCOSYL TRANSFERASES

Several of the transferases act reciprocally, that is, each have identical acceptors, but the product of one transferase is often a much poorer acceptor for another transferase, or not an acceptor at all. Fig. 1 shows the biosynthetic interactions of three transferases, the β1,4 galactoside α2,6 sialyl transferase (I), the N-acetylglucosaminide α1,3 fucosyl transferase (II) and the β-galactoside α1,2 fucosyl transferase (III).

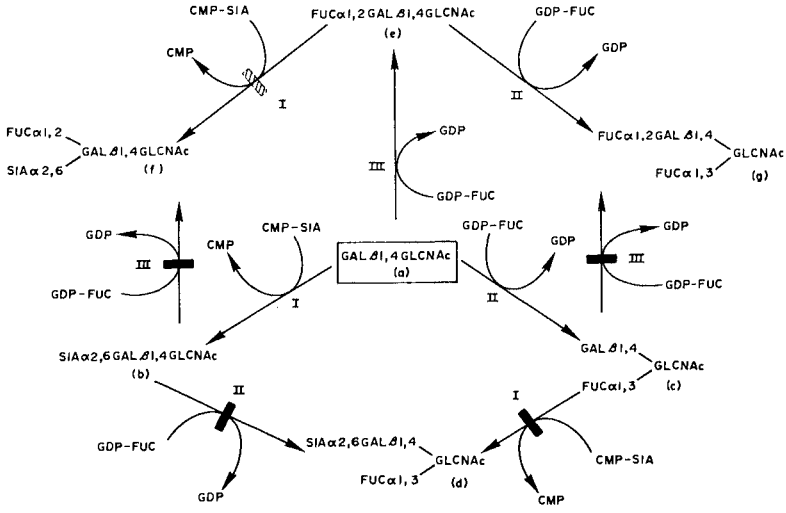


Fig. 1. The proposed biosynthetic pathway for the synthesis of oligosaccharides formed by the combined actions of I, β-galactoside α2,6 sialyl transferase; II, N-acetylglucosaminide α1,3 fucosyl transferase and III, β-galactoside α1,2 fucosyl transferase. The solid bars indicate that the reaction as written does not proceed, and the hashed bars indicate that the reaction indicated proceeds very slowly. The letters (a) through (g) refer to the different structures shown.

Each of these enzymes can use as an acceptor substrate the Galβ1,4GlcNAcβ1,2Man sequence at the non-reducing end of the two oligosaccharide groups of asialo-transferrin (Table 1). Furthermore, the products formed by the action of one transferase are potentially acceptors for the other two transferases, and the product formed by the combined action of any two of the transferases is potentially an acceptor substrate for the third transferase. The products formed by combinations of these transferases could be synthesized by alternate paths, but studies with the purified enzymes demonstrate that some of the pathways diagrammed in Fig. 1 are effectively forbidden. For example, the sialyl transferase I cannot employ Galβ1,4[α1,3Fuc]GlcNAc as an acceptor, nor can fucosyl transferase II use NeuAcα2,6Galβ1,4-GlcNAc as an acceptor (12). This indicates that structure (d) is unlikely to be found in naturally occurring animal oligosaccharides, unless other transferases than I and II are responsible. Structure (d) has not been reported in the literature to our knowledge. Moreover, the formation of the difucosyl structure (g) requires that fucosyl transferase III act before fucosyl transferase II. This observation is of interest since structure (g) should possess Lewis blood group D (Le^d) activity (25,26), indicating that synthesis of Le^d active substances perhaps results from the sequential addition of fucose, first to the galactosyl residue and then to the N-acetylglucosaminyl residue. Likewise, the formation of structure (f),NeuAcα2,6[Fucα1,2]Galβ1,4GlcNAc, can be formed only if the fucosyl transferase III acts

Note a. S. Kornfeld, personal communication.

before the sialyl transferase I. This structure also has not yet been reported in glycoproteins.

The reciprocal actions of two sialyl transferases and a β-galactoside α1,2 fucosyl transferase (III) were also examined with the acceptor substrate, Galβ1,3GalNAcα1→OSer/Thr, which is the sole disaccharide in an Antarctic fish antifreeze glycoprotein that was used in the studies summarized in Fig. 2.

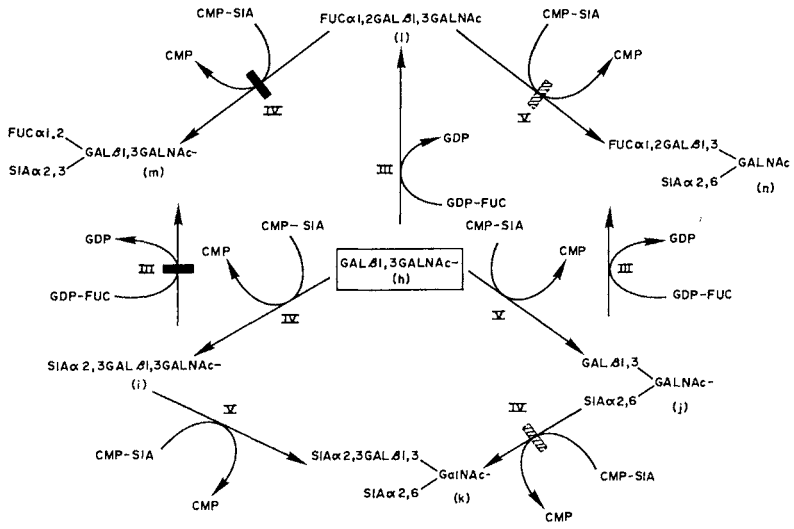


Fig. 2. The proposed biosynthetic pathway for the synthesis of oligosaccharides formed by the combined actions of IV, β1,3 galactoside α2,3 sialyl transferase; V, N-acetylgalactosaminide α2,6 sialyl transferase and III, β-galactoside α1,2 fucosyl transferase. The solid bars indicate that the reaction as written does not proceed, and the hashed bars indicate that the reaction proceeds very slowly. The letters (h) through (n) refer to different structures shown.

This structure is a precursor of the O-linked oligosaccharides in many glycoproteins, including glycophorin, porcine submaxillary mucin, fetuin, and the Thomsen-Friedenreich antigen. In some cases, the prior action of one transferase inhibited or prevented the subsequent action of another transferase. For example, structures (i) and (l) could not be converted to structure (m) by either fucosyl transferase III or sialyl transferase IV, respectively. This suggests that structure (m) may not occur in animal oligosaccharides, and to our knowledge it has not been reported in the literature. Structure (n), however, which contains the same sugars as structure (m) is synthesized by the combined action of transferases V and III, but conversion of structure (l) to (n) by the sialyl transferase is much slower than conversion of structure (j) to (n) by the fucosyl transferase. Structure (n) is one of the oligosaccharide groups of porcine mucin (27), thus its biosynthesis is likely to proceed by the formation of Galβ1,3[NeuAcα2,6]GalNAc (structure j) followed by the formation of Fucα1,2Galβ1,3[NeuAcα2,6]GalNAc (structure n). Similarly, structure (k), the prosthetic group found in glycophorin and fetuin (27) can be synthesized by the concerted action of two sialyl transferases, with either transferase IV or V acting initially followed by the other. However, the conversion of structure (j) to structure (k) is about ten times slower than conversion of structure (i) to structure (k). Thus, the preferred biosynthetic pathway is the action of the β-galactoside α2,3 sialyl transferase followed by that of the N-acetylgalactosaminide α2,6 sialyl transferase.

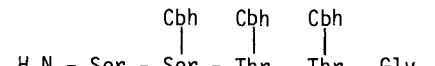
The observed glycosyl transferase catalyzed reactions, summarized in Figs. 1 and 2, may indicate not only what types of structures are permitted in animal oligosaccharides, but also the preferred pathways of biosynthesis of certain oligosaccharides. They may also indicate why some oligosaccharides in glycoproteins exhibit structural heterogeneity, since if one transferase acts on a precursor oligosaccharide to form a product which is a poor acceptor for another transferase, then incompletely glycosylated structures could result. Of course, the present studies were performed in vitro under conditions that differ markedly from those in vivo, and additional studies will be required to assess whether the preferred biosynthetic pathways shown are correct.

GLYCOSYL TRANSFERASES AS REAGENTS FOR MODIFICATION OF OLIGOSACCHARIDES IN GLYCOPROTEINS

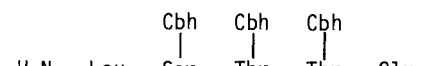
Although glycosidases are frequently used for structural analysis of oligosaccharides and for the assessment of the structure-function relationships of glycoconjugates, glycosyl transferases have only become available for this purpose in recent years. Some of the first reports employing transferases for structure-function studies were concerned with the ABO (H) blood group antigens of human erythrocytes, and the use of specific, but impure, transferases to convert O_h (Bombay) cells to O cells (11) and O cells to either A or B cells (14, 15,16,17). Similar studies with erythrocytes are summarized below for the MN blood group antigens and viral adsorption to erythrocytes. There are now a few other reports that have employed transferases to probe the structures or assess the structure-function relationships of oligosaccharides in individual glycoproteins. The β-galactoside α2,6 sialyl transferase (Table 1) was particularly useful in assessing the molecular basis for inactivation of the rabbit hepatic galactose binding protein by neuraminidase (28). The binding protein is itself a glycoprotein that contains the oligosaccharide sequence Siaα2,6Galβ1,4GlcNAc (29). Digestion with neuraminidase produces non-reducing terminal galactose residues, allowing the binding protein to complex with its own oligosaccharides rather than with those of other glycoproteins. Resialylation of the asialo-binding protein restores its original binding activity by substituting the terminal galactose residues. Since the sialyl transferase employed in these studies requires acceptors with the sequence Galβ1,4GlcNAc at their non-reducing termini, the extent of incorporation of sialic acid by the transferase indicates the amount of galactose in this sequence (30). Studies with asialo derivatives of α_1-acid glycoprotein, the rabbit liver galactose binding protein, and human blood coagulation Factor VIII show that the sialyl transferase incorporates sialic acid equivalent to 88%, > 90% and 63% respectively of the galactose content of each protein. This technique provides a direct measure of the Galβ1,4GlcNAc content of these glycoproteins and is particularly attractive since very small quantities of both enzyme and glycoprotein acceptor substrates are required. Similarly, the α1,2 fucosyl β-galactoside α1,3 N-acetylgalactosaminyl transferase has been used to detect the presence of the α1,2 fucosyl β-galactoside group in glycoproteins (11). For example, the extent of incorporation of N-acetylgalactosamine by this transferase was equal to the fucose content of procine submaxillary mucin (A^- blood group), indicating that all of the fucose in this mucin is linked α1,2 to galactose. Similarly, human Factor VIII was found to have 10% of its galactose content present as α1,2 fucosyl galactose, providing the first indication that this protein had ABO (H) blood group active oligosaccharides. The N-acetylglucosaminide α1,3 fucosyl transferase has been particularly useful in the demonstration of a heretofore unrecognized hepatic binding protein activity in mouse, rabbit and rat hepatocytes (31). Considerable evidence has been obtained showing that the uptake of lactoferrin from blood by mouse liver was mediated by lactoferrin oligosaccharides, and that the structure Galβ1,4[Fucα1,3]GlcNAc in lactoferrin is specifically required. Furthermore, asialo-transferrin, which is not rapidly removed from blood by the liver, is rapidly cleared after its oligosaccharide chains have been fucosylated by the N-acetylglucosaminide α1,3 fucosyl transferase (Table 1) to produce the same fucosylated oligosaccharide structures that are found in lactoferrin. Based on the success of such studies to date, glycosyl transferases will most likely be valuable reagents in similar kinds of structural analyses and structure-function problems in the future.

GLYCOSYL TRANSFERASES AS REAGENTS FOR MODIFICATION OF OLIGOSACCHARIDES ON CELL SURFACES

The Role of Sialic Acid in the Expression of the Human MN Blood Group Antigens. Glycophorin, the major sialo-glycoprotein of the erythrocyte membrane, is known to carry the MN blood group antigens in the following amino acid sequences, which are the first five residues from the NH_2 terminus (32,33,34).

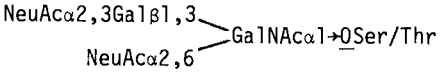


M-active



N-active

The carbohydrate groups (Cbh) in both types of glycophorin have the following structure (35).



Since the M and N active glycophorins differ in sequence only in the first and fifth amino acids, it has been suggested that MN allelic genes determine both the amino acid sequences of glycophorins and their blood group specificity (32,33,34). Yet it has been recognized for some time that removal of the sialic acid from intact erythrocytes or from glycophorin, or other treatments that structurally alter the disialo-tetrasaccharide groups of glycophorin, destroy both M and N activities (36,37,38). Thus, the contribution of sialic acid-containing oligosaccharides to the antigenicity of M and N glycopeptides remains unexplained. Others have proposed that M and N antigens differ from one another in the sialic acid groups attached to the core disaccharide, Galβ1,3GalNAc, of glycophorin, and that a sialylated N antigen is a precursor of M antigen (39).

With the availability of three pure sialyl transferases (Table 1), including two which form the Siaα2,3Gal and the Siaα2,6GalNAc structures of glycophorin, it has now been possible to evaluate more exactly the role of sialic acids in determining the MN antigenic activity of erythrocytes (40). In these studies, erythrocytes from MM and NN individuals were desialyzed with Vibrio cholera neuraminidase to destroy the MN antigenic activities and then resialylated with the three pure sialyl transferases, either singly or in combination. Restoration of the blood group activity to the erythrocytes was determined with human and rabbit anti-M and anti-N antisera. Table 3 summarizes results obtained in these studies with one of the anti-N and two of the anti-M antisera employed.

TABLE 3. The Extent of Incorporation of Sialic Acid Into Asialo-Erythrocytes By Sialyl Transferases and the Extent of Restoration of MN Antigenicity

Cell Type	Sialyl Transferase (ST) used	Sialic Acid Content (nmol/mL)	Agglutination Titer		
			Anti N	Anti M1	Anti M2
Native NN	None	544	512	< 2	< 2
Asialo NN	None		2	< 2	< 2
	GalNAcα2,6ST	49	2	< 2	< 2
	Galα2,3ST	104	16	< 2	< 2
	Galα2,6ST + GalNAcα2,6ST	224	512	< 2	< 2
	Galα2,3ST + Galα2,6ST + GalNAcα2,6ST	335	256	< 2	< 2
Native MM	None	628	16	512	64
Asialo MM	None		< 2	< 2	< 2
	GalNAcα2,6ST	53	< 2	< 2	< 2
	Galα2,3ST	100	< 2	< 2	2
	Galα2,6ST + GalNAcα2,6ST	233	8	64	16
	Galα2,3ST + Galα2,6ST + GalNAcα2,6ST	333	8	1024	16

From these studies the following conclusions may be drawn. 1) Both types of blood group activity were restored by resialylation, but the antigenicity of resialylated cells always corresponded to that of the native cells from which they were prepared. Cells were never converted on resialylation to a MN phenotype different from that of the cell donor. 2) Either of two of the transferases restored MN activity to the cells. One formed the Siaα2,3-Gal structure and the other the Siaα2,6GalNAc structure, each of which is found in the disialo-tetrasaccharide groups on residues 2, 3, and 4 of glycophorin. The sialyl transferase that forms the Siaα2,6 galactose structure in asialo-glycoproteins did not restore blood group activity. 3) Because either of two transferases can restore both M and N activity, carbohydrate structures cannot be the basis for the structural differences between M and N antigens. Moreover, there was no evidence for a precursor-product relationship of the MN antigens during resialylation. 4) Each transferase incorporated different amounts of sialic acid, but when acting together, ∿ 60% of the sialic acid removed by neuraminidase was replaced on the cells. Sodium dodecyl sulfate gel electrophoresis of the erythrocyte ghosts after resialylation showed that sialic acid had been incorporated into several different integral membrane proteins, but the majority (∿ 90%) was found in glycophorins A and B. 5) Although these results are consistent with the proposal that MN alleles determine the polypeptide sequences of glycophorin, sialic acid in glycophorin appears to be important for the interaction of the MN antigenic determinants with specific anti-N and anti-M sera. 6) Evidence was obtained (not shown) indicating a striking variability in the reactivities of individual anti-M and anti-N antisera, which suggests that native M and N antigens may contain several determinants.

Restoration of Myxovirus Receptors in Human Asialo-erythrocytes by Incorporation of Sialic Acid With Sialyltransferases. Ortho- and paramyxoviruses bind sialic acid on erythrocyte membranes causing agglutination of the cells (41). Although the major sialic acid containing membrane protein is glycophorin, it is unclear whether the sialic acid mediated binding of myxoviruses to cells is dependent upon either of the two types of sialyl linkages in the disialo-tetrasaccharide of glycophorin, or upon yet another type of linkage of sialic acid found in minor amounts in glycophorin or in other glycoproteins of the cell. With pure sialyl transferases of known substrate specificity (Table 1), and erythrocytes resialyzed after neuraminidase treatment (Table 3), it was possible to assess the types of sialic acid linkages in erythrocytes that are required for adsorption of different myxoviruses (42). The adsorption of viruses was determined by their ability to agglutinate desialyzed and resialylated erythrocytes. Four different viruses were employed, two paramyxoviruses, Sendai and Newcastle disease viruses, and two orthomyxoviruses, human influenza A2 virus and equine influenza A virus. The results of these studies are summarized in Table 4.

TABLE 4. Restoration of Myxovirus Receptors to Neuraminidase Treated Erythrocytes By Resialylation With Sialyl Transferases

Cells Tested (Sialyl Transferase Treatment)	Sialic Acid Content (nmol/ml cells)	Viral Agglutination Titer			
		Sendai	Newcastle Disease	Influenza (human)	Influenza (equine)
Native	544	1024	4096	1024	2048
Asialo	0	< 2	< 2	< 2	< 2
Asialo (Galα2,6)	49	< 2	< 2	1024	2
Asialo (GalNAcα2,6)	103	< 2	< 2	128	< 2
Asialo (Galα2,3)	222	1024	4096	1024	2048
Asialo (Galα2,6, GalNAcα2,6, Galα2,3)	332	256	2048	1024	512

Each of the four viruses failed to agglutinate desialyzed cells. However, the hemagglutination titers of all four viruses were restored to original levels on incorporation of ∿ 40% of the sialic acid present in native cells by the β-galactoside α2,3 sialyl transferase. This transferase specifically forms the Siaα2,3Galβ1,3GalNAc structure, which contains one of the three types of sialic acid linkages present in glycophorin. About 90% of the sialic acid incorporated by this enzyme into asialo-erythrocytes was found in glycophorin as judged by sodium dodecyl sulfate gel electrophoresis of membranes from resialylated erythrocytes. Incorporation of sialic acid into cells by the other two transferases did not restore the binding of either the Sendai, Newcastle disease or equine influenza A viruses. These two transferases form the Siaα2,6Galβ1,4GlcNAc or Siaα2,6GalNAc sequences. However, the human influenza A2 virus was able to agglutinate cells that were resialylated by transferases that form these linkages in addition to the transferase forming the Siaα2,3Galβ1,3GalNAc sequence. Thus, the human influenza A2 virus may bind to any sialic acid containing structure, although the Sendai, Newcastle disease and equine influenza A viruses appear to recognize quite specifically the Siaα2,3Galβ1,3GalNAc structure. The availability of additional sialyl transferases may eventually permit the demonstration of even more subtle differences in the specificity of binding of viral hemagglutinins.

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Present Addresses - J. C. Paulson, Department of Biological Chemistry, University of California Medical Center, Los Angeles, California 90024, USA; J.-P. Prieels, Laboratoire de Chimie Générale I, Faculté des Sciences, Université Libre de Bruxelles, 50 Ave. F. D. Roosevelt, B1050, Brussels, Belgium.

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ENZYMES FOR SPLITTING AND SYNTHESIZING THE GLYCOSIDIC LINKAGES IN CARBOHYDRATE DETERMINANTS OF GLYCOPROTEINS

A. Ya. Khorlin, N. D. Gabrielyan,
S. E. Zurabyan and M. L. Shulman

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow 117988, GSP-1, USSR

Abstract - Comparing recently obtained and literary data a connection has been traced between mechanism of action, specificity, and sorption properties of active sites of some glycosidases (hexosaminidases and neuraminidase), as well as acceptor specificity of sialyltransferases from frog and rat liver has been studied. A suposition has been made that the sorption properties of glycosidase active sites were in intimate connection to the mechanism of action, while the sorption properties of aglyconic sites were responsible for aglyconic specificity of the enzymes. Acceptor specificity of sialyltransferases isolated from liver of evolutionary distant animals is quite near in case of low-molecular substrates but differs for polymeric substrates. The fact may be explained by existence of two levels of substrate recognition by enzyme.

Proteins of three types are united by ability for recognizing carbohydrate structure and formation of specific carbohydrate-protein complexes. These proteins are a) viral, bacterial, plant, and animal lectins, hemagglutinins, and toxins; b) glycosyltransferases - enzymes responsible for biosynthesis of oligosaccharides or oligosaccharide determinants of glycoconjugates; and c) O-glycoside-hydrolases - enzymes splitting glycosidic bonds.

The named proteins differ in their biological role, however realization of the biological function in all cases can be considered as a response to specific carbohydrate-protein complex formation. Study of biological function requires individual approach in each case. However, the complex formation itself points to common peculiarities in the recognition mechanism. These studies present practically one of the aspects of the investigation of connections between the structure, function, biosynthesis, and catabolism of carbohydrate determinants of glycoconjugates.

The present paper is devoted to some results of the studies on glycosidases and glycosyltransferases carried out in our laboratory.

Biosynthesis of carbohydrate components of glycoconjugates is well known not to proceed *via* template way (1); the oligosaccharide determinant structure is controlled by specificity of the two complementary systems: glycosyltransferases and glycosidases. Though glycosidases and glycosyltransferases catalyze different processes there must be certain similarity of their specificities and mechanisms of action (*cf.* Ref. 2). Glycosyltransferases are catalysts for the transfer of glycosyl residue from donor to acceptor, revealing both donor and acceptor specificities. Glycosidases catalyze hydrolytic splitting of monosaccharide off oligosaccharide or oligosaccharide determinant and reveal glyconic and aglyconic specificities. Active sites of the enzymes should have certain similarity in their properties regulating the mechanism of recognition of an acceptor in the glycosidic linkage formation as well as an aglycone in the splitting of this linkage. The problem we raised was to trace connections between mechanism, specificity, and properties of active sites of glycosyltransferases and glycosidases by studying mechanism of action and specificity of these enzymes.

Necessary substrates - oligosaccharides imitating or coinciding with carbohydrate determinant of glycoproteins became available after elaboration of the next methods of synthesis of heterooligosaccharides:

1. Oxazoline method using 2-methyl-glyco-(2',1':4,5)-2-oxazolines as glycosylating agents (3,4);
2. Diphenylcyclopropenyl method based on glycosylation of 2,3-diphenyl-2-cyclopropen-1-yl ethers of saccharides by glycosyl perchlorates; the method was used to synthetise previously difficulty obtainable linear and branched heterooligosaccharides (5,6), α-L-fucose terminal oligosaccharides (7) among them;
3. Synthesis of sialodisaccharides carried out under Koenigs-Knorr's reaction conditions using methyl ester of acetochloroneuraminic acid as a glycosylating agent (8).

The connection between sorption properties of active site, mechanism, and specificity was traced for N-acetyl-β-D-hexosaminidase B_1 isolated as homogeneous fraction from boar epididymis.

Data on sorption properties of hexosaminidase active site were obtained by studying interaction of the enzyme with a number of substrates and inhibitors with modified structural elements essential for sorption in the active site. The summary data are presented in Table 1.

TABLE 1. K_i, K_m, V, and $-\Delta G°$ for substrates, analogues, and inhibitors (Ref. 9)

Combinations	K_i or K_m (mM)	V (mcmol.min^{-1}.mg protein^{-1})	$-\Delta G°$ (kcal. mol^{-1})
GlcNAcβ-O$C_6H_4NO_2$	1.1	1870	6.7
6-deGlcNAcβ-O$C_6H_4NO_2$	1.7	200	6.4
4-deGlcNAcβ-O$C_6H_4NO_2$	12.5	360	5.2
3-deGlcNAcβ-OPh	11.0	45	5.3
GlcNAcβ-OPh	2.1	390	6.2
Glcβ-OPh	400*	nonhydrolyzed	3.1
1,5-anhydro-N-acetylglucosaminitol	1.6*		6.5
4-MeOGlcNAcβ-O$C_6H_4NO_2$	8.7	222	5.4
6-MeOGlcNAcβ-O$C_6H_4NO_2$	2.6	62	6.2
GlcNAc	4.9*		5.8
GlcNAcβ1→4GlcNAc	13.9*		5.1
GlcNAcβ1→4GlcNAcβ1→4GlcNAc	27.8*		4.7
GlcNAcβ1→6GlcNAc	5.9*		5.7

* K_i values were obtained by enzymatic hydrolysis of p-nitrophenyl N-acetyl-β-D-glucosaminide.

Contributions due to N-acetylglucosamine residues and its functional groups to the Gibbs' energy of sorption at the catalytic site were calculated from values for affinity, as well as contributions due to monosaccharide residues were calculated for Gibbs' energy of substrate sorption at aglyconic subsites of the active site. The calculated values, characterizing sorption properties of subsites of the active site of the hexosaminidase are shown in Diagrams 1 and 2, in comparison with respective values for the active site of N-acetyl-β-D-hexosaminidase from Aspergillus oryzae (10).

Before all, attracts attention to itself the similarity of sorption properties of the catalytic sites(A) of hexosaminidases and their difference from those of catalytic site of lysozyme for sorption of a monosaccharide residue at the D-site of lysozyme is disadvantageous (ΔG = +2.9 kcal.mol^{-1}) (Ref. 11).

DIAGRAM 1. Contributions due to functional groups of 2-acetamido-2-deoxy-β-D-glucopyranose residue to Gibbs' energy ($\delta\Delta G°$, kcal.mol^{-1}) of sorption at catalytic site of pig (Ref. 9) and A. oryzae (Ref. 10) hexosaminidases (in parentheses)

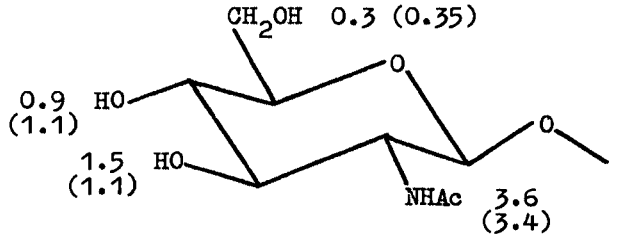

DIAGRAM 2. Results of mapping of active sites of pig (Ref. 9) and A. oryzae (Ref. 10) hexosaminidases

	Sorption sites			
	A	B	C	
Pig hexosaminidase	GlcNAcβ1→6GlcNAc -6.5	+0.6		kcal.mol^{-1}
	GlcNAcβ1→4GlcNAc -6.5	+1.4		kcal.mol^{-1}
	GlcNAcβ1→4GlcNAcβ1→4GlcNAc -6.5	+1.4	+0.4	kcal.mol^{-1}
Hexosaminidase from A. oryzae	-5.6 (-5.95)	-2.6	-0.6	kcal.mol^{-1}

The differences in sorption properties of hexosaminidases and lysozyme seem to reflect differences in their mechanisms of action. Thus, results of measuring α-secondary isotopic effect in hydrolysis of phenyl N-acetyl-β-D-glucosaminides by pig hexosaminidase (k_H/k_D = 1.04) are in line with the mechanism of double substitution at the anomeric center of the splitted monosaccharide. The other glycosidases, namely β-glucosidase (12,13) and β-galactosidase (14) have been shown to reveal the similar mechanism. On the contrary, the mechanism involving glycosyl cation formation (12) has been shown for lysozyme. Thus, sorption properties of the catalytic sites are in near connection with the mechanism of catalysis.

The data presented in Diagrams 1 and 2 permit revealing not only similarity but also differences in properties of active sites of hexosaminidases from various sources. Differences in sorption properties of aglyconic sites (B and C) are, apparently, the main reason for differences in aglyconic specificity of the hexosaminidases. As for as the enzyme from A. oryzae hydrolyzes chitin oligosaccharides much faster than pig hexosaminidase (cf. Refs. 9 and 10) it yields conclusion that aglyconic specificity increases with the increase in sorption at aglyconic sites. This conclusion seems correct for the interaction of one and the same enzyme with a number of substrates differing in aglycone structure. Thus, the rate of hydrolysis of chitobiose by pig hexosaminidase is lower than that for 1→6-linked disaccharide (9).

The similar connection between sorption properties of aglyconic sites in the enzyme-substrate complex formation and aglyconic specificity can be traced for neuraminidase from Vibrio cholerae. Table 2 presents values, characterizing affinity of the enzyme active site to sialooligosaccharides and values of the enzymic hydrolysis of the latter.

Taking contribution due to catalytic site equal to the change in ΔG of sorption of N-acetylneuraminic acid at the active site it is obviously seen that in this case high sorption ability of the aglyconic site correlates to high aglyconic specificity.

TABLE 2. K_i, K_m, V (Ref. 8), and $\Delta G°$ of the interaction of neuraminidase from Vibrio cholerae with N-acetylneuraminic acid and sialooligosaccharides

Combinations	K_i or K_m (mM)	V (nmol.min^{-1}.mg protein^{-1})	$-\Delta G°$ (kcal, mol^{-1})
NeuNAc	4.9*	–	5.7
NeuNAcα2→6Gal	5.9	100	5.6
NeuNAcα2→6Glc	6.7	66	5.6
NeuNAcα2→3Glc	3.3	800	6.0

* K_i values were obtained by enzymic hydrolysis of p-nitrophenyl N-acetyl-α-neuraminoside (15).

Aglyconic specificity of glycosidases is, apparently, analogous to acceptor specificity of glycosyltransferases. Our data on acceptor specificity of sialyltransferases, terminating biosynthesis of oligosaccharide determinants of sialobiopolymers prove the correctness of the supposition.

By the beginning of our study existence of different sialyltransferases terminating biosynthesis of only definite oligosaccharide structures was pointed out in literature (16-19). However there were no data on comparative characteristics of deliberately different sialyltransferases in respect to the same low-molecular and polymeric substrates. We think just this approach provides maximal information on common and differing features of the specificity. To realize the approach we chose evolutionary distant objects, namely membrane forms of sialyltransferases from frog liver (amphibia) and rat liver (mammal). Besides, membrane forms of enzyme isolated from rat liver in stage of fast proliferation (regenerating liver) have been studied.

Low-molecular acceptors used are presented in Table 3. The oligosaccharides did not differ in monosaccharide sequence from oligosaccharide terminals of native substrates of sialyltransferases, however some structural features of acceptors varied, namely a) position of glycosidic linkages between monosaccharide units, b) anomeric configuration of glycosylated residues of galactose, c) size of the oligosaccharide chain, and d) structure of the terminal monosaccharide. Such a choice of substrates permitted revealing characterous peculiarities of acceptor specificity. Table 3 shows data on specificity of the studied sialyltransferases.

Acceptor specificity may be characterized as follows:

1. β-D-Galactose is optimal terminal monosaccharide.
2. Gal→GlcNAc and Gal→Glc sequences are optimal for recognition. Acceptor activity of disaccharides with these monosaccharide sequences depends upon the position of the glycosidic linkage between monosaccharide units (1→4 > 1→3 > 1→6).
3. Lactose or N-acetyllactosamine terminals are necessary but insufficient forfull recognition of the substrate structure by the enzyme (see results of the sialylation of the tetrasaccharide).

The studied sialyltransferases differ in the breadth of acceptor specificity, the most observed for the frog liver sialyltransferase, the least for the transferase from regenerating rat liver. Moreover, they differ in the structural trend of the reaction. Thus, sialyltransferases from frog and rat livers synthesised α2→6 sialotrisaccharides mainly (α2→3 by-product was 8-10 and 1-2% respectively). Regenerating rat liver sialyltransferase produces α2→6 and α2→3 isomers in approximately equal proportion.

The mentioned features remained after solubilyzation of the membrane-bound enzymes by action of non-ionic detergent (Triton X-100).

The found distinctions in properties of sialyltransferase systems could be explained by the presence of different sets of sialyltransferases (21,22) in the membrane-bound preparations. Therefore a comparative study of the

TABLE 3. Acceptor specificity of sialyltransferases from animal liver*

Acceptors	Acceptor activity of substrates**		
	Frog liver	Rat liver	Regenerating rat liver
Gal 1 4 GlcNAc	100	100	100
Gal 1 3GlcNAc	85	85	30
Gal 1 6GlcNAc	25	15	14
Gal 1 4Glc	100	50	48
Gal 1 3Glc	55	-	-
Gal 1 6Glc	1	1	1
Gal -OMe	1	1	1
Gal -OMe	85	-	-
Gal 1 4(Gal 1 6)GlcNAc	100	-	-
Gal 1 4GlcNAc 1 6Gal 1 4Glc	55	-	-
Glc 1 4Glc	1	-	-
GalNAc 1 6Glc	1	-	-
GalNAc 1 3Glc	1	-	-
GalNAc 1 6Gal	30	-	-

* Conditions for enzymic sialylation see Ref. 20.

** Incorporation of ^{14}C-NeuNAc into acceptor in percents of that into N-acetyllactosamine (parallel experiment, 1500-3000 c.p.m.).

lyltransferases has been carried out after solubilyzing and separation h the aid of isoelectric focusing.

multiple forms of sialyltransferase were isolated from solubilyzed rosomal fraction of frog liver (Fig. 1). Four multiple forms of the yme having pI 4.5, 4.9, 6.45 and 8.6 were isolated from normal rat li-. All these forms from frog liver showed the same acceptor activity in pect to both lactose and N-acetyllactosamine. The latter was found to effective acceptor for all forms of the rat sialyltransferase. $\alpha 2 \rightarrow 6$ mers were in all cases main products of the reaction.

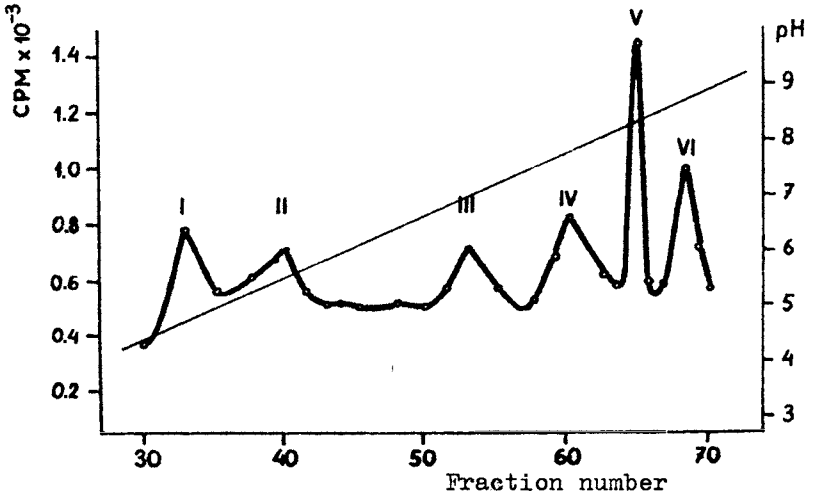

Fig. 1. Isoelectrofocusing of solubilyzed sialyltransferase system from frog liver. pI: I - 4.46; II - 5.26; III - 6.91; IV - 8.10; Y - 8.62; VI - 9.06. Experimantal conditions correspond to reported (23) using 2% amfoline solution (pH 3.5 - 10.0) in sucrose gradient 0 - 40%, 500 v.

Thus, sialyltransferases showed relatively similar specificity in respect to low-molecular substrates. However, study of interactions of sialyltranferases with polymeric substrates revealed considerable distinctions in their acceptor specificities (Table 4).

TABLE 4. Acceptor specificity of sialyltransferases from normal (I), regenerating (II) rat liver, and frog liver (III)*

Acceptor	I	II	III
Asialo-PSM	5	4	<0.5
Asialofetuin	100	187	5
Lactosyl-lysozyme**	2	10	5

* Sialylating conditions: incubation mixture (50 mcl) containing 0.12 mcmol CMP-^{14}C-NeuNAc ($1.2 \cdot 10^6$ c.p.m.), 80-200 nmol acceptor sites of asialoglycoprotein (estimated by galactose oxydase method and by amount of sialic acid in mother glycoprotein), 0.15% of Triton X-100 in 0.1M sodium-phosphate buffer), pH 7.0, 1.0 mg (I and III) or 0.7 mg protein of the enzymic sample (II) was temperature controlled for 60 min at 37° C. Incorporation of ^{14}C-NeuNAc into asialofetuin in sialylating by enzyme from the normal rat liver (4400 c.p.m.) was considered 100.

** The sample of lactosyl-lysozyme was a gift from prof. G. Moczar, Laboratoire de Biochimie du Tissu Conjonctif, Faculté de Médecine Université de Paris (France).

The data presented in Table 4 show frog sialyltransferases to have quite low activity in respect to asialofetuin contrary to rat sialyltransferases. This distinction was observed when the respective forms were compared and remained under various conditions of sialylation. This result can scarcely be explained when taking into consideration only the structure of the disaccharide terminal of carbohydrate determinants of asialofetuin - Galβ1→4GlcNAcβ1→ and Galβ1→3GalNAcβ1→OSer(Thr) (Ref. 24). It testifies that complete recognition of sialyltransferase acceptor structure requires the more prolonged fragment of the oligosaccharide chain.

The considered results of the studies on aglyconic specificity of glycosidases and acceptor specificity of sialyltransferases are related exclusively to the recognition of the aglycone or acceptor structures by active sites of the enzymes. However, the second level of recognition cannot be excluded which is controlled by more complex interactions between polymeric substrate and enzyme and regulated by variuos protein and lipid effectors (25-28). Recognition of this type should be taken into consideration in mechanistic studies of protein-carbohydrate interaction.

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A VALINOMYCIN ANALOGUE CONTAINING ONLY NATURALLY-OCCURRING AMINO ACIDS

L. G. Pease,[a] D. Baron,[b] K. R. K. Easwaran[c] and E. R. Blout

Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115, USA

Abstract - Cyclo(L-Val-Gly-Gly-L-Pro)$_3$ has been synthesized. This homodetic cyclic dodecapeptide is an ionophore and was designed both to be a valinomycin analogue and to bridge the gap between ionophores which exist in bacterial systems and those proteins which perform similar functions in eukaryotic membranes. Other peptide analogues of valinomycin have been reported, but these contain non-eukaryotic components, i.e., D-amino acids. The factors which may affect the functional and structural behavior of cyclo(L-Val-Gly-Gly-L-Pro)$_3$ compared with valinomycin are: (1) increased conformational flexibility, (2) reduced hydrophobic character, (3) additional NH groups, and (4) all amide linkages.

Data on the binding constants of several cations to cyclo(L-Val-Gly-Gly-L-Pro)$_3$ are given and compared with those of valinomycin and other valinomycin analogues. Cyclo(L-Val-Gly-Gly-L-Pro)$_3$ forms three types of peptide(P)-cation(C) complexes, i.e., PC (1:1 complex), P_2C (peptide-sandwich complex), and PC_2 (ion-sandwich complex). In acetonitrile solution the stability of the peptide-K^+ complex is comparable to the valinomycin-K^+ complex. The P_2C complexes are the major species found for small cations. Other findings are that the stabilities of the PC complexes are correlated with the diameter of the cation in the series $Mg^{2+} < Ca^{2+} < Ba^{2+}$, and that a divalent cation is more strongly bound by a large factor than a monovalent cation of comparable diameter.

Conformational data (obtained from ^1H nuclear magnetic resonance (nmr) investigations) are presented, and compared with data from valinomycin and another peptide analogue, cyclo(L-Val-D-Pro-D-Val-L-Pro)$_3$ (B. F. Gisin and R. B. Merrifield, J. Amer. Chem. Soc. 94, 6165 (1972)). While the free conformation of our analogue in chloroform or methylene chloride solution is of a new type, distinct from the free conformations of valinomycin or the other peptide analogue, the conformations of the cation complexes of all three compounds compared are structurally analogous.

Preliminary results of membrane and two-phase extraction studies are described which indicate that cyclo(L-Val-Gly-Gly-L-Pro)$_3$ behaves largely as a valinomycin-type carrier, with selectivity properties similar to both valinomycin and the other peptide analogue.

INTRODUCTION

Throughout the last twenty years it has been repeatedly demonstrated that studies on compounds which model naturally-occurring molecules may give insight into the relationship between structure and function in the native biochemical system. Of particular interest recently have been those species which are able to interact effectively with biomembranes, thus conferring on the membrane a specific property, e.g., enhanced conductivity towards a particular cation. Among the compounds which accomplish this latter function (ionophores), valinomycin

Note a. Present address: Department of Chemistry, Amherst College, Amherst, Massachusetts 01002, USA.

Note b. Present address: LASIR, CNRS, 94320 Thiais, France.

Note c. Present address: Molecular Biophysics Unit, Indian Institute of Science, Bangalore, 560012, India.

has emerged as an extraordinary molecule, due to its very high selectivity for K^+ over Na^+, and to the mechanism of its membrane activity, which is considered to be archetypal of carrier-mediated transport. Total synthesis of valinomycin by Shemyakin and coworkers (1,2) unequivocally proved its structure: cyclo(L-Val-D-HyIv-D-Val-L-Lac)$_3$. Several analogues of valinomycin have been designed and synthesized, and their properties -- notably, conformation, ion binding, and membrane activity -- studied (3-6).

We have reported (7) the synthesis and ion binding of cyclo(L-Val-Gly-Gly-L-Pro)$_3$, a valinomycin analogue which was designed as a potential model to bridge the gap between ionophores which arise from bacterial systems and those proteins which may perform similar functions in eukaryotic membranes. Hence, we chose to use only amino acids which occur naturally in eukaryotes, i.e., no D-residues or ester linkages. The possibility of conformational analogy with valinomycin was maintained through the incorporation of glycines in the sequence where D-residues occur in valinomycin. The principal factors which may affect the structural and functional behavior of cyclo(L-Val-Gly-Gly-L-Pro)$_3$ as opposed to valinomycin are: (1) increased conformational flexibility due to greater available conformational space, (2) reduced hydrophobicity of residues, (3) additional N-H's, and (4) presence of all amide linkages. By comparison with other valinomycin analogues which have been examined (3-6), the main distinction in our compound is the presence of glycines, the concomitant lack of any D-residues and reduction in hydrophobic side chains.

We have organized this presentation so as to draw comparisons among valinomycin, cyclo(L-Val-Gly-Gly-L-Pro)$_3$, and a few representative other valinomycin analogues. First, we will discuss the interaction of these compounds with ions. Then, solution conformations will be considered. Finally, we describe preliminary results of membrane studies and two-phase extraction experiments for cyclo(L-Val-Gly-Gly-L-Pro)$_3$.

INTERACTIONS WITH IONS

Results of a circular dichroism study of cyclo(L-Val-Gly-Gly-L-Pro)$_3$ and its complexes with some alkali metal and alkaline earth cations in acetonitrile solution have been described in detail elsewhere (7). Table 1 summarizes the binding constants which were obtained through the use of a computer fit of titration curves.

TABLE 1. Complexation behavior of valinomycin and analogues

Ion:		Li^+	Na^+	K^+	Mg^{+2}	Ca^{+2}	Ba^{+2}
Diameter (Å):		1.20	1.90	2.66	1.30	1.98	2.70
Valinomycin	K_1						
CH_3OH[a]		<5	4.7	8×10^4	<5	5×10^2	2.2×10^3
CH_3CN[b]		-	$<10^3$	3×10^5	-	-	-
cyclo(L-Val-Gly-Gly-L-Pro)$_3$[c]							
CH_3CN	K_1	1.1×10^3	5.8×10^1	1.7×10^5	2.4×10^3	3×10^8	2×10^{10}
	$K_{1/2}$	5.5×10^6	6.9×10^5	2.3×10^5	1.0×10^6	8×10^5	2×10^6
	K_2	2.4×10^2	d	d	1.5×10^2	5.5×10^5	1.5×10^1

Where the constants describe the following equilibria: K_1: P + C = PC; $K_{1/2}$: PC + P = P_2C; K_2: PC + C = PC_2

a. From Reference 3.
b. From M. C. Rose and R. W. Henkens, Biochim. Biophys. Acta 372, 426 (1974).
c. From Reference 7.
d. No indication of any formation of these species was found.

Figure 1 shows an example of the curves obtained. In brief, three different stoichiometries

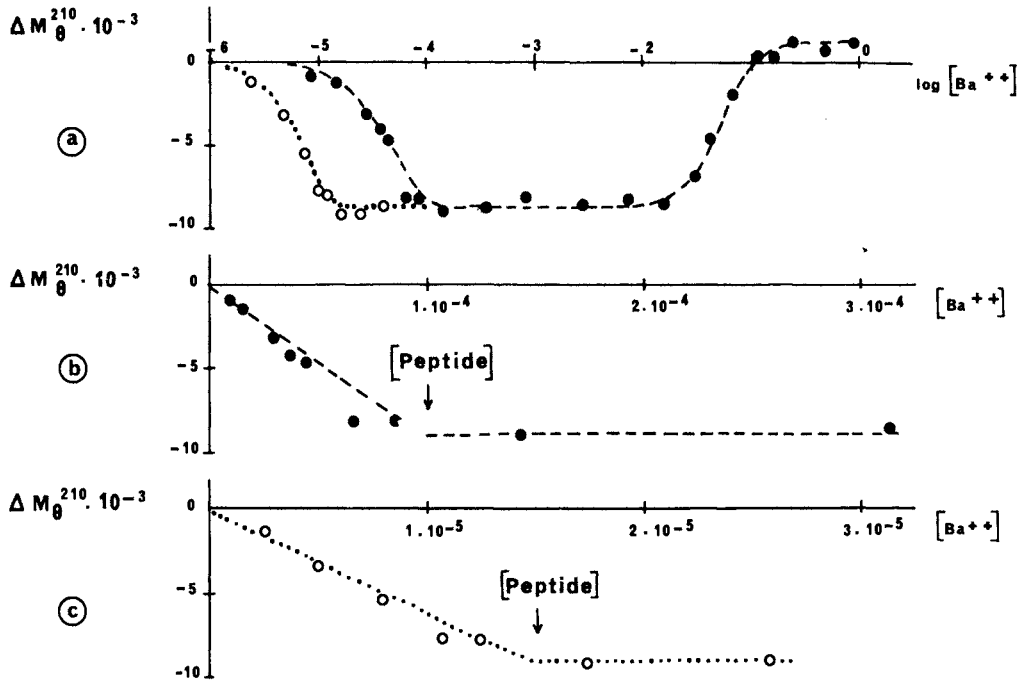

Fig. 1. Titration curves ($\Delta\underline{M}^{\lambda}_{\theta} = \underline{M}^{\lambda}_{\theta}$ peptide plus ion $- \underline{M}^{\lambda}_{\theta}$ free peptide) of cyclo(L-Val-Gly-Gly-L-Pro)$_3$ in CH_3CN:(peptide) $\simeq 10^{-4}$M (o) or 10^{-5}M (•); dashed or dotted lines are calculated curves: (a) $\Delta\underline{M}^{210}_{\theta}$ vs. log (Ba^{2+}); (b) $\Delta\underline{M}^{210}_{\theta}$ vs. (Ba^{2+}); (c) $\Delta\underline{M}^{210}_{\theta}$ vs. (Ba^{2+}) (From Ref. 7).

of complexation were observed: 1:1, 1:2, and 2:1, peptide:cation. Among the 1:1 species association was more favorable the larger the cation (up to K^+ and Ba^{2+}, the largest ions examined, ~2.7 Å in diameter), and was stronger for a divalent cation by a large factor than for a monovalent cation of a corresponding size. Na^+ was seen to form the weakest complexes among the monovalent ions, suggesting that two types of 1:1 complex may occur: one with the cation interacting with six carbonyls (larger cations), and the other with three carbonyls (high charge density, small cation). Na^+ would not be expected to bind well in either of these modes. The "peptide sandwich", P_2C, was observed to form to a significant extent with all cations studied, and the association constant was relatively invariant. This finding may be a result of opposing influences: first, the steric interference between two approaching peptides which should be reduced for larger cations, and second, the likelihood of occurrence of a 1:1 species in which the cation interacts with only three carbonyls (i.e., not really in the cavity), which would be greater for small cations, and might lead more readily to P_2C species. PC_2 species were observed to have the highest constants of association for the smaller ions, not unexpectedly, as small ionic size would make two sites sterically accessible.

By comparison with the above, valinomycin shows a strong preference for monovalent cations (see Table 1). The magnitude of the 1:1 binding constants for K^+ of our analogue and of valinomycin are approximately the same in acetonitrile. Selectivity for K^+ over Na^+ is somewhat less for our analogue, and binding to Li^+ is considerably better with the analogue than with valinomycin. Valinomycin has also been reported (8) to form sandwich complexes -- of the type V_2K^+.

Stability constants of K^+ complexes of many other valinomycin analogues have been summarized by Ovchinnikov et al. (3). Among those analogues in which the basic ring size was not varied, the complexation with K^+ appeared to be less favorable with ligands which had more conformational freedom, and conversely, introduction of N-substituted amino acids into the valinomycin sequence in place of the acid residues, hence conferring more rigidity, led to significantly stronger complexation. Ovchinnikov and coworkers attributed this finding to a lessened relative stability of the free ligand. The same explanation could be applied to the finding of enhanced complexation by another peptide analogue of valinomycin, cyclo(L-Val-D-Pro-D-Val-L-Val)$_3$ (4,5), as suggested in studies using two-phase systems (5) and in nmr analysis of complexes (6, see Note d).

Note d. Approximate stability constants in methanol can be read from a plot in Ref. 6: Li^+, <10; Na^+, 10^4; K^+, 10^8.

The most salient difference between the complexation behavior of valinomycin and cyclo(L-Val-Gly-Gly-L-Pro)$_3$ is the very strong binding of divalent cations by the latter compound. This result is most likely attributable to the relatively thin "overcoat", and hence large Born free energy term contributing to the stability of the cyclo(L-Val-Gly-Gly-L-Pro)$_3$-C^{2+} complexes (3,9,10). In contrast, the presence of hydrophobic side chains at all residues of valinomycin results in a thicker "greasy" shell around the cation, and hence a weaker contribution from the Born free energy of solvation, in particular to the complexes of the doubly charged cations. The other distinction relative to valinomycin noted in all the analogues which utilize amide carbonyls for binding is that binding to the smaller monovalent ions (Li^+, Na^+) is not so markedly reduced relative to that to K^+. This may be due to the greater polarizability and enhanced point charge of amide carbonyl oxygens as compared to that of ester carbonyl oxygens (3).

Lastly, it appears that stronger binding will be observed, given the same eventual conformation of the complexes, when the ionophore adopts a rigid free conformation (which is close to the bound conformation), than in cases of a highly flexible free ionophore. The results with cyclo(L-Val-D-Pro-D-Val-L-Pro)$_3$ and with valinomycin analogues with N-substituted amino acids (3) support this suggestion. However, cyclo(L-Val-Gly-Gly-L-Pro)$_3$ in acetonitrile, despite its expected greater conformational freedom, binds only slightly less strongly than valinomycin in the same solvent. This result may reflect in part the fact that the conformation adopted by valinomycin in solvents of medium polarity (e.g., CH_3CN or methanol) is somewhat flexible (3).

SOLUTION CONFORMATIONS OF FREE AND COMPLEXED FORMS

Results of a ^1H nmr study of cyclo(L-Val-Gly-Gly-L-Pro)$_3$ and its cation complexes (11) have revealed some interesting similarities with and differences from the natural ionophore (see Table 2). In all cases, as with valinomycin, the spectra reveal that C_3 symmetry exists on

TABLE 2. ^1H nmr parameters for valinomycin and analogues. N-H resonances in free and complexed forms[a] (solvents as indicated)

Valinomycin[b]		L-Val - D-HyIv -	D-Val - L-Lac
Free, $CDCl_3$	δ	7.75	7.85
	$^3J_{NH-CH}$	6.2	8.0
	$\Delta\delta/\Delta T$	6.8×10^{-3}	5.5×10^{-3}
With K^+, $CDCl_3$	δ	8.25	8.40
	$^3J_{NH-CH}$	4.8	4.8
	$\Delta\delta/\Delta T$	2.5×10^{-3}	2.5×10^{-3}

Cyclo(L-Val-Gly-Gly-L-Pro)$_3$[c]		L-Val -	Gly -	Gly -	L-Pro
Free, CD_2Cl_2	δ	6.78	7.47	7.87	
	$^3J_{NH-CH}$	8.45	4.2,4.2	4.6,4.6	
	$\Delta\delta/\Delta T$	5.8×10^{-3}	2.0×10^{-3}	2.0×10^{-3}	
Free, CD_3CN	δ	6.90	7.55	7.80	
	$^3J_{NH-CH}$	-	-	-	
	$\Delta\delta/\Delta T$	3.0×10^{-3}	3.0×10^{-3}	3.0×10^{-3}	
With K^+, CD_3CN	δ	7.88	7.40	8.77	
	$^3J_{NH-CH}$	6.1	7.7,4.8	7.0,3.0	
	$\Delta\delta/\Delta T$	1.0×10^{-3}	3.0×10^{-3}	1.0×10^{-3}	

Cyclo(L-Val-D-Pro-D-Val-L-Pro)$_3$[d]		L-Val -	D-Pro -	D-Val -	L-Pro
Free, $CDCl_3$	δ	7.8		7.8	
	$^3J_{NH-CH}$	10 - 11		10 - 11	
	$\Delta\delta/\Delta T$	3.0×10^{-3}		3.0×10^{-3}	
With K^+, $CDCl_3$	δ	8.1		8.1	
	$^3J_{NH-CH}$	4.8		4.8	
	$\Delta\delta/\Delta T$	3.0×10^{-3}		3.0×10^{-3}	

a. Chemical shifts (δ) are ppm downfield from internal TMS; coupling constants are in Hz; and temperature dependences ($\Delta\delta/\Delta T$) are in ppm/deg.
b. From D. G. Davis and D. C. Tosteson, Biochemistry 14, 3962 (1975).
c. From Reference 11.
d. From Reference 6.

the nmr time scale. Analysis of temperature and solvent dependences of N-H resonances for our dodecapeptide in CD_2Cl_2 or $CDCl_3$ leads to a proposed conformation with the N-H's of all of the glycines intramolecularly hydrogen-bonded. Further, the $^3J_{NH-CH}$ values of 4.2, 4.2 and 4.6, 4.6 Hz for the two Gly's are only consistent with ϕ angles of $\sim 180^o$ (12, see Note e). These principal findings led to a proposed structure which was also consistent with the rest of the ^1H nmr data (see Note f). The ϕ,ψ angles are listed in Table 3. A molecular

TABLE 3. Proposed solution conformations of valinomycin and peptide analogues (solvents are indicated)

A. FREE CONFORMATIONS								
Valinomycin[a]		L-Val	-	D-HyIv	-	D-Val	-	L-Lac
Non-polar, e.g., $CHCl_3$	ϕ	-80		120		90		-120
	ψ	90		0		-90		0
Med. polar, e.g., CH_3OH	ϕ	-85		120		110		-120
	ψ	100		0		90		120
Polar, e.g., $(CD_3)_2SO$	Many interconverting.							
Cyclo(L-Val-Gly-Gly-L-Pro)$_3$[b]		L-Val	-	Gly	-	Gly	-	L-Pro
CD_2Cl_2 or $CDCl_3$	ϕ	-90		180		180		-60
	ψ	30		50		60		-60
CD_3CN	Many interconverting.							
Cyclo(L-Val-D-Pro-D-Val-L-Pro)$_3$[c]		L-Val	-	D-Pro	-	D-Val	-	L-Pro
$CDCl_3$	ϕ	-60		60		60		-60
	ψ	120		0		-120		0
Polar	Mixture of above and probable cis bond conformer.							
B. COMPLEXES								
Valinomycin-K^+[a]		L-Val	-	D-HyIv	-	D-Val	-	L-Lac
$CDCl_3$	ϕ	-68		94		68		-94
	ψ	120		5		-120		-5
Cyclo(L-Val-Gly-Gly-L-Pro)$_3$[b]		L-Val	-	Gly	-	Gly	-	L-Pro
$-K^+$, CD_3CN	ϕ	-70		60		50		-70
	ψ	110		45		-110		-60
$-Ba^{2+}$, CD_3CN	ϕ	-65		20		50		-60
	ψ	140		90		-170		-70
Cyclo(L-Val-D-Pro-D-Val-L-Pro)$_3$[c]		L-Val	-	D-Pro	-	D-Val	-	L-Pro
$-K^+$, $CDCl_3$	ϕ	-60		60		60		-60
	ψ	120		0		-120		0

a. From Reference 13.

b. From Reference 11.

c. From Reference 6.

Note e. For an explanation of conventions used in dihedral angle nomenclature, see: Biochemistry 9, 3471 (1970).

Note f. Examination of ^{13}C nmr spectra demonstrates that all X-Pro bonds are trans.

model is shown in Figure 2. An unusual pattern of hydrogen bonding occurs in this proposed

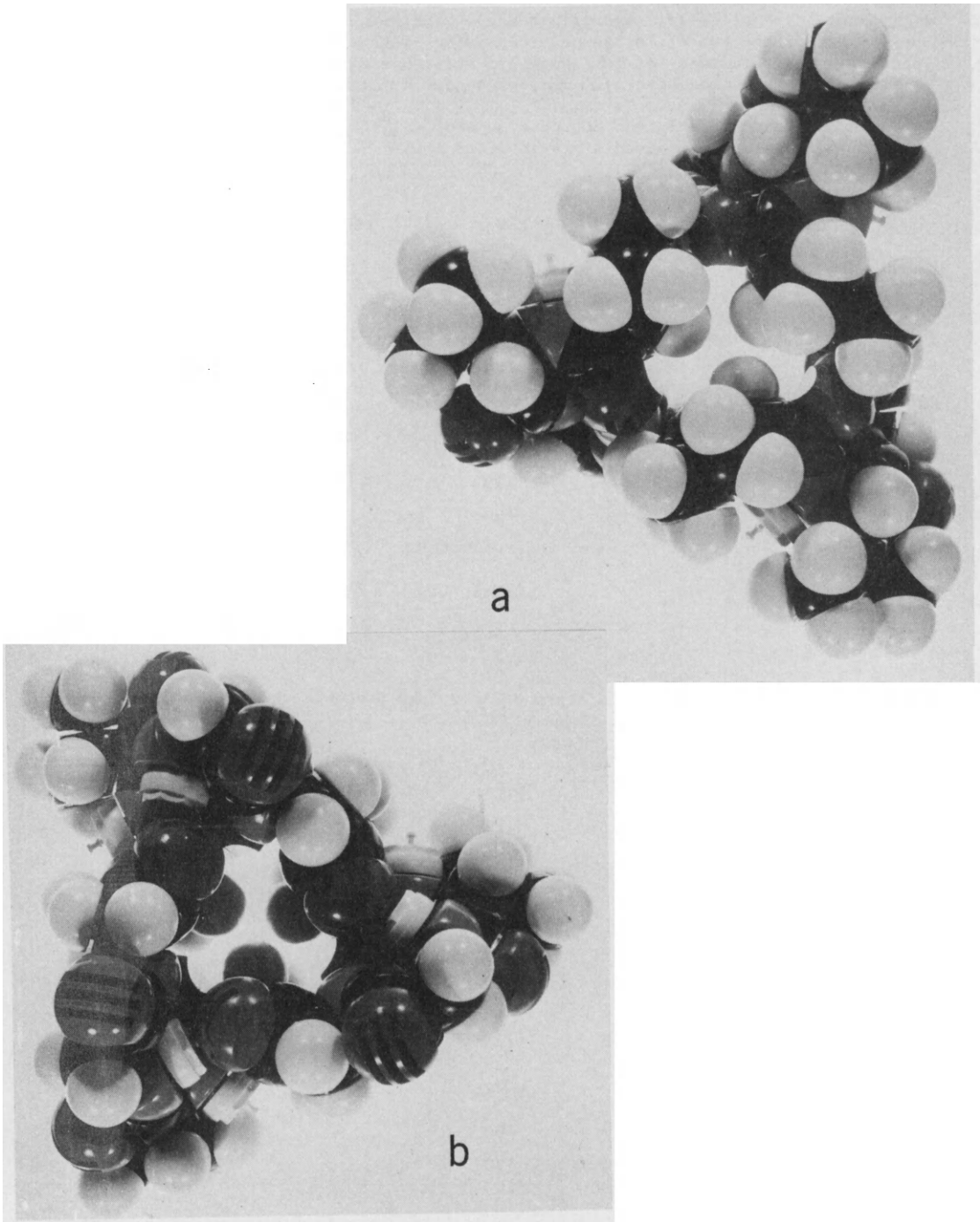

Fig. 2. Photograph of a CPK model of the proposed conformation of free cyclo(L-Val-Gly-Gly-L-Pro)$_3$ in CD_2Cl_2. a and b show opposite faces of the molecule.

conformer. Namely, there are two hydrogen bonds in each repeat unit, one (1←4) between the N-H of Gly(2) and the C=O of Pro, and the other (1←4) between the N-H of Gly(1) and the C=O of the Gly(2) of the preceding (L-Val-Gly(1)-Gly(2)-L-Pro) unit. This arrangement is shown

schematically in Figure 3 (see Note g).

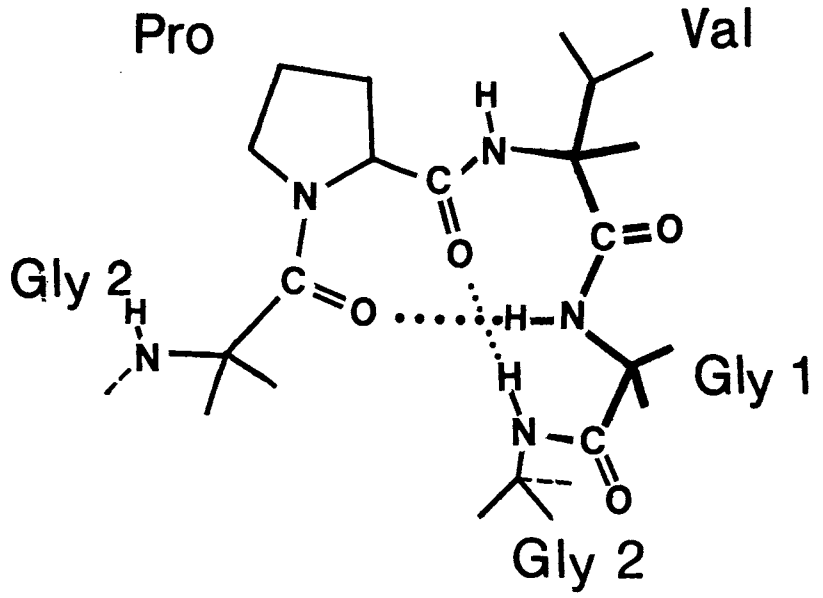

Fig. 3. Diagrammatic representation of hydrogen-bonding scheme in cyclo-(L-Val-Gly-Gly-L-Pro)$_3$. Free conformation in CH_2Cl_2, showing two 1←4 intramolecular hydrogen bonds between the N-H's of Gly 1 and Gly 2 and the C=O's of Gly 2 and Pro, respectively.

The proposed conformation of cyclo(L-Val-Gly-Gly-L-Pro)$_3$ in CD_2Cl_2 and $CDCl_3$ is different from that of free valinomycin in nonpolar solvents (2,13), where a bracelet-type structure with six intramolecular hydrogen bonds formed by the L-Val and D-Val N-H's, and with alternating Type II and Type II' β-turns, is observed. This conformation is now believed to be of the A_2 type, i.e., similar to the conformation of the K^+ complex (13). With free cyclo-(L-Val-Gly-Gly-L-Pro)$_3$, in each Gly(2)-Pro-Val-Gly(1)-Gly(2) unit there are essentially two loops of right-handed 3_{10} helix (i.e., two distorted β-turns), since hydrogen bonding occurs between residues 1 and 4 and residues 2 and 5. The ϕ,ψ angles indicate that the helix is imperfect (a true right-handed 3_{10} helix has $\phi,\psi = -60^o,30^o$). The difference may arise from the presence of the proline side chain or from the constraints of cyclization. As anticipated, the presence of an additional N-H per repeat unit relative to valinomycin (or the other peptide analogue, cyclo(L-Val-D-Pro-D-Val-L-Pro)$_3$) has made possible additional conformational possibilities.

In CD_3CN solutions of cyclo(L-Val-Gly-Gly-L-Pro)$_3$, the ^1H nmr data suggest that no one rigid conformation with a specific pattern of intramolecular hydrogen bonding occurs. Temperature dependences of the N-H's are all the same, and the N-H signals do not show well-defined coupling patterns. This result is similar to findings for valinomycin in polar solvents such as aqueous methanol, dimethyl sulfoxide, or trifluoroethanol/water (1/2). In contrast, in acetonitrile valinomycin adopts a C_3-symmetric conformer with three β-turn (1←4) hydrogen bonds rather than six such bonds that occur in the bracelet form. This is the so-called "propeller" conformation (see ϕ,ψ angles in Table 3, nmr data in Table 2).

The other peptide analogue of valinomycin, cyclo(L-Val-D-Pro-D-Val-L-Pro)$_3$, has also been examined by ^1H nmr (6). The data showed that more than one conformation which interconvert slowly on the nmr time scale were present. These workers interpret their findings in terms of populations of (1) a conformer which is analogous to the valinomycin A_2 bracelet structure, and hence has S_6 symmetry and all N-H's intramolecularly hydrogen bonded (see Table 3 for ϕ,ψ angles), and (2) an asymmetric conformer which most likely contains one or more cis Val-Pro bonds. The ratio of these populations varies with solvent. Evidence for cis peptide bond-containing conformers was also obtained for cyclo(L-Val-Gly-Gly-L-Pro)$_3$, but only in dimethyl sulfoxide solution.

Note g. In a recent X-ray structure determination of the amino terminal tetrapeptide of alamethicin, a strikingly similar conformation was observed, including the two distorted β-turns. See: N. Shamala, R. Nagaraj and P. Balaram, Biochem. Biophys. Res. Comm. 79, 292 (1977).

Upon addition of equimolar quantities of perchlorates of K^+, Ba^{2+}, or Tl^+ to acetonitrile solutions of cyclo(L-Val-Gly-Gly-L-Pro)$_3$, marked changes are observed in the ^1H nmr spectrum (see Table 2 and Figure 4). The new spectra are consistent with formation of 1:1 complexes

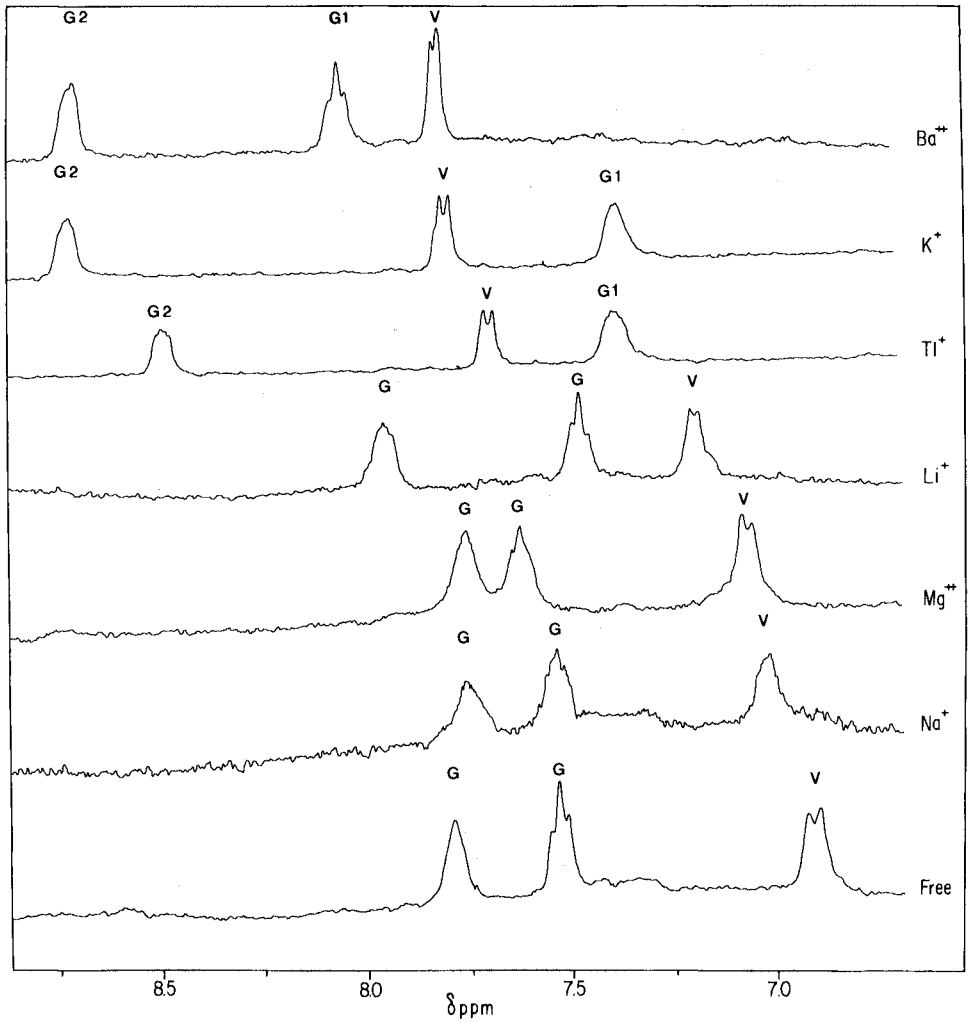

Fig. 4. The amide region of the 270 MHz ^1H nmr of cyclo(L-Val-Gly-Gly-L-Pro)$_3$ and its 1:1 complexes. Ba^{2+}, K^+, Tl^+, Li^+, Mg^{2+}, and Na^+ ions in CD_3CN. Temperature, 28°. (From Ref. 11).

with approximately the same conformations as the valinomycin-K^+ complex (see Table 3 for proposed ϕ,ψ angles), which is of the bracelet type. Hence, the complex conformation would contain alternating Type II Val-Gly and Type II' Gly-Pro β-turns, and corresponding

intramolecular hydrogen bonding of the Val N-H and the N-H of one of the Gly's (Gly(2) in the sequence) (see Figure 5). In the Ba^{2+} complex, where it appears that some minor

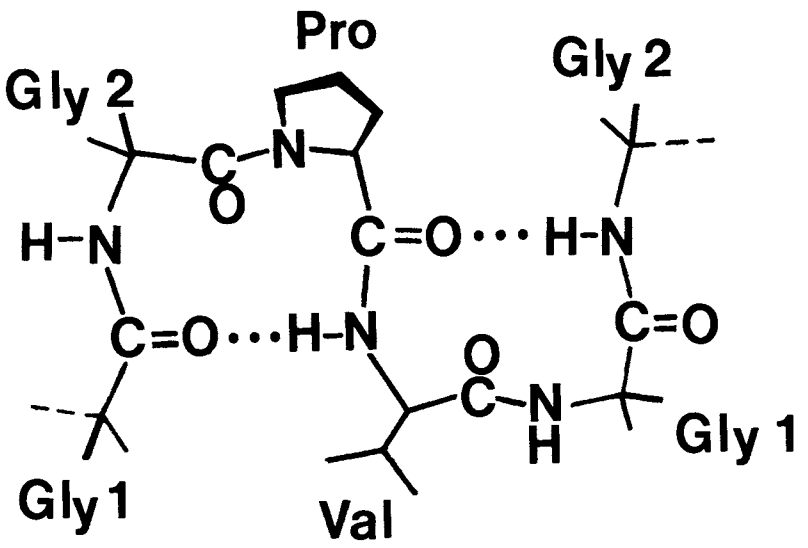

Fig. 5. Diagrammatic representation of hydrogen-bonding scheme in cyclo-(L-Val-Gly-Gly-L-Pro)$_3$. Binding conformation, showing two 1←4 intramolecular hydrogen bonds between the N-H's of Val and Gly 2 and the C=O's of Gly 1 and Pro, respectively. Note that the carbonyl oxygens of Gly 2 and Val point into the plane of the paper.

conformational adjustments have occurred to optimize the carbonyl interaction with the doubly charged cation, some changes are seen in nmr parameters, and hence in the derived ϕ,ψ angles (Table 3).

As can be seen also in Table 3, the proposed conformation of the cyclo(L-Val-D-Pro-D-Val-L-Pro)$_3$ complex is also very close to those of valinomycin and cyclo(L-Val-Gly-Gly-L-Pro)$_3$. The similarities in the conformations of the cation complexes of these ionophores are not surprising, given their related sequences, yet in marked contrast are the relatively diverse free conformations. One may therefore hope to further the goal of achieving an understanding of structure-function relationships by comparing results with a series of such compounds.

IMPLICATIONS FOR STRUCTURE-FUNCTION RELATIONSHIPS IN IONOPHORES

The mechanism of the transport activity of valinomycin in membranes has been discussed in detail on many other occasions (3). In brief, the effectiveness of this ion-carrier appears to derive from its high rate of association-dissociation with cations at the membrane interface, together with its highly specific and favorable complex formation in the membrane phase.

In comparison, the peptide analogue cyclo(L-Val-D-Pro-D-Val-L-Pro)$_3$ has a much reduced (by 10^4) membrane activity. The explanation put forth for this result is that the association-dissociation reactions at the interface are very slow, so that transport is effected instead by formation in the aqueous phase of complexes which then must diffuse across the unstirred layers and the membrane (5,6). The reasons for the occurrence of this less efficient transport mechanism appear to be: (1) formation of a complex which is too stable, thus limiting the rate of its dissociation, and (2) lack of conformational sensitivity at the interface to favor a release of cation.

We have not completed studies of the transport activity of cyclo(L-Val-Gly-Gly-L-Pro)$_3$ in bilayers. Preliminary results suggest that overall it behaves as a valinomycin-type (14). Namely, Eisenman finds it forms stoichiometric (1:1) complexes with sodium ion, and displays in glycerol monooleate-hexadecane membranes, according to the current-voltage behavior, "equilibrium domain" transport, arising from a high rate of loading and unloading, and a favorable affinity for the cation. Similar observations were made for Li^+ and NH_4^+. K^+ is more than 100 times more permeable, but leads to a transient increase of conductance in the presence of sodium, followed ultimately by a decrease in total conductance. A likely explanation for this finding is that potassium ion depletes the free carrier in the aqueous

phase (and hence in the membrane) through formation of complexes with more than one peptide molecule per K^+ (for example, P_2C type complexes). Good conductance was observed when cyclo(L-Val-Gly-Gly-L-Pro)$_3$ was added to the aqueous phase at rather low concentrations (10^{-6}M), despite a highly unfavorable partitioning of the free peptide between water and hydrocarbon-like solvents. This result suggests that the complexed peptide may partition more favorably into the membrane than the free form. In addition, the analogies observed in transport properties between cyclo(L-Val-Gly-Gly-L-Pro)$_3$ and valinomycin support the conclusion that their selectivity and the properties of their complexed forms with monovalent ions are quite comparable.

Results of two-layer extraction measurements (15) between water and methylene chloride reinforce the above-mentioned ideas. The relative extraction constants for cyclo(L-Val-Gly-Gly-L-Pro)$_3$ in this system are (normalized to K^+): Li^+ (0.0035) $\approx$ Na^+ (0.0035) < Cs^+ (0.10) < Tl^+ (0.45) < K^+ (1.0) $\approx$ Rb^+ (1.0). A study of selectivity using membrane transport measurements led to a slightly different order for valinomycin -- and for the other peptide analogue, cyclo(L-Val-D-Pro-D-Val-L-Pro)$_3$ (16), namely: $Li^+ < Na^+ < Tl^+ < NH_4^+ < Cs^+ < Rb^+ \approx K^+$. On the other hand, binding to cations in methanol by valinomycin gave the order of selectivity (values normalized to K^+) (17): Na^+ (0.037) < Cs^+ (0.33) < Tl^+ (0.37) < K^+ (1.0) < Rb^+ (2.1). This order directly parallels the two-phase extraction results with cyclo-(L-Val-Gly-Gly-L-Pro)$_3$.

CONCLUSIONS

Our studies of a peptide analogue of valinomycin have yielded comparisons of conformational, ion-binding, and membrane transport properties of the natural depsipeptide and a synthetic model peptide which contains only amino acid residues which occur naturally. The analogue may, therefore, model membrane proteins which perform ion-binding and transport functions.

We find that cation complexes of our peptide ionophore in acetonitrile are isostructural with valinomycin cation complexes. A salient difference in ion-binding properties between the two is that cyclo(L-Val-Gly-Gly-L-Pro)$_3$ binds divalent cations much more strongly than monovalent cations (1:1 complexes), while the reverse holds for valinomycin. This finding can be attributed to the reduced thickness of the hydrophobic shell around the cation in the peptide complexes.

The free conformation of our peptide analogue (in chloroform and methylene chloride) is different from any observed with valinomycin and other analogues. It is C_3-symmetric and contains six intramolecular hydrogen bonds in three short sections of distorted 3_{10} helices. This pattern of hydrogen bonding is made possible by the presence of an additional N-H in each repeat unit, relative to valinomycin.

Preliminary results of membrane and two-phase extraction studies indicate that the peptide analogue functions as a valinomycin-type carrier, though its effectiveness relative to the natural ionophore is reduced by its less favorable partitioning between the aqueous and membrane or organic solvent phases. The selectivity series of our analogue and valinomycin appear to be similar, although the two have not been measured using identical experimental conditions.

Another peptide analogue, cyclo(L-Val-D-Pro-D-Val-L-Pro)$_3$, studied by Gisin and coworkers, is reported to display transport properties distinct from valinomycin (5). It appears that some degree of conformational flexibility, and a tendency for the cation complex to dissociate at a membrane interface, are necessary characteristics for valinomycin-type ionophoric activity.

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TOXIC COMPONENTS FROM *BUTHUS EUPEUS* AND *LUCOSA SINGORIENSIS* VENOMS

E. V. Grishin

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow 117988, GSP-1, USSR

Abstract - Eight polypeptides toxic to mammals and four toxins to insects have been isolated from the venom of the Middle-Asian scorpion Buthus eupeus. All mice toxins and one insectotoxin I_2 were shown to have molecular weight of about 8000. Three other insectotoxins have molecular weight of 4000. The total amino acid sequences of two insectotoxins I_1 and I_2 were determined. There has been carried out an investigation of some properties of the Middle-Asian tarantula Lycosa singoriensis venom. There has been also isolated and partially characterized the toxic component with 10000-11000 molecular weight.

INTRODUCTION

At present the investigation of the complex mechanism of the signal conductance between nerve cells and their target organ is closely connected with application of neurotoxins as valuable tools for the study of important sites involved in nerve transmission. The utilization of neurotoxins enables to bring some light to ionic channels function and, in some cases, even to isolate the correspondent membrane receptor (Refs. 1,2). So, the research of new toxins is extremely important so long as it can extend the number of unique and highly specific tools for membrane studies. This paper presents the investigation results of toxic components from the scorpion Buthus eupeus and tarantula Lycosa singoriensis venoms.

SCORPION NEUROTOXINS

The particular interest is attributed to neurotoxins from scorpion venoms which have been found to slow down the inactivation rate of fast sodium channels of the excitable membrane. According to voltage clamp analysis data these neurotoxins extremely specifically and practically irreversibly bound to the functional components of sodium channels (Refs. 3-5). They are bound by the receptor with the dissociation constants of about 10^{-7} to 10^{-9} M but the action of scorpion neurotoxins is considerably dependent on the magnitude of the membrane potential being very small at zero potential (Refs. 4,5). The venom of the scorpion Buthus eupeus obtained by electrical stimulation of telsons is a complex mixture of at least 25 components and has no proteolytic activity. Its toxic components represent a small part of the crude venom and 12 different neurotoxins can be isolated by means of the combination of gel-filtration on Bio-Gel P-10 and ion exchange chromatography on both CM-cellulose CM-32 and DEAE-Sephadex A-50 (Ref.6). One can divide all obtained toxins into two groups: polypeptides toxic to mice and toxins possessing paralizing activity for insects. The yield of toxins was from 0.2 to 2%. The minimal paralizing activity of insectotoxins was estimated to be of about 1 μg per cockroach, Nauphoeta cinerea. LD_{50} of mice toxins was found to be 100-500 μg per kg of body weight. It should be emphasized that mice toxins do not demonstrate any paralizing activity against insects, and insectotoxins are not lethal to mice. The amino acid composition of all isolated scorpion toxins is shown in Table 1. The letters I and M indicate insectotoxins and the mice toxins respectively.

TABLE 1. The amino acid composition of scorpion toxins from *Buthus eupeus* venom

	I_1	I_2	I_3	I_4	M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8
ASP	2	11	3	5	11	10	9	10	11	9	13	10
THR	2	2	3	2	2	2	1	2	2	2	2	1
SER		6			4	3	3	3	3	3	4	3
GLU	3	1	2	1	6	4	5	6	4	2	4	3
PRO	3	2	1	2	6	3	4	5	6	3	4	5
GLY	4	7	5	4	6	5	6	6	8	6	9	7
ALA	2	2	1	1	7	4	5	5	6	4	6	6
1/2CYS	8	8	8	8	8	8	8	8	8	8	8	8
VAL		1			2		3	2	2	3	1	1
MET	3		2	3								
ILE		3			4	2	2	3	4	4	2	2
LEU	1	3	1	1	3	2	1	2	2	3	2	2
TYR	1	4	1		4	3	6	4	5	4	4	4
PHE	2	1	2	2			1	1		2	2	
HIS			1	1	2	3		4	2	2	2	2
LYS	2	8	1	3	5	5	8	5	9	5	3	5
ARG	3		5	2	2	3	2	2	2	2	2	2
TRP		3			3	3	4	2	4	2	5	3
TOTAL	36	62	36	35	75	60	68	70	78	64	73	64
N-TERM.	MET	ALA	MET	MET	ALA	ALA	VAL	ALA	ALA	ALA	ALA	ALA

According to amino acid composition all mice toxins and insectotoxin I_2 are rather like known scorpion toxins from *Androctonus australis Hector* and *Centrurodies sculpturatus* venoms (Refs. 7-8). The toxins of this group have polypeptide chains from 60 to 78 amino acid residues with four disulfide bridges, contain tryptophan and have no methionine. On the other hand it is obvious that there is another structural group of the scorpion toxins. The toxins of this group consist of a single polypeptide chain of 35-36 amino acid residues with four disulfides. Moreover in insectotoxins I_1, I_3 and I_4 two or three methionines and no serine, valine isoleucine and tryptophan residues were found.

```
                                                  10
MET-CYS-MET-PRO-CYS-PHE-THR-THR-ARG-PRO-ASP-MET-ALA-GLN-GLN-
                  20                                      30
CYS-ARG-ALA-CYS-CYS-LYS-GLY-ARG-GLY-LYS-CYS-PHE-GLY-PRO-GLN-
                        36
CYS-LEU-CYS-GLY-TYR-ASP
```

Fig. 1. The amino acid sequence of insectotoxin I_1.

The total amino acid sequence of insectotoxin I_1 is shown in Fig. 1. It is composed of 36 amino acid residues including 8 half-cystines and does not have any common features with the scorpion toxins studied up to now (Ref.9). One can suppose that insectotoxins I_3 and I_4 are the structural analogs of the I_1. So, the first eight N-terminal amino acid residues of I_4 are absolutely identical to those of I_1. But nevertheless there are unequal replacements: Asx instead of Arg, Arg instead of Met, etc. (Fig. 2).

```
MET-CYS-MET-PRO-CYS-PHE-THR-THR-ARG-PRO-ASP-MET-ALA-GLN-

MET-CYS-MET-PRO-CYS-PHE-THR-THR-ASX-ASX- X -ARG-ALA-LYS-
```

Fig. 2. N-Terminal amino acid sequences of insectotoxins I_1 and I_4

Fig. 3 shows the amino acid sequence of the insectotoxin I_2. This toxin has polypeptide chain of 62 amino acids with four disulfide bridges. Comparing the structure of I_2 to sequences of toxins from Centrurodies sculpturatus venom it is evident that the greatest extent of homology appears at the positions of the first 22 amino acid residues.

```
                                                    10
ALA-ASP-GLY-TYR-VAL-LYS-GLY-LYS-SER-GLY-CYS-LYS-ILE-SER-
                          20
CYS-PHE-LEU-ASP-ASN-ASP-LEU-CYS-ASN-ALA-ASP-CYS-LYS-TYR-
     30                                                40
TYR-GLY-GLY-LYS-LEU-ASN-SER-TRP-CYS-ILE-PRO-ASP-LYS-SER-
                               50
GLY-TYR-CYS-TRP-CYS-PRO-ASN-LYS-GLY-TRP-ASN-SER-ILE-LYS-
                          62
SER-GLU-THR-ASN-THR-CYS
```

Fig. 3. The amino acid sequence of insectotoxin I_2

One may conclude that insectotoxin I_2 belongs to the homologous group of toxins from Centrurodies sculpturatus venom but it is not toxic to mice. Unfortunately now nothing is known about the localization of the "active sites" in toxins as well as about the role of its single amino acid residues and so it is impossible to elucidate those structural particularities that determine the species specificity of scorpion neurotoxins.

TOXIC COMPONENT OF THE TARANTULA VENOM

Recently a rather comprehensive information has been accumulated about physiological and biochemical characteristics of crude spider venoms. However respective individual toxins data are very limited (Refs 10-11). We carried out the isolation and characterization of toxic component of tarantula Lucosa singoriensis venom and studied some properties of the crude venom. Tarantula venom obtained by water extraction of poison glands proved to be the mixture of at least 12 proteins of high molecular weights mainly. Its LD_{50}, determined by intravenous injections to mice, is 15 mg/kg of body weight, the crude venom being nontoxic to insects. Tarantula venom does not influence whatsoever on the blood serum creatinphosphokinase activity. It does not possess any phospholipase, proteolytic and haemolytic activities but displays a high hyaluronidase activity. Crude venom changes neither frequency nor amplitude of miniature potentials of the endplate. No necrosis were detected when the venom is subcutaneously injected, but the capillary permeability of blood vessels was shown to rise highly. Tarantula venom ($5 \cdot 10^{-5}$ g/ml) induces the sharp contraction of the guinea pig ileum fibers. This effect appears to be connected with the increase of the influx of calcium ions into the myoplasm of both normal and depolarized muscle cells. The separation of the crude venom on Bio-Gel P-10 followed by the ion exchange chromatography on CM-cellulose CM-32 allow to isolate the individual toxic component. The yield of toxin was about 0.4% and its toxicity was twice as much as that of the crude venom. This could be explained either by inactivation during the separation or by sinergism with some other components of the venom. According to the amino acid analysis data tarantula toxin consists of 104 amino acid residues and has the following composition: Asx-10, Thr-5, Ser-3, Glx-14, Pro-2, Gly-6, Ala-8, Cys-10, Val-5, Ile-5, Leu-6, Tyr-2, Phe-4, His-5, Lys-14, Arg-5. The obtained protein contains neither tryptophan residues nor free SH-groups. Its N-terminal amino acid residue is blocked and C-terminal residue is alanine. The preliminary data indicate that tarantula toxin in concentration of $5 \cdot 10^{-7}$ g/ml possesses the action of the crude venom. Thus toxin appeared to be the highest analog of wasp kinins and bradikinine.

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PHYSIOLOGICAL SIGNIFICANCE OF THE CONSTITUENT POLYPEPTIDE CHAINS OF RICIN D

M. Funatsu

Faculty of Agriculture, Kyushu University, Fukuoka 812, Japan

Ricin D is a highly toxic glycoprotein to animals even orally and consists of two different polypeptide chains bound by a single disulfide bond. These polypeptide chains denominated Ile and Ala chain, respectively, after their N-terminal amino acid residues, were possibly separated by the reductive cleavage of the intermolecular disulfide bond with β-mercapto-ethanol at pH 8.2 in the absence of urea.
Ricin D showed a cytoagglutinating action on sarcoma ascite tumor cells simultaneously with toxic function. These physiological functions of ricin D as well as the Ile and Ala chains and the affinities of these proteins for galactose were examined.

TABLE 1. Physiological properties of ricin D, Ile and Ala chains

Physiological properties	Ricin D	Ile chain	Ala chain
MLD_{48}	0.001	0.08	0.3
Inhibition of cell growth	+++++	+++	-
Inhibition of amino acid uptake by the cells	+++++	+	+++
Inhibition of amino acid uptake by cell free system	+++++	+++++	-
Cytoagglutination	+++++	-	+++++

MLD : ug N of ricin protein / g body weight of mouse

TABLE 2. Cytoagglutinating activities of ricin D, Ile and Ala chains

Protein + sugar	Protein concentration (ug/ml) 4	7	8	10	20	65	100	200	300
Ricin D	-	-	±	+	+++	+++	+++	+++	+++
" + 0.2 M glucose	-	-	±	+	+++	+++	+++	+++	+++
" + " galactose	-	-	-	-	-	-	-	-	-
" + " lactose	-	-	-	-	-	-	-	-	-
" + " N-Ac-galactosamine	-	-	-	-	-	-	-	-	-
Ala chain	-	±	+	++	+++	+++	+++	+++	+++
" + 0.2 M glucose	-	±	+	++	+++	+++	+++	+++	+++
" + " galactose	-	-	-	-	-	-	-	-	-
" + " lactose	-	-	-	-	-	-	-	-	-
" + " N-Ac-galactosamine	-	-	-	-	-	-	-	-	-
Ile chain	-	-	-	-	-	-	-	-	-

As shown in Table 1, ricin D strongly inhibited the growth of cells as well as the amino acid uptake by the intact cells and also the uptake by the cell free system. On one hand, the Ile chain slightly affected the growth of cells and not the amino acid uptake by the intact cells. However, it inhibited remarkably the amino acid uptake by the cell free system. On the other

hand, the Ala chain was hardly effective on these inhibitions. Nevertheless, it has a strong cytoagglutinating potency and a remarkable affinity for galactose similarly to ricin D, whereas the Ile chain has not such functions.

Table 2 shows the inhibitory effects of various sugars on the cytoagglutinating activities of ricin D, Ile and Ala chains.

It is obvious from the table, ricin D and the Ala chain brought about the cytoagglutination depending upon their concentrations in the absence of sugar, but not the Ile chain. However, the cytoagglutination was completely inhibited by either galactose or lactose and or N-acetyl galactosamine, except glucose.

In the other experiment, it was found that ricin D and the Ala chain were absorbed through Sephadex column.

From the evidences mentioned above, it seems reasonable to conclude that the Ile chain is the toxic principle of ricin and the Ala chain contributes to the specific binding of ricin to the surface of cells. These two polypeptide chains, therefore, are essential for the toxicity of ricin D.

RECOGNITION OF PEPTIDE HORMONES AND KININS: MOLECULAR ASPECTS OF THE PROBLEM

G. Chipens, F. Mutulis and S. Galaktionov

Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR, Riga, USSR

Abstract - Some aspects of space structure complementarity of oligopeptide hormones and receptor sites and the possible contribution of intramolecular ionic-type interactions to the stability of the "biological" conformation of peptide bioregulator molecules have been discussed. The theoretical conformational analysis of the bradykinin molecule performed earlier revealed close proximity of the C-terminal carboxyl group and the guanino group in the arginine residue; a cyclic analogue of bradykinin has been synthesized, in which the close location of the two groups was stabilized by covalent bonding. The CD spectra of the synthesized compound were identical with those of bradykinin, and the new compound was active in eliciting a strong and prolonged depressor effect in rats.

Interaction of molecules or complex systems thereof underlies most regulatory processes operative in the living cells and organisms. According to our present knowledge, interaction of a low-molecular components - "effector" (hormones, antigens, various modulators, substrates or inhibitors of enzymatic reactions, etc.) with high-molecular components - "receptor system" (cell membrane receptors, antibodies, enzymes, etc.) results in a mutually-induced alteration of the stereo-electronic structure of the components and, consequently, in altered functional properties of the resultant complex. Regulatory processes are known to be characterized by high specificity, owing to the correspondence of geometrical forms and the appropriate spacing of functional groups in the effector-receptor pair. As evidenced by X-ray analysis, specific recognition and binding occuring in the course of protein-protein interaction is provided by a small area on their surface characterized by rigid structure with loops or ledges on the effector molecule and hollows - "pockets" or "slots" on the receptor (Refs.1-4). The active centres of protein effectors (enzyme inhibitors, antibodies, etc.) comprise, on the average, 6-8 amino acid residues (Refs. 1-5). Geometry of these "recognition and binding" centres is spatially stabilized by means of loops or β-bends and is "cemented" by the overall space structure of the protein globule. The ends of the "active" fragments (especially in the case of mini-proteins) are frequently immobilized by means of disulphide bonds (Refs.6 & 7).

The concept of biochemical universality makes it conceivable to expect that the process of "recognition" and binding of peptide hormones and kinins to receptors located on the cell membrane would be equally determined by similar "active" regions (containing 6-8 amino acid residues) on the peptide molecules. However, in contrast with the proteins, the space structure of peptide effectors in solution is not so well-defined: it is most likely that there exists an equilibrium of several equally stable conformers in solution, the best suited of them being "selected" by the receptor. The existence of such a limited set of conformers, characterized by relatively rigid space structures seems to be a prerequisite for the purposeful transfer of information to occur at the molecular level and for the effector-receptor interaction involving peptide effectors to be specific (Ref.8).

Extremely interesting, in this respect, are the peptide space structure data obtained using semi-empirical conformational analysis, which provides evaluation of intramolecular conformational energy for each possible molecular conformation as a measure of its stability (Refs.9-10). Having acquired similar data for a number of peptide molecules, it may be possible to establish the common principles shared by the space organization of the peptides. In fact, our studies along this line have led us to the establishment of a number of characteristic features inherent in the structural organization of low-molecular peptides which are well-correlated with our earlier findings on their functional organization (Ref.8). Thus, the conformational calculations performed in our laboratory for the molecules of biologically active peptides - bradykinin, angiotensin, Met-enkephalin, tuftsin, reveal the presence in all cases of a more or less limited set of stable conformers characterized by compact structures with N- and C-terminal parts located in close proximity, hence, quasicyclic structure being a prominent feature of their space organization (Refs.11-14). In each case, the observed structure of the molecule is determined by the overall system of intramolecular interactions as a whole; one can discern, however, several structural elements, each of them contri-

buting to the stability of the quasicyclic molecular structures. The first to be mentioned in this connection are the glycin and proline residues, distinquishable from the rest of the natural amino acid residues present in the amino acid sequence of the above peptides, owing to the characteristic steric conditions of their backbone (Ref.15). The presence of proline in the peptide chain is known to substantially limit conformational lability of the peptide backbone, the effect not being confined to the position occupied by proline itself, but being also present in the preceding position (ref.16); consequently, proline plays the role of a conformationally rigid element in the peptide chain. Conversly, the glycine residue exhibits enhanced conformational lability and is a kind of "conformational joint-hinge" providing close spacing of N- and C-terminal parts of the peptide chain. This fact is in good agreement with the results of calculations performed on the glycine-containing peptides bradykinin and Met-enkephalin, as well as the recent results with luliberin (Ref.17): the most stable backbone conformations in all these peptides are characterized by the glycine residue conformation which is sterically inconsistent for any other type of amino acid residues. The specific role of glycine and proline residues in peptide molecules is also indicated by their increased relative content (~12%), as compared with proteins, in the total amino acid composition of short (up to 30 amino acid residues) peptides (Ref.18).

The compact quasicyclic structures of peptides mentioned are stabilized predominantly by non-bonded interactions; an additional factor contributory to their stability is present in all the cases described above (Table 1), viz.strong electrostatic interaction between the functional groups carrying alternative charges - guanidyl group of arginine residue, ε-amino group of lysine residue or α-amino group and C-terminal carboxyl group. Such interaction occurs between ionogenic groups in the molecule on their close spacing, the process being accompanied by the appropriate hydrogen bonding. It is notable that in aqueous solution the above interactions are weakened by hydration, whereas in non-polar medium, i.e. in the course of effector-receptor complex formation they are significantly enhanced leading to increased relative content of quasicyclic conformers of the peptide effector molecule. The aforesaid gains indirect substantiation from the protein-protein interaction kinetics studies on enzymes binding to their specific inhibitors. The results of these studies demonstrated the occurance of desolvation of the interacting surfaces and charged side groups in amino acids during the early stages of interaction (Ref.1). Furthermore, according to the results of our quantum chemical calculations carried out in vacuo, the energy of interaction between guanidyl and carboxyl ions was estimated to be about -55 kcal/mole, the value comparable with the dissociation energy of covalent disulphide bond.

TABLE 1. Quasi-cyclic structures of oligopeptides, as characterized by the data of semi-empirical conformational analysis

Peptide	Type of interaction	Size of quasi-cycle	Ref.
Bradykinin ArgProProGlyPheSerProPheArg	$Arg^1\delta\text{-}G^+$ † ... $^-OOC(Arg^9)$	1-9	(11)
Angiotensin AsnArgValTyrValHisProPhe	$Arg^2 2\delta\text{-}G^+$ ... $^-OOC(Phe^8)$	2-8	(12)
Met-Enkephaline TyrGlyGlyPheMet	$\alpha\text{-}NH_3^+$ ... $^-OOC(Met^5)$	1-5	(13)
Tuftsin ThrLysProArg	$Lys^2\varepsilon\text{-}NH_3^+$ ... $^-OOC(Arg^4)$	2-4	(14)
BPP pGlyTryProArgProGluIleProPro	$Arg^4\delta\text{-}G^+$ ... $^-OOC(Pro^9)$	4-9	(23)

† G^+ - delta guanidyno group: $-NH=\overset{\oplus}{C}(NH_2)(NH_2)$

Thus, the results obtained using semi-empirical conformational analysis suggest important implication of ionogenic groups in the formation and maintenance of the space structure in the peptide molecules, especially during the process of their interaction with receptors. It is appropriate to recall, in this connection, that we had postulated previously the presence of typical elements in amino acid sequences in the molecules of biologically active low-molecular peptides. These sequences contained an ionogenic basic amino acid incorporated between proline and valine residues, on the one side, and acidic amino acid and glycine, on the other side (Refs.8,20,21). These fragments were also shown to exhibit a rather wide range of non-specific biological action, but their conjugation with the "shortest" peptide fragments induced a significant rise in the specific activity of these fragments (Ref.22). However, there is no clear-cut evidence, at present, demonstrating direct implication of these fragments in the activation of receptor during effector-receptor interaction. At the same time, the finding concerning the space organization of the peptide effectors described above serve, in our

opinion, to emphasize the structural role of the detected fragments in the oligopeptide molecules.
The localization of ionogenic side chains, which participate in the interactions responsible for the closure of quasicyclic structures in non-cyclic peptides is somewhat similar to the localization of disulphide bonds, responsible for the closure of ring structures in cyclic peptides (see hypothesis on the equifunctionality of ionic and disulphide bonds (Refs.8,27). This can be viewed as another indication of the important role reserved for quasicyclic (or cyclic) structures in the molecules of biologically active peptides comparable size values (approximately, 6-8 amino acid residues) of functionally active disulphide cycles and tentative quasicycles in the molecules of some peptides (Refs.8,27).
The above consideration lead to assume that high selectivity and specificity observed during the processes of mutual "recognition" and peptide effector binding to the receptor are due to the interaction of conformationally rigid quasi-cyclic sturctures of the effectors with the receptor "pockets", providing large interacting areas and multiple contact sites. It can be expected, therefore, that covalent fixation of the stable quasi-cyclic structures of the effector molecule "selected" by the receptor will result in increased interaction efficiency. The hypothesis was put to trial using bradykinin, a peptide endowed with high specific activity, exhibiting a variety of biological effects. The stable conformations calculated for this compound demonstrated, in agreement with spectroscopy data (ref.24), considerable prevalence of 4 types of quasi-cyclic structures for the peptide backbone (2 of them are depicted in Fig.1) in which guanidyl group of Arg^2 is located close to C-terminal carboxyl group.

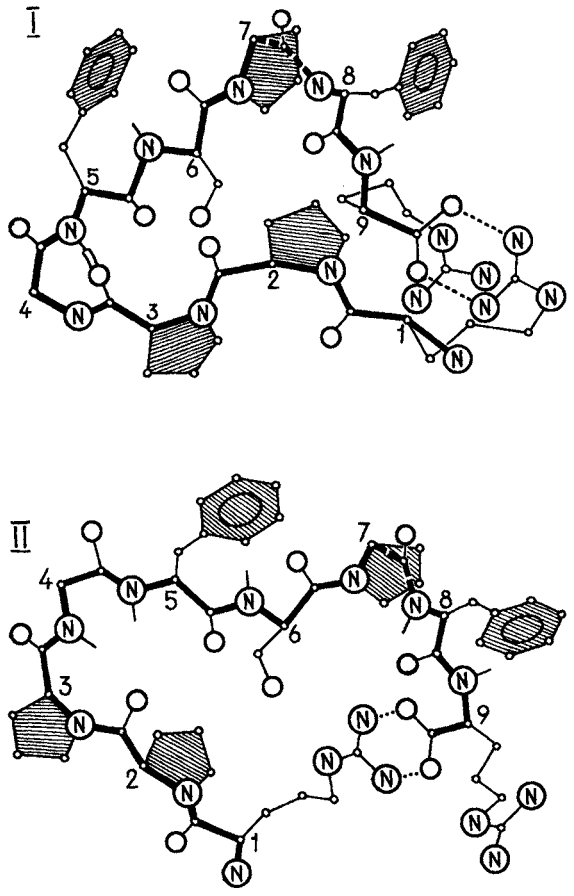
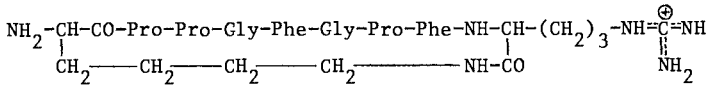


Fig. 1. Two of the four types of the most stable quasi-cyclic bradykinin structures (Ref.24).

According to calculations, these structures show highest stability also in the absence of electrostatic interaction of the above mentioned ionogenic groups. Bearing these results in mind, we undertook an attempt to synthesize bradykinin analogue in which mutual location of N- and C-terminal groups, as predicted by the calculations, was stabilized by covalent bon-

ding. The model compound, cyclo-(N-ε'-lysine, 6-glycine)-bradykinin:



was synthesized using routine methods of peptide synthesis and was purified to produce a homogeneous substance, as evidenced by chromatographic assays, with predicted molecular weight and amino acid and elementary composition. Comparison of CD spectra of bradykinin with those of its cyclic analogue in aqueous solution revealed their complete identity, thus confirming the hypothesis claiming likeness of their stable structures. Further evidence in favour of this hypothesis was presented by the results of biological testing which appeared to be even more telling (see Note a).

The mode of bradykinin action on the smooth muscles is known to be subject to considerable variation depending on the tissue and species of origin and other factors (ref.25). It is noteworthy that competition studies performed on a large number of bradykinin analogues failed to provide so far any conclusive evidence of antagonistic relationship (refs.25 & 26). Peculiarity of bradykinin, as compared with other peptide compounds exhibiting myotropic activity, is further substantiated by the results of biological experiments carried out on its cyclic analogue. It must be noted, first and foremost, that the chemical modification caused a considerable change in the scope of its action. For example, bradykinin and cyclobradykinin were found to produce in rats depressor effect characterized by equal magnitude (Fig.2),

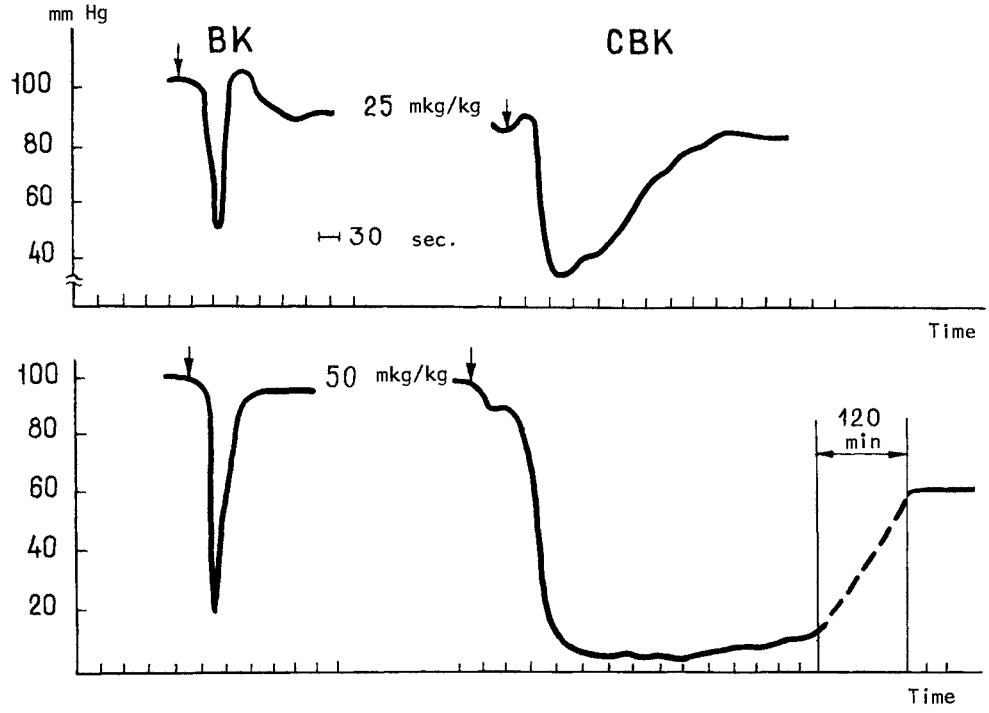


Fig. 2. The effect of bradykinin (BK) and cyclobradykinin (CBK) on arterial pressure of urethane-anaesthetized rats.

the effect being, however, more prolonged in the case of the latter compound. Depressor action of "linear" bradykinin, applied at 50 mg/kg was abolished 1-5 min following the administration, whereas cyclobradykinin at the same dose induced a sustained reduction in blood pressure by 30-40 mm Hg lasting for 2 hr; full recovery was not attained for several consecutive hours thereafter. On the other hand, cyclobradykinin appeared to be completely inactive when administered to guinea-pigs under the same conditions. Increase in vascular permeability in response to cyclobradykinin administration to rats was 25 times weaker than in the case of bradykinin. In vitro experiments with colon ascendens of the rat demonstrated lack of myotropic activity in cyclobradykinin; no antagonism towards bradykinin action was also noted in these experiments.

It is obvious that the results presented above are far from being complete; nonetheless, characteristic mode of biological action found for the cyclic analogue of the non-cyclic peptide demonstrates feasibility of approach aimed at purposeful searching of "conformatio-

Note a. Preliminary biological testing of cyclobradykinin was performed in our laboratory by a team of workers led by Dr.V.Klusha.

nally limited" analogues of peptide molecules.
It is known that the early attempts to carry out covalent fixation of particular fragments of the molecule which are brought in close proximity, met with little success (Ref.28). However, recently there has been a report by Veber et al. who synthesized a highly active cyclic analogue of somatostatin (Ref.29). It is hoped that new analogues of other peptide hormones and kinins will be obtained in due course on the basis of our knowledge of the three-dimensional structure of their molecules.

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SYNTHESIS AND STRUCTURE-FUNCTIONAL STUDIES ON HEME PEPTIDE MODELS OF NATURAL OXYGEN-BINDING HEMOPROTEINS

R. P. Evstigneeva

Lomonosov Institute of Fine Chemical Technology, Moscow 119831, USSR

Abstract - The synthesis of amino acid sequences 57-100 human hemoglobin β-subunit fragments was achieved. The complexes of hemin with peptide were obtained and tested on oxygen binding. The interaction of heme peptide complexes with phospholipid bilayer membranes was studied. The possibility of liposome using for preparation of reversibly oxygenated ferrous porphyrin models was shown.

INTRODUCTION

To study the influence of the nearest amino acid surroundings of heme on their affinity for oxygen the synthesis of heme peptide complexes was accomplished. The peptide synthesis was elaborated for obtaining these compounds. In the discussion to be presented here the routes to synthesis of amino acid sequences 70-100 of human hemoglobin β-subunit fragments are considered.

SYNTHESIS OF PEPTIDE FRAGMENTS

The synthesis of peptide fragments was carried out by classical and solid-phase methods. The fragments 57-69, 70-77 and 78-100 of human hemoglobin β-chain (Fig. 1) were synthesized using mainly active ester and azide methods and partial protection of the trifunctional amino acids (Ref. 1). The synthesis of the amino acid sequence 70-100 was achieved also by solid-phase method utilizing condensation of peptide blocks on the polymer support. This synthesis requires the most detailed consideration. In accordance with the general plan of the synthesis (Scheme 1), amino acid sequence 70-100 was divided into four fragments: 70-73, 74-81, 82-88 and 89-100 so as the N-terminal amino acids be less inclined to racemization. The break of peptide bond between Asp 73 and Gly 74 was made to avoid transpeptidation in the acidic conditions of peptide synthesis and in the course of peptide separation from the polymer. The peptide bond between these amino acid residues was formed on the final stages of the synthesis. t-Butyloxycarbonyl

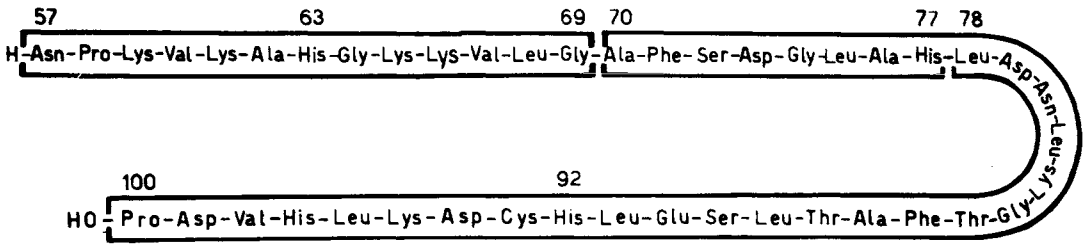
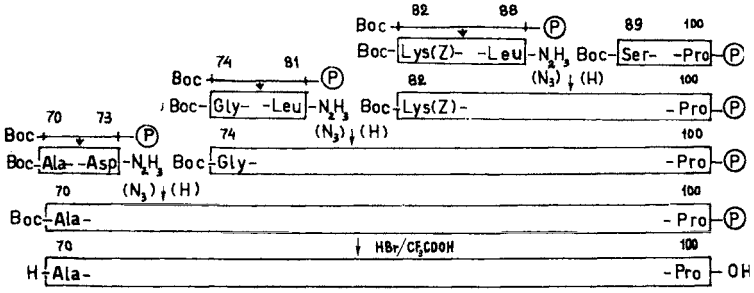


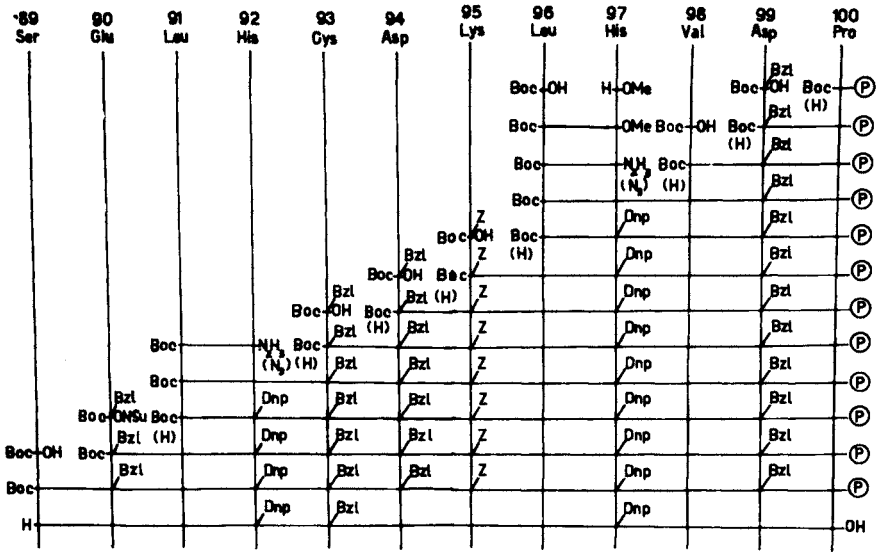
Fig. 1. Amino acid sequence 57-100 of human hemoglobin β-chain. His 63 is distal to heme, His 92 is proximal to heme



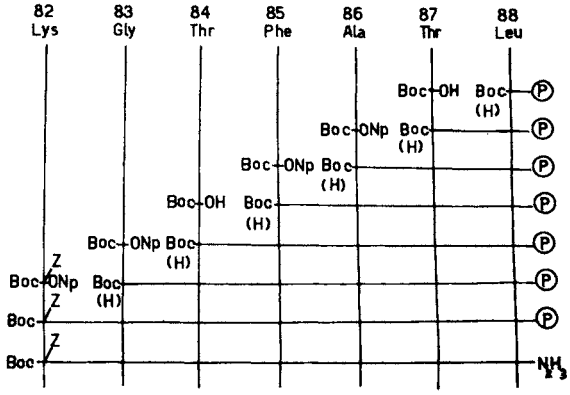
Scheme 1. Synthesis of polypeptide 70-100 by fragment condensation on the polymer

(Boc) group was utilized for the protection of α-amino group of lysine and benzyl group for serine 72, β- and γ-carboxyl of aspartic and glutamic acids. Side chains of aspartic acids 73 and 79, serine 89, histidine 77, asparagine and threonine were not protected. Imidazole rings of histidine 92 and 97 were blocked by 2,4-dinitrophenyl(Dnp) protection (Ref. 2). Chloromethylated copolystyrene-divinylbenzene (2%) resin (4.5% Cl) was used as a polymer support. C-Terminal amino acids were attached to the resin by treatment of resin with equimolar (to chlorine) quantity of cesium salt of Boc-amino acids in dimethylformamide at 50° for 20 hours. The substitution level was 0.5-0.6 mmol of Boc amino acid/g of resin.

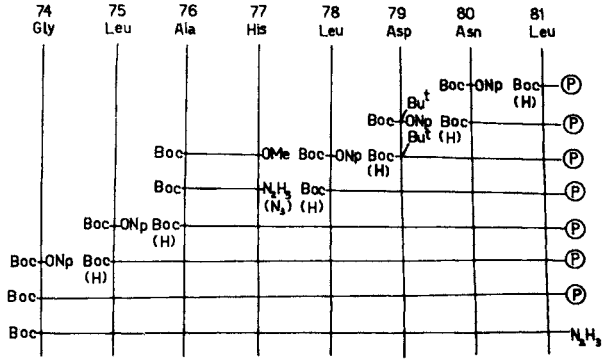
The chain elongation from C-terminal amino acid to N-terminus was carried out by using active ester, dicyclohexylcarbodiimide and mixed anhydryde methods. 50% solution of trifluoroacetic acid in methylene chloride was used for removing the Boc groups. The peptide resins were cleaved by hydrazinolysis. The coupling of fragments was carried out by azide method on the polymer. Dodecapeptidyl resin, containing amino acid sequence 89-100 with proximal histidine was obtained according to the scheme 2 (Ref. 3). The routes of synthesis of heptapeptide 82-88-, octapeptide 74-81- and tetrapeptide 70-73-resins are shown in schemes 3-5, respectively. Attachment to polypeptide 89-100-resin of peptide fragments 82-88, 74-81 and 70-73 was achieved by azide method. The use of succinimide and pentafluorophenol in azide condensation allowed to increase the yields of polypeptides. Resulting henetriacontapeptide resin was obtained in 75% yield. The cleavage of polypeptide 70-100 from the resin was carried out by treatment with hydrogen bromide in trifluoroacetic acid in the presence of anisole. The removal of N^{im}-2,4-dinitrophenyl blocking group was accomplished with 2-mercaptoethanol at pH 8. The purification of polypeptide was carried out by gel chromatography on Sephadex G-25 and G-75 followed by ion exchange chromatography on CM-cellulose with the gradient of pH and buffer concentration. The homogeneity of the purified polypeptide was ascertained by paper electrophoresis and paper chromatography. The amino acid composition was determined by amino acid analysis of the hydrolysate, and N-terminal amino acid - by dansyl method. Since unprotected trifunctional amino acids were used in the synthesis, the possibility of side reaction was tested. The examination of lactonization side reaction involving neighbouring unprotected hydroxy groups and carboxyl functions of dicarboxylic acids was carried out using model peptides Boc-Ala-Phe-Ser(Bzl)-Asp-Ⓟ and Boc-Ser-Glu(Bzl)-Leu-Ⓟ. There was no lactonization under the conditions of peptide synthesis for sequences Ser-Asp (72-73) and Ser-Glu (89-90). We examined the possibility of O-acylation side reactions of unprotected threonine and serine hydroxy groups the conditions of peptide synthesis involving p-nitrophenyl esters and dicyclohexylcarbodiimide in the presence of 1-hydroxybenzotriazole, as well as upon interaction with p-nitrophenylacetate used for blocking unreacted amino groups on the polymer. With this purpose a number of N-protected serine and threonine containing model peptide resins were synthesized and their reactivity towards acylating reagents was tested. The efficiency of acylation of serine and threonine was controled by quantitative amino acid analysis of peptide resins. O-Acetylpeptides were detected by comparing chromatographic mobilities of products cleaved from initial and acetylated peptide resins. There was no O-acylation of serine and threonine in the course of peptide synthesis or upon p-nitrophenylacetate treatment of the peptide resins. However, even small excesses of p-nitrophenylacetate or Boc-amino acid p-nitrophenyl esters acylated hydroxy groups of serine and threonine in the presence of 1-hydroxybenzotriazole. Full acetylation was observed with large excesses of p-nitrophenyl acetate, 1-hydro-



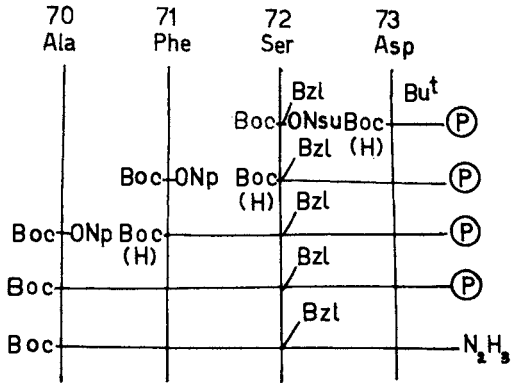
Scheme 2. Synthesis of the fragment 89-100



Scheme 3. Synthesis of the fragment 82-88



Scheme 4. Synthesis of the fragment 74-81

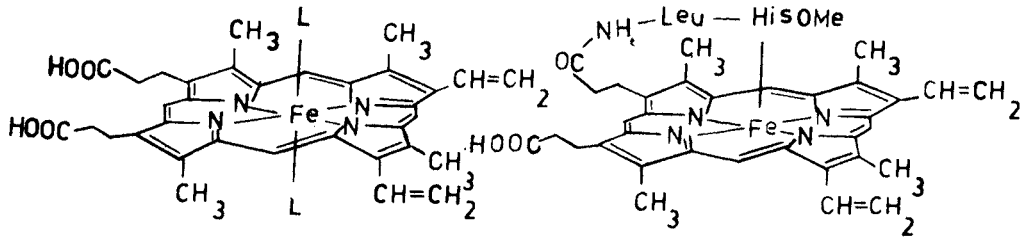


Scheme 5. Synthesis of the fragment 70-73

xybenzotriazole and triethylamine for at long reaction time. We did not detect side reaction at unprotected β-carboxyl group of aspartic acid by treating H-Asp(OH)-(P) with p-nitrophenylacetate or its mixture with 1-hydroxybenzotriazole. Thus it is possible to use p-nitrophenylacetate as acetylating agent for blocking the unreacted amino groups on the polymer in the presence of unprotected hydroxy and carboxy groups of amino acids. Optical purity of synthesized peptides was checked by gas liquid chromatography on the optically active stationary phases (Ref. 4). The peptides cleaved from the resins by hydrogen bromide in trifluoroacetic acid were hydrolized in acidic media. The resulting amino acid mixture was transformed into the mixture of N-TFA-derivatives of amino acid isopropyl esters. Racemization was not observed in the case of peptide bond formation by dicyclohexylcarbodiimide method in the presence of 1-hydroxybenzotriazole, active p-nitrophenyl ester method with addition of 1-hydroxybenzotriazole and azide method in the presence of pentafluorophenol. The racemization extent in the method of mixed anhydrides was less than 1%.

SYNTHESIS OF HEME PEPTIDE COMPLEXES

For studying complexing of hemin with peptides a several human hemoglobin β-chain fragments containing proximal and distal histidine were chosen. The influence of peptide properties and of its binding with heme on iron electronic configuration was tested. By interacting hemin with peptides in chloroform solution the six-coordinate complexes (I) were easily obtained and their low-spin state was proved by ESR and Mössbauer spectroscopy data. Complex formation was retarded as a result of imidazole hydrogen substitution for 2,4-dinitrophenylgroup. The same effect was observed if sterically hindered amino acid such as leucine was neighbouring to histidine. In these cases high-spin species (20%) were detected, which indicated the presence of mixture of five- and six-coordinate complexes. The five-coordinate complexes (II) were obtained by covalent binding of hemin with peptide.



The structure of the complexes was elucidated by electronic spectra. The oxygenation becomes easier with the increase of the peptide length and their hydrophobicity. Rapid irreversible oxidation was observed in the case of short peptides.
Bilayer phospholipid membrane was used by us for formation of hydrophobic environment (Ref. 5). Vesicular membrane also allowed solubilization of water-insoluble hemin derivatives. The influence of phospholipid membrane on the oxygenation was studied with simple models of hemin and 6(7) N-leucylhistidylprotohemin IX methyl ester. Phosphatidyl choline employed for preparation of vesicular membrane was obtained from egg yolk. Hemin and their complexes with peptides were incorporated into liposomes without disturbance their entity, as was verified by NMR-spectra (signal of $N^{+}(CH_3)_3$ group was split into two peaks). Sodium dithionate was used for the reduction of ferri- to ferrocomplexes. Oxygenation was carried out at room temperature and controlled by electronic spectroscopy. Heme oxidated easily in these conditions and heme peptide complex oxygenated. Thus we constructed the model of oxygen carrier which can be studied in physiological condition.

Acknowledgements - I am most grateful to all my collaborators whose names appear in the references. I wish to thank Dr. S.V.Vitt for analysis of optical purity of synthesized peptides.

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PROTEINS OF THE COTTONSEED

P. Kh. Yuldashev, M. A. Kuchenkova, T. S. Yunusov and Sh. S. Azimova

Institute for Chemistry of Plant Substances, Uzbek SSR Academy of Sciences, Tashkent, USSR

Abstract - A study has been made of some proteins of the cottonseed, including the structural determination of 7S globulin, of subunit C of 11S globulin and of an esterase, all isolated from the aqueous extract of the seeds.

Cotton is the major agricultural plant of Uzbekistan. Not only is it valuable for its fibers; its seeds are used in feed and are potential sources for food protein. Some of its proteins are endowed with enzymatic properties.

We are currently investigating proteins of the cottonseed from four aspects: a) isolation, purification and structure; b) physicochemical properties; c) enzymatic properties; d) utilization as food products. We believe that such study in combination with that of their formation and functioning will facilitate deeper penetration into molecular processes in the plant and thus pave the way for their directed modification so as to better utilize its potentialities.

We have studied the water soluble fraction of the cottonseed protein which constitutes ca. 5% by weight of the oil-free seeds (Ref. 1), and in which we have found a variety of enzymes:proteases (Refs. 2,3), malate dehydrogenases (Refs. 4,5) , aspartate aminotransferases (Ref. 6) and lipases (Refs. 7-10). It was also shown that the leaves of the cotton plant manifest phenol oxidase activity (Ref. 11).

A scheme for the isolation of these enzymes and their purification to the homogeneous state has been developed and the chemical and enzymic properties of the purified specimens have been determined.

Of the proteins, about 20% were found to be globulins. Besides being one of the major components of the cottonseed food proteins, plant globulins are of interest in the ability of some of them to exert specific effects on animal cells.

We have found five globulins in the protein extract. Of these the two major components comprised over 90% of the overall protein. They had sedimentation coefficients of 7S and 11S, corresponding to molecular weights of 130,000 and 280,000, respectively.

Treatment of the 7S globulin with urea and with 1% sodium dodecylsulfate (SDS) showed it to be an aggregate of polypeptide chains, evenly divided into two subunits,, each with four polypeptide chains of MW 16.000 and 18.000, respectively. By comparing the peptide maps of the original protein, its subunits and the glycoprotein fraction it could be seen that the difference between the two subunits lies in the glycopeptide region (Ref. 12). Each polypeptide chain contains a disulfide bridge and one tryptophan residue.

The above-said was substantiated by direct determination of the amino acid sequence of the protein and non-carbohydrate polypeptide, both substances in the reduced, carboxymethylated (CM) state, effected by chymotryptic degradation followed by gel filtration of the resultant peptides through a Sephadex G-25 column and subsequent ion-exchange and paper chromatography, and thin-layer electrophoresis on cellulose. The amino

acid sequence of the tryptic peptides was determined by Edman degradation, the amino acid residues being identified as their phenylthiahydantein and/or dansil derivatives (Ref. 12). To reassemble the protein from the peptides in the hydrolyzates, overlapping peptides were obtained by chymotryptic hydrolysis (Ref. 13) of the CM-globulin, with identification of the fragments as in the tryptic case. The tryptic peptides of both the non-carbohydrate subunit and protein complex were found to be of the same composition and to contain the same N-terminal amino acid residues, while differing in the C-terminus of the polypeptide chain which carried the carbohydrate moiety. The latter comprising 4-5 mannose and 3-4 glucosamine residues was attached to the polypeptide at only one point, the amide group of Asn-129. The only other differences were in the dicarboxylic amino acid residues in positions 46, 83, 106, 125 and 127, the carboxyl groups of which were free in the case of the carbohydrate-containing polypeptides and in the form of amide groups in the non-carbohydrate subunit.

The primary structure of the carbohydrate-containing subunit exemplifying the amino acid sequence of the entire 7S globulin is thus as follows:

```
                                                  10
Arg-Gln-Gln-Lys-Ser-Ala-Pro-Gln-Gly-Phe-Gln-Leu-Asn-Arg-
                          20
Val-Pro-Val-Ala-Gly-Phe-Thr-His-Gln-Asn-Lys-Val-Ser-Gln-His-
 30                                                  40
Pro-CmCys-Leu-Ala-Arg-Phe-His-Asn-Gly-Gln-Arg-Phr-Gly-Ile-
                               50
Asn-Phe-Glu-Arg-Leu-Gly-Tyr-Gly-Ser-Gln-Arg-His-Ser-Asn-Gln-
      60                                                  70
Trp-Gln-Arg-Ser-Gly-Gln-Tyr-Phe-Ala-Pro-Gln-Asn-Leu-Val-Met-
                               80
Asn-Asn-His-Gln-Ile-Arg-Leu-Ala-Asn-Glu-Asn-Lys-CmCys-Phe-
          90                                             100
Tyr-Gln-Ser-Gly-Asn-Arg-Val-Arg-Leu-Ala-Gln-Thr-Arg-Gly-Ala-
                                    110
Val-Lys-Leu-Glu-Ala-Phe-Val-Gly-Ser-Arg-Gln-Asn-Phe-Leu-Val-
     120
Gly-Gln-Asn-Ser-Ala-Lys-Phe-Glu-Gln-Asp-Val-
     130
Asn-Ile-Thr-Ser-Ala-Leu-Val
```

$(GlcNHx)_{3-4}(Man)_{4-5}$

The other protein from cottonseed, the structural determination of which will be described here is the 11S globulin. Treatment with 8M urea similar to that for 7S globulin revealed that the 11S globulin in contrast consisted of subunits (A, B and C) of differing amino acid composition (Ref. 14). Initially, on filtration through DEAE-cellulose in 8M urea two peaks were obtained, one of A and B together and the other of subunit C. The subunits A and B separated on filtration through Sephadex G-75 in 0.1% SDS. Subunit C comprised about 60% of the protein complex and contained a carbohydrate moiety consisting of 1 glucosamine and three mannose residues. In order to determine the amino acid sequence of subunit C it was also subjected to the above-described tryptic and chymotryptic degradation (Refs. 15,16). Isolation and purification of the resultant peptides was carried out as usual, while the insoluble part of the tryptic hydrolyzate was subjected to additional partial acid hydrolysis. The amino acid sequence of all the peptides was determined by Edman degradation, using the dansyl derivatives, and in the case of peptides with amidated dicarboxylic acids by the direct Edman method (Ref. 17). The subunit C does not contain methionine, but cyanogen bromide cleavage led to rupture of the Asp-Pro bond, thereby facilitating reassembly of the polypeptide chain (Ref. 18). The total amino acid sequence of this subunit consisting of 135 amino acid residues was thus completely determined. Investigation of the acid hydrolysis and periodate oxidation products of the methylated subunits S showed that its carbohydrate moiety was attached to the protein through the glucosamine residue, while analysis of the overall data led to the conclusion that the point of attachment to the protein is at Asn-20.

The structure of the subunit is thus:

10
His-Asn-Gln-Trp-Glu-Glu-His-Gly-Asn-Phe-Arg-Gly-Asp-Ala-
20 *
Glu-Glu-Leu-Val-Ile-Asx-Ser-Thr-Pro-Arg-Val-Gln-Gly-Asn-
30 40
Gln-Arg-Leu-Ile-Ser-Phe-Val-Ala-Asx-Glx-Arg-Val-Thr-His-
50
Lys-Asp-Gln-Arg-Gly-Gln-Glu-Ser-Arg-Gln-Ile-Asn-Gly-Phe-
60 70
Leu-Glu-His-Glu-Asn-Arg-Glx-Als-Gly-Val-Thr-Glu-Ala-Asx-
80
Gly-Leu-Glx-Glx-Thr-Phe-Ser-Glx-Arg-Gln-Phe-His-Gln-Asn-
90
Arg-Lys-Phe-Ile-Glx-Glu-Asn-Arg-Ile-Pro-Gln-Ala-Ser-Ala-
100 110
Arg-Gln-Asn-Pro-Gln-Asn-Gln-Val-Leu-Gln-Arg-Glu-Thr-Phe-
120
Gln-Ser-His-Gln-Asn-Arg-Gln-Glu-Gly-Asp-Ile-Val-Ala-Leu-
130
Gly-Glu-Gln-Asp-Arg-Ser-Gln-Gln-Asn

(* - Glc-NHX - Man $\begin{matrix}\text{Man}\\\text{Man}\end{matrix}$)

Of the enumerated cottonseed enzymes, here will be described our determination of the quaternary and primary structure of an esterase that was isolated by precipitation with ammonium sulfate from an aqueous phosphate buffered (pH 7.4) extract of ground, de-oiled cotton seed kernels. It was reprecitated from the same kind of solution and purified by twofold chromatography on a DEAS-cellulose column and gel filtration through Sephadex G-75. The enzyme has pH optima at 25° of 7.4 for tributyrin and 9.5 for triacetin. It was completely inhibited by diisopropylfluorophosphate (Ref. 19). The apparent molecular weight of the enzyme was determinated to a first approximation by gel filtration and ultracentrifugation to be about 40,000 at pH 7.4. On lowering the pH to 4.5 the enzyme dissociated into two active subunits each of molecular weight ca. 20,000, as again determinated by gel filtration and ultracentrifugation. Treatment of the subunits with 2:1 methanol-chloroform mixture showed them to contain lipids. The latter must have been instrumental in holding together two inactive equal molecular weight protomers of the subunit which is dissociated in the process of the extraction. The similarity of the four polypeptide chains comprising the esterase molecule was confirmed by chromatoelectrophoresis.

Since the enzyme contained four methionine residues, seven arginine residues, and four lysine residues, as found from determination of its total amino acid content on an LKB analyzer, it was decided to carry out its sequencing by tryptic hydrolysis, cyanogen bromide cleavage and thermolytic and chymotryptic splitting of the cyanogen bromide fragments. The peptides were separated by gel filtration, ion exchange, and paper chromatography, and with the aid of peptide maps. Analysis of the sequence of the isolated peptides was carried out by direct Edman degradation and in combination with dansylation. As a result the primary structure of a subunit of the enzyme comprising 116 amino acid residues was found to be the following:

10
Met-Arg-Gly-Asp-Gly-Asn-Ser-Glu-Ala-Met-Thr-Lys-Gln-Ala-
20
Cys-Asp-Gln-Ser-Phe-Ala-Pro-Asp-Val-Ala-Asn-Gly-Gln-Gly-Thr-
30 40
Gln-Lys-Arg-Ala-Gly-Gln-Ser-Ile-Asp-Ala-Pro-Phe-Ser-Arg-Gln-
50
Gly-Gln-Leu-Thr-Asp-Leu-His-Cys-Met-Pro-Trp-Asp-Thr-Arg-Ser-
60 70
Ala-Gly-Asn-Ser-Pro-Gln-Ala-Leu-Glu-Phe-Arg-Gly-Gly-Asp-Ser-
80
Gly-Pro-Tyr-Ile-Ala-Val-Thr-Gln-Glu-Leu-Lys-Met-His-Ser-Asp-
90 100
Gly-Cys-Thr-Ile-Arg-Thr-Arg-Pro-Gly-Asp-Thr-Gln-Ser-Val-Ala-
110
Glu-Leu-Lys-Phe-Val-Pro-Gln-Gly-Ala-Leu-Asp-Tyr

It is noteworthy that the 70-76 sequence of the chain coincided with that of the active center for serine proteinases (Ref. 20).

Besides the chemical study of the cotton seed proteins we briefly note that methods have also been developed for obtaining nutrient proteins from cotton seed cake. Comparative data were obtained on the nutrient values of the protein extracted from the cake and from cotton seed de-oiled by various methods in the laboratory. The dependence of the composition and yields of nutrient proteins on the method of de-oiling and changes in the technological scheme has been investigated in detail.

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INSULIN-DEPENDENT CYTOPLASMIC REGULATOR. SEPARATION, IDENTIFICATION AND INVESTIGATION OF INSULIN-LIKE ACTION

J. Ch. Turakulov and M. Ch. Gaynutdinov

Institute of Biochemistry of the Academy of Sciences, UzSSR, Tashkent, USSR

Abstract - Thermostable glycolipoprotein was isolated and purified from rat liver cytoplasm. The glycolipoprotein in small concentrations causes the increase in the maximum amount of calcium ions, which can be accumulated in rat liver mitochondria. Glycolipoprotein activity in cytoplasm is increased by insulin injection. The amino acid composition of ICR (insulin-dependent cytoplasmic regulator) differs from the amino acid composition of insulin. ICR inhibits the transport of oxidized substrates in mitochondria. The ICR introduction results in a great reduction in blood sugar in rats with alloxan diabet. The ICR also inhibits glucose output from the liver slices. It is supposed that the ICR is a mediator of insulin action on the liver metabolism.

Identification on cyclic 3'5' AMP as a mediator of peptide hormones action on metabolism (Refs. 1,2) was a mighty stimulus of the research development with the aim of identification and clarification of the intracellular regulators. Now calcium ions (Ref. 3), prostaglandins (Ref.4) and peptides (Ref. 5) are found to be the mediators of hormones action apart from cyclic nucleotides. It has been shown in our previous work that insulin action on mitochondria depends on the increase of the low-molecular thermostable regulator activity in cytoplasm. This regulator was called insulin-dependent cytoplasmic regulator-ICR (Ref. 6). The purification of ICR, its identification and study of the insulin-like effects of ICR at the mitochondria and cellular levels is the aim of the present work.

Separation of the insulin-dependent cytoplasmic regulator. The ICR activating effect on the calcium ions translocation in liver mitochondria served as a test of the ICR activity during its isolation and purification. The ICR adding caused the increase in the maximum amount of calcium ions, which can be accumulated in mitochondria (Ref. 6). Liver cytoplasm of the rats, which were given insulin 45 minutes before the animals were killed, was prepared by centrifuging of the liver homogenate (45 g) at 30 000 g for 20 min. Thermostability of the insulin-dependent regulator of the mitochondria function has been previously shown (Ref. 6). That is why the first stage of purification was heating the cytoplasm at 95° for 7 min. The heated cytoplasm was centrifuged in the cold at 30 000 g. The supernatant was concentrated at the rotor evaporator up to 1-2 ml and then it was placed on the column with Sephadex G-25 (400x25 mm) which was eluted by 0,1 M KCl. In all fractions the activity was determined in accordance with the effect on the calcium transport in the liver mitochondria. The conditions of activity determination were indicated in Figure 1. Figure 1 shows that the activity was found in the peak, next to the high-molecular proteins peak, eluted just after passing the free volume of the column.

It is interesting that after gelfiltration on Sephadex G-25 the total activity of the material was greatly increased. The concentration dependence of the action of purified fractions has a cupolalike character (Figure 2). Therefore the activity can be found only when an eluate from the column is diluted 100 times.

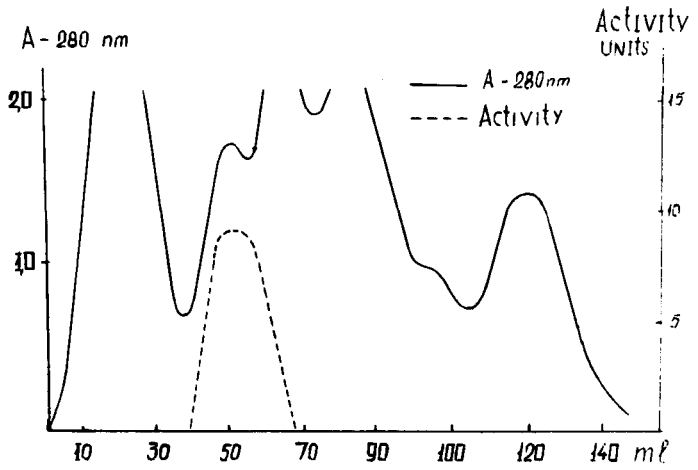


Fig. 1. Separation of heated cytoplasm on the column with Sephadex G-25. Volume each of fraction is 8 ml.

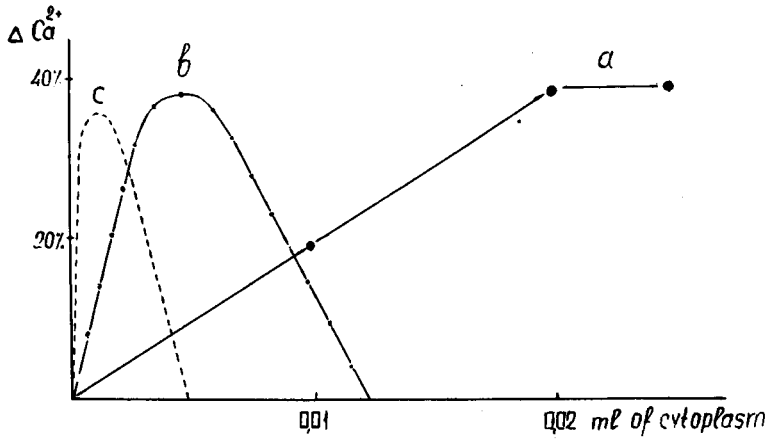


Fig. 2. Concentration dependence of ICR action on the calcium transport in mitochondria. A - nonpurified cytoplasm; B - fraction after gelfiltration on Sephadex G-25; C - fraction after rechromatography on DEAE cellulose. ICR activity was measured in the incubation medium containing 0,1 M KCl, 5 mM Tris Hcl., pH 7,3, succinate 5 mM, NaH_2PO_4 - 5.10^{-4} m, rotenone - 0,5 ug/ml.

Chromatography on DEAE cellulose was used at the next stage of purification. After gelfiltration the active material from rat liver was diluted 10 times with distilled water, pH 9,0, then the material was applied to the column with DEAE cellulose (15x250 mm). The activity was found in the fraction which was eluted from the column when the KCl gradient reaches 0,4 M (Fig. 3).

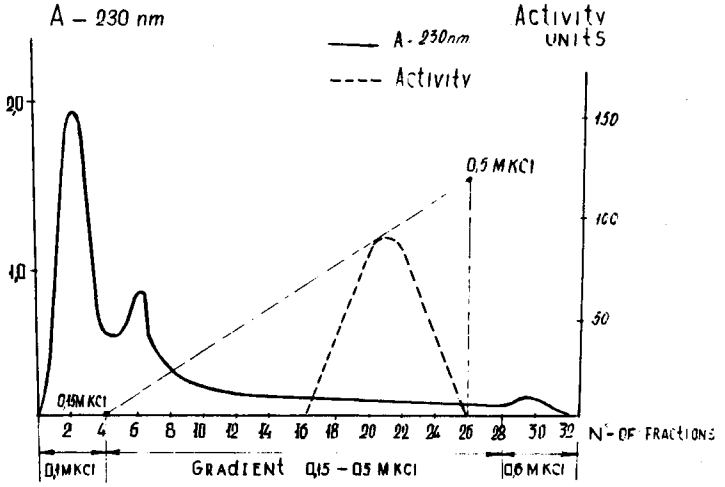


Fig. 3. Separation of ICR on the column with DEAE-cellulose.

The fraction containing the activity was subjected to rechromatography on the same column and under the same conditions. Figure 2 shows the concentration dependence of the ICR action on the Ca^{2+} transport in mitochondria at different stages of purification. The cupolalike dependence is typical only to the purified specimens action but not to unpurified cytoplasm. It is interesting that the total activity of the specimen during purification increases several times. It can be explained by the fact that the larger part of ICR in the cytoplasm is in the bound inactive state, moreover, ICR can be separated from the binding components during purification process. The cupolalike character of the concentration dependence of the purified specimens and the total activity change during the process of purification cause great difficulties while determining the activity, as the optimal dilution of the fractions taken from the columns during gelfiltration and ion-exchange chromatography is chosen empirically. When determining the activity the dilution of specimens (for DEAE cellulose the dilution is 50000 times) is made in 0,1 M KCl, as when the ionic strength of the medium is diminished aggregation of the material takes place easily with the loss of activity. This aggregation was reversed when 0,1 M KCl was added in the specimen. The purified material was desalted on the column with Sephadex G-10. Before desalting the active fraction was concentrated on the rotor evaporator up to 1 ml. During the desalting the material is separated in two fractions, one of the fraction eluted before the salt, another together with KCl, and both fractions were active. It is interesting that rechromatography of the second fraction at the same column again results in separation of the fraction which is washed away from the column before the salt, moreover, the fraction is in aggregated condition. The results of the experiment show that when the column with Sephadex G-10 is eluted with distilled water the aggregated part of the specimen is separated from the salt on the column, moreover, the aggregation degree grows after separation of the salt and monomeric part of the specimen is not separated from the salt under the same conditions. Similar results were obtained when ICR was desalted by dialysis. ICR is divided into two fractions during dialysis, and both fractions are active. Redialysis of the fraction, which has already passed the cellophane membrane, again causes the division into two fractions. So, the only form that passes the cellophane membranes is the monomeric form of ICR.

Identification of ICR chemical character. The purified specimen of ICR has no triptophan and nucleotides in accordance with UV-spektrum data. Table 1 shows the results of determination of ICR amino acid composition. The lack of sulfur-containing amino acids is characteristic. The ICR amino acid composition is unlike the insulin amino acid one.

TABLE 1. Amino acid composition of ICR

Amino acid	mμM/0.1 mg of protein	Molar ratio of amino acid relative to leucine	Nearest integre
Aspartic acid	61,5	2,94	3
Threonine	31,0	1,47	1
Serine	150,0	7,14	7
Glutamic acid	131,5	6,2	6
Proline	95,5	4,55	5
Glycine	133,5	6,26	6
Alanine	65	3,09	3
Valine	21	1,000	1
Isoleucine	21,5	1,02	1
Leucine	21	1,00	1
Tyrosine	13,5	0,6	1
Phenylalanine	20,5	0,98	1
Histidine	20,5	0,98	1
Lisine	108,5	5,14	5
Arginine	26,5	1,2	1

After rechromatography on DEAE cellulose ICR was electrophoretically homogeneous in the presence of 8 M urea. When the staining with toluidine blue and Schiff reagent was made one band appeared, moreover, it was shown that the eluate (0,1 M KCl) of this gel zone contains ICR activity. ICR staining with toluidine blue and Schiff reagent indicated to the carbohydrate component presence in ICR, and that was proved by finding the large amount of carbohydrate (in accordance with Dubois (Ref. 7) after 4-hrs hydrolysis in 4M HCl, at 110°). A very low solubility in the solutions with low ionic strength is characteristic of ICR. The possible explanation is the presence of hydrophobic site in ICR molecule. Really, the components of lipid nature can be extracted with chloroform-methanol from ICR (neutral lipids and cardiolipin in accordance with preliminary data). Fig. 4 shows infra red spectrum of ICR.

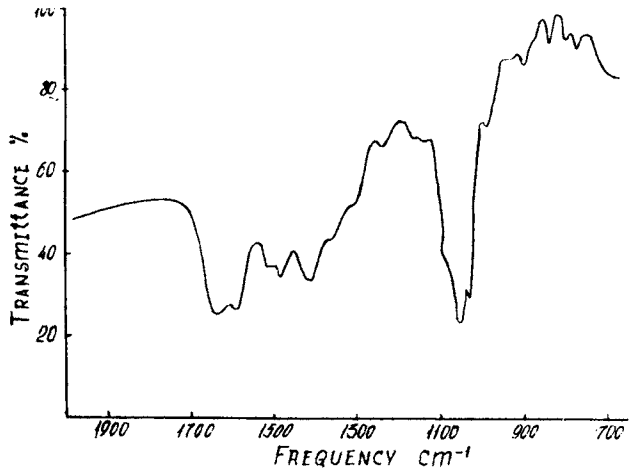
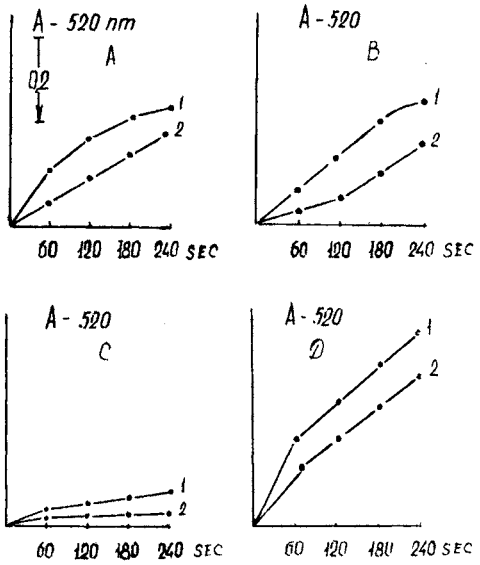


Fig. 4.

The intensive strip in the spectrum with maximum 1045 sm^{-1} can possible be explained by the skeletal fluctuation of P-O-C group. The quantitative analysis of inorganic phosphorus after exhaustive acidic hydrolysis showed that there was 45 mg phosphate per 1 g of protein in ICR. The presence of peptide links in ICR was proved by characteristic stripes in spectrum (stripe Amide 1 with maximum at 1670 sm^{-1}, Amide 2 with maximum at 1600 sm^{-1}). Three stripes in the spectrum with maximums at 785, 855, 930 nm show the presence of carbohydrates in molecule (930 nm-ring oscillation, 855 nm-deforming oscillation (ecvatorial), 766+10 - pulsating oscillation of the ring). The data received show that ICR is glycolipoprotein with a comparatively small molecular weight, this finding is proved by the data on the ability of the monomeric active form of ICR to diffuse through cellophane membranes, and by the data on the determination of molecular weight (5000 daltons) by means of gelfiltration. ICR ability to produce effects at the membrane level seems to be explained on the one hand by the presence of hydrophobic site in the molecule (lipid components of ICR), on the other hand by the solubility in the water solution with ionic strength equal to that of cytoplasm.

ICR influence on the mitochondria. It has been previously mentioned that low ICR concentration (about 10^{-7} g/ml) increases the mitochondria ability to accumulate calcium ions. Cupolalike dependence of the ICR concentration is characteristic of this effect.

At a concentration of about 5.10^{-5} g/ml ICR inhibits pyruvate and succinate oxidation in mitochondria. As soon as the effect takes place under the conditions when the oxidation rate is limited by the substrate entrance in the mitochondria, it was supposed that ICR inhibits the substrates transport through mitochondria membrane. While examining this supposition the rate of substrate transport was estimated according to the rate of deenergised mitochondria swelling in the isotonic solutions of ammonia salts of the oxidation substrates. It is well known that the swelling rate under these conditions is limited by anion transport (Ref. 8). Figure 5 shows that ICR inhibits mitochondria swelling in the isotonic solution of ammonium pyruvate, when deenergisation of mitochondria is caused by the adding of rotenon and 2,4 DNP.



1 - CONTROL
2 - ICR

Fig.5. The influence of ICR on the swelling of deenergised rat liver mitochondria in the isotonic solutions of ammonium salts of the oxidizable substrates. Incubation medium contained 10^{-4} M 2,4 DNP, rotenone 0,5 μg/ml, Tris HCl 5 mM, pH 7,5 and isotonic solutions: a)ammonium succinate + antimicin A-0, 1 μg/ml; b)ammonium pyruvate; c) ammonium citrate; d) ammonium glutamate. 1 - control; 2 + 3.10^{-6} g/ml ICR.

This result is interpreted as the inhibition of pyruvate transport in mitochondria. It is well known that metabolite transport through mitochondria membrane is fulfilled by special carriers, which are specific as regards the corresponding substrate. The ICR action is specific not only as regards monocarboxylates carrier, but also as similar inhibiting action of ICR showed as regards transport of ammonium salts solutions of glutamate, aspartate, succinate and citrate. So the activity of at least three carriers of metabolites is inhibited when ICR is added to the suspension of liver mitochondria. On the other hand the inhibition with ICR adding is not characteristic of the transport of all anions in mitochondria since in case of phosphate ICR showed the activated action. So insulin action mediated by ICR was found in the substrate transport inhibition from cytosol to mitochondria.

The hypoglycemic action of ICR. Treating ICR as the mediator of insulin action we supposed it to be able to act like insulin at cellular level and in vivo.

It is well known that insulin inhibites the glucose exit from the liver in vivo and in vitro, and this is one of the basic causes of the hypoglycemic effect of insulin. In our tests with liver slices ICR inhibited glucose output in adrenalin presence. The optimal concentration of ICR was 0,2 ug/ml (Fig. 6).

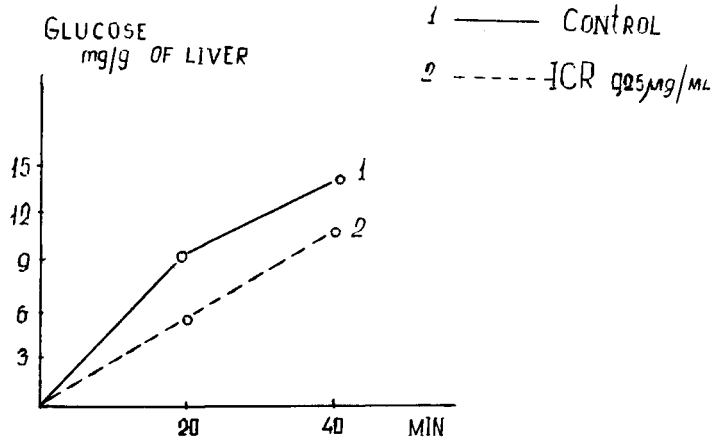


Fig. 6. Influence of ICR on the glucose output from liver slices in the presence of 5.10^{-6} M adrenaline. Liver slices (500 mg) with diametre 2 mm were incubated in 10 ml Krebs-Ringer bicarbonate buffer, pH 7,4. 1 - control; 2 - 0,25 µg/ml ICR.

In the tests in vivo ICR introduction (50 ug/100 g of weight) caused great reduction of blood sugar in rats with alloxan diabet (Table 2).

TABLE 2. Action of intravenously injected ICR on the content of sugar in the blood of rats.

	Number of rats	Sugar in blood (mg%) prior ICR injection (50 µg/100 g)	Sugar in blood (mg%) through 60 min. after ICR injection	Sugar in blood through 100 min. after ICR injection
Intact rats	8	108,3±5,6	85,7±7,3	98±8,7
Alloxane (50 mg/kg) in 24 hours	12	630±51,3	432±32,7	210,6±23,8

In accordance with the idea that the transfer of pyruvate from cytosole to mitochondria is the limited stage of glucose synthesis in liver cells the hypoglycemic action of ICR in vivo (Ref.9)is well explained by the ICR inhibition of the pyruvate transport through mitochondria membrane. So our data about the action of purified specimens of ICR on mitochondria, liver slices and in vivo proves the supposition about ICR role as the mediator of insulin action on the liver metabolism.

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PROGRESS IN THE CHEMICAL SYNTHESIS OF PROINSULIN

H. Zahn and E. E. Büllesbach

Deutsches Wollforschungsinstitut, Technische Hochschule Aachen, D-5100 Aachen, Federal Republic of Germany

Abstract - Reduced proinsulin, and insulin A-chain and B-chain were selectively and quantitatively converted to hexa-S-trityl-proinsulin, tetra-S-trityl-A-chain and di-S-trityl-B-chain. Amongst the various deprotection methods studied, treatment of the hexa (S-trityl)-derivative in trifluoroacetic acid using thiophenol as reversible scavenger was found to be the best. This method will also ensure the single step deprotection of the fully protected synthetic proinsulin derivative to hexa-thiol proinsulin as the other protecting groups are also labile under these conditions. This material was converted to the corresponding hexa S-sulfonate in 85 % yield. This was then reduced and oxidized to native proinsulin. The yield based on the S-sulfonate was 45 % after purification by Sephadex G-50 chromatography in 30 % acetic acid.

INTRODUCTION

Insulin has been known for many years but is still fascinating not only to the medical world but also to Physicists, Chemists and Molecular Biologists. 1967 saw the discovery of the one chain precursor Proinsulin by Steiner (Ref. 1).
This single polypeptide chain has been identified as a ribosomal product in the microsomal fraction from islet tissues. The newly synthesized peptide chain, after folding and thiol oxidation, is transferred to the Golgi apparatus where it begins to undergo proteolytic processing to insulin (fig. 1) and packaging into secretory granules.

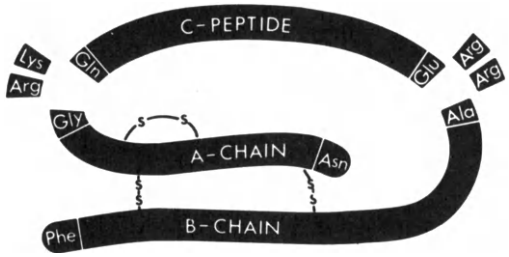

Fig. 1. Products of the conversion of proinsulin to insulin in the ß-cells

Human proinsulin is a tri-disulfide protein comprising 86 amino acids (Ref. 2 & 3). For its synthesis the strategy of fragment condensation and acid labile protection have been followed. t-Butyl protection (t-Bu^t/Boc) was used for carboxyl, hydroxyl and N$^\varepsilon$-amino functions whilst thiol functions were protected with trityl groups. Biphenylisopropyloxycarbonyl and trityl groups provided temporary protection at the α-amino functions of the intermediates. Four main fragments (1-23, 24-45, 46-70 and 71-86)

and two "big" fragments (1-45 and 46-86) were synthesized (Fig. 2). All the fragments were soluble in dimethylformamide. These were purified by gel chromatography on Sephadex LH 20 and counter current distribution techniques and characterized by thin layer chromatography and amino acid analysis (Ref. 4).

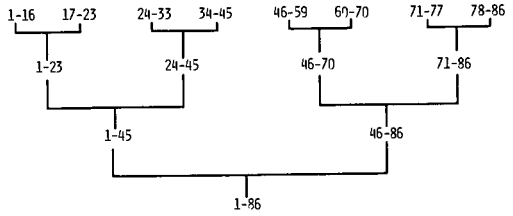

Fig. 2. Fragments and subfragments for the chemical synthesis of human proinsulin

Parallel to these studies, we also investigated and optimized the removal of S-trityl groups from tritylated native bovine proinsuline, and the subsequent reduction-oxidation steps to regenerate proinsulin. These reactions constitute the last steps in the total synthesis and are the object of this communication (Fig. 3).

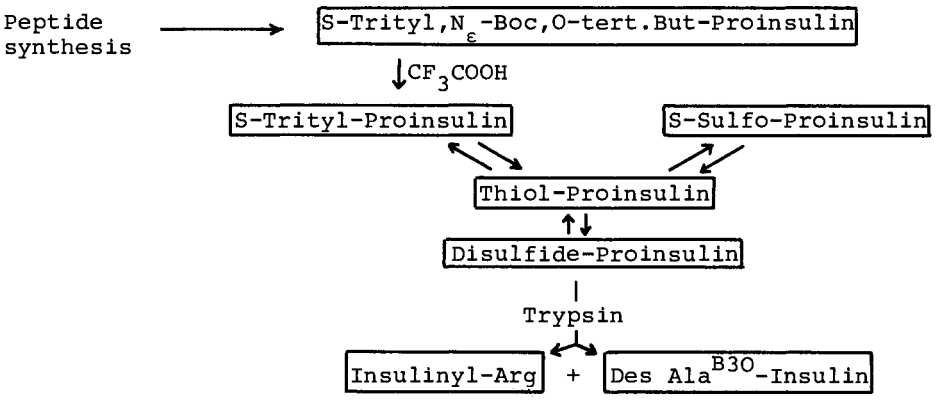

Fig. 3. Conversion of protected proinsulin to proinsulin and Des-Ala-insulin

S-Tritylation Reaction

The S-trityl group can be readily introduced into a cysteine containing pentapeptide with tritylcarbinol in trifluoroacetic acid according to 1970 Photaki et al. (Ref. 5). The trityl-cation generated in presence of trifluoroacetic acid reacts with the thiol group and the resulting S-trityl derivative exists in equilibrium with the trityl cation (Fig. 4). The removal of trifluoroacetic acid shifts the reaction equilibrium to the S-tritylated derivative. In the presence of excess of tritylcarbinol this reaction proceeds quantitatively.

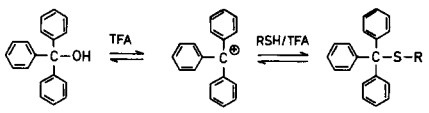

Fig. 4. The pathway of S-tritylation reaction

Proinsulin as well as insulin A and B chains were obtained in the thiol form by reduction of the corresponding S-sulfonated chains. These thiol derivatives were S-tritylated in trifluoroacetic acid in which they were readily soluble. The S-trityl derivatives were isolated as colourless amorphous powders in yields of 78 % for the A-chain, 91 % for the B-chain, and 95 % for proinsulin.
The introduction of the hydrophobic trityl residues (two in the B-chain, four in the A-chain, and six in proinsulin) rendered all three S-trityl-derivatives insoluble in aqueous solution even in 8 M urea at all pH values.
Therefore, the usual analytical procedures (i.e. electrophoresis, gel chromatography, ion exchange chromatography and isoelectrofocussing) could not be carried out. In additional the S-trityl chains were only poorly soluble in aqueous-organic mixtures and organic solvents, except dimethylsulfoxide (5-10 mg/ml).
In view of the poor solubility of the S-tritylated chains we had to modify the procedures for the estimation of thiol and disulfide groups carried out in an heterogeneous system and therefore, the accuracy of the method diminished. Ellman thiol analysis (Ref. 6) of the tritylated chains showed no free thiol groups (Table 1). Disulfide bonds were estimated by the reduction of the chains followed by reaction with iodoacetic acid in aqueous solution (Ref. 7). If the tritylated chains contained S-S-bonds this would convert such bonds into their S-carboxymethylated derivatives. Thin layer chromatography indicated the products to be homogenenous with higher Rf-values than the corresponding S-carboxymethylated chains. Amino acid analysis of the reduced and carboxymethylated chains were identical and traces of S-carboxymethyl cysteine was observed. These studies clearly indicated that the S-tritylation was nearly quantitative.

TABLE 1. Analytical data of the S-tritylated A- and B-chains and proinsulin

Estimation of Groups	Tritylated chains		
	di S-Trt B-chain	tetra S-Trt A-chain	hexa S-Trt proinsulin
SH	0	0	0
S-S	0.017 eq	0.042 eq	0.065 eq
Trt	2.06±0.2 eq	3.36±0.4 eq	6.18±0.6 eq

Quantitative determination of trityl residues was accomplished by the difference ^{1}H-NMR spectroscopy of the trityl and corresponding S-sulfonate chains. The values of the extent of tritylation agree with the theoretical values within the limits of error (Table 1).

S-Detritylation Studies

As mentioned earlier all the S-tritylated chains were extremely insoluble. To achieve quantitative deblocking it is necessary to work in homogeneous systems otherwise partially deblocked derivatives are likely to be formed. Up to now various detritylation studies have been made on synthetic protected peptides using the following methods

1. oxidation with iodine or dithiocyanogen (Ref. 8)
2. treatment with silver or mercury salts followed by HCl or H_2S (Ref. 8)
3. treatment with trifluoracetic acid or HBr/acetic acid containing water, phenol or anisol (Ref. 8)
4. reduction with liquid ammonia
5. electrolytic reduction (Ref. 9) and
6. reaction with 2-nitrophenylsulfenylchloride and reduction to thiol (Ref. 10).

Fujino and Nishimura recently described the removal of t-butyl and p-methoxybenzylthioether groups, with mercuric acetate in trifluoroacetic acid (Ref. 11). This procedure was investigated with di-(S-trityl)-B-chain and hexa-(S-trityl)-proinsulin. The mercaptide was treated with ß-mercaptoethanol and the thiol derivatives isolated after gel chromatography on Sephadex G-25 in 30 % acetic acid. They were converted to S-sulfonate derivatives. Electrophoresis of these derivatives showed two trace impurities which necessitated the further purification of S-sulfonate by ion-exchange chromatography on SP-Sephadex (Fig. 5).

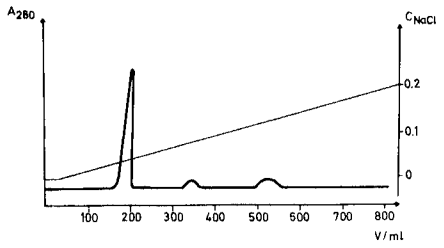

Fig. 5. Purification of hexa-S-sulfonate proinsulin (5 mg) on a SP Sephadex column (1.5 cm x 12 cm)
Starting buffer 400 ml 7M urea 1.5 M acetic acid
limiting buffer 400 ml 7M urea 1.5 M acetic acid 0.2 M NaCl
flow rate 25 ml/h
fraction 5 ml

The advantage of the methods employing trifluoroacetic acid is the simultaneous deblocking of the acid labile protecting groups. However the tetra-(S-trityl)-A-chain is insoluble in the mercaptide form.
Another method of detritylation is treatment with trifluoroacetic acid and a cation scavenger e.g. water, phenol or anisol (Ref. 11). This method had been applied to small peptides only and as the scavengers were inefficient, repeated treatments were necessary.
We therefore investigated this deblocking procedure with the aim of optimizing the S-detritylation. It was known that the detritylation results from the formation of an equilibrium reaction between the S-trityl derivatives and the respective thiol and trityl cation. This equilibrium can be shifted by the presence of excess cation scavenger setting free the thiol group:

$$\text{-Cys(Trt)-} \xrightleftharpoons{+\,H^{\oplus}} \text{-Cys(H)- + } Trt^{\oplus}$$

$$Trt^{\oplus} + RSH \xrightleftharpoons{-\,H^{\oplus}} R\text{-S-Trt}$$

$$\text{-Cys(Trt) + RSH} \rightleftharpoons \text{-Cys(H) + RS-Trt}$$

Trans-S-tritylation occurs if thiols are used as cation scavengers as they take part in the equilibrium reaction (Refs. 12 & 13).
We demonstrated the trans-S-thioetherification by treating Cys(Trt) with trifluoroacetic acid and thiophenol, whereby trityl-thiophenol was isolated as an odourless crystalline product. It was identified and characterized by comparing it to a standard sample (Ref. 14).
We next demonstrated the S-detritylation of the S-tritylated insulin chains.
The scavengers used in the detritylation studies were:
2-mercaptoethanol, p-nitrothiophenol, thiocresol, cysteine and thiophenol. This latter in trifluoroacetic acid gave the best results.
Using this method the yields for the S-sulfonate derivatives of A-chain, B-chain and proinsulin were 70 %, 72 % and 85 % (Table 2) respectively. These yields include the oxidative sulfitolysis of the thiol-chains leading to the S-sulfonate derivatives. Electrophoresis of the S-sulfonates at pH 2.2 and 4.8 showed them to be homogeneous.

TABLE 2. Removal of trityl groups from S-tritylated chains with trifluoroacetic acid in the presence of 120 equivalents thiol per S-trityl group followed by sulfitolysis

scavenger	yield of		
	B-chain di-S-sulfonate	A-chain tetra-S-sulfonate	proinsulin hexa-S-sulfonate
cysteine	10 %	10 %	-
2-mercapto-ethanol	34 %	-	20 %
p-nitro-* thiophenol	-	-	40 %
thiocresol*	10 %	10 %	10 %
thiophenol	72 %	70 %	85 %

* poorly soluble in trifluoroacetic acid

Regeneration of proinsulin from the S-trityl derivative

We have shown that S-trityl groups can be introduced into and removed from A- and B-chains, and proinsulin.
With synthetic S-sulfo-proinsulin, additional steps such as reduction to thiol-proinsulin followed by oxidation to proinsulin in its disulfide form will be necessary. Therefore, we subjected hexa-(S-trityl)-proinsulin to all steps necessary for the production of proinsulin from the synthetic protected proinsulin chain (see Fig. 6).

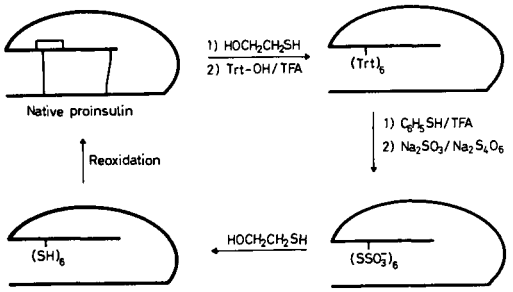

Fig. 6. Resynthesis of proinsulin from S-tritylproinsulin, S-sulfo-proinsulin and thiol-proinsulin

The S-trityl-proinsulin was converted to the corresponding S-sulfonate as described earlier. It was reduced quantitatively to the thiol-proinsulin with 2-mercaptoethanol and isolated by Sephadex G-25f chromatography in 30 % acetic acid. Aerial oxidation (190-200 h) was done at a concentration of 1 - 1.1 mol/l ammoniumhydrogencarbonate at pH 8.4 - 8.8. The mixture was then purified on Sephadex G-50 f in 30 % acetic acid. Two peaks were obtained, the first of which was shown to be a polymer and the second to be electrophoretically identical with native proinsulin (Figs. 7 & 8).

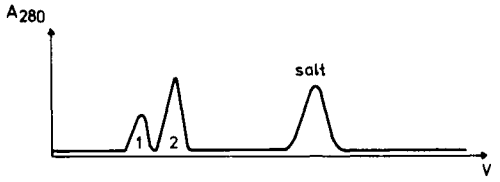

Fig. 7. Separation of crude regenerated proinsulin on Sephadex G-50f (1.5 cm x 150 cm) in 30 % acetic acid
1 = Polymer, 2 = Proinsulin (45 % yield)

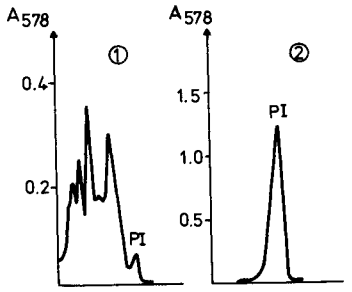

Fig. 8. SDS gelelectrophoresis
Staining with Amidoblack 10B and scanning at 578 nm
1) Fraction 1 of the gelchromatography
2) Fraction 2 of the gelchromatography and similar to proinsulin

The oxidation yield was 45 % starting from sulfo-proinsulin. The same results were obtained with reduced proinsulin.
The digestion of synthesized proinsulin with trypsin gave a mixture of des-B^{30}-insulin and insulinyl(B30)-arginine, as was obtained with native proinsulin (Fig. 9). Similarly subtilisin digestion of the synthesized and native proinsulin gave identical "fingerprints". This shows that the disulfide bonds have been correctly formed in the resynthesized proinsulin.

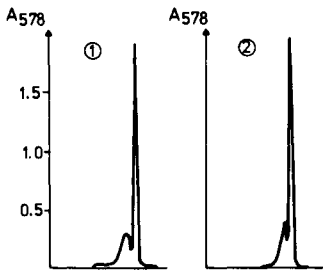

Fig. 9. Digestion of 1) resynthesized proinsulin
2) native proinsulin
with trypsin followed by disk electrophoresis.
Staining with Amidoblack 10B and scanning at 578 nm.
The first peak is insulinyl-Arg, the second des-ala-insulin

Prospects for the total synthesis of proinsulin

Let us quote the famous sentence in the first publication by Dixon and Wardlaw (Ref. 15) on resynthesis of insulin from the separate chains:

"However, even with the present yield, it can be said that if chemically synthesized A and B chains were available in mgm. amounts it should be possible to obtain insulin by the above method and thereby provide the terminal step in the total synthesis of a protein with biological activity.

We all know that Dixon and Wardlaw were right and that using their method synthetic insulin in fact was obtained. We should like to translate the prophecy of Dixon and Wardlaw to the case of proinsulin: After having completed the succesful conversion of S-Tritylproinsulin to proinsulin in a yield of nearly 40 % we are confident that we have only to wait for a sample of synthetic S-trityl proinsulin for demonstrating the effectiveness of the new detritylation method with thiophenol and trifluoroacetic acid to perform the last two steps of the chemical synthesis of proinsulin.

Acknowledgement - Thanks are due to Dr. Helbig for providing samples of pure proinsulin, prepared from b-component kindly provided by Eli Lilly Company, Indianapolis, Indiana, USA. This work was supported by a grant from the Fonds der Deutschen Chemischen Industrie.

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MITOCHONDRIAL MEMBRANE-BOUND STEROID HYDROXYLATING SYSTEMS: STRUCTURE AND FUNCTIONS

A. A. Akhrem, V. N. Lapko, A. G. Lapko, S. P. Martsev, V. M. Shkumatov and V. L. Chashchin

Institute of Bioorganic Chemistry, Byelorussian Academy of Sciences, Minsk, USSR

Abstract- A phenomenon of self-association has been shown for in vitro steroid hydroxylating system from adrenal cortex mitochondria. The data obtained testify to the presence of independent specific sites for binding with adrenodoxin reductase and cytochrome P-450 in the adrenodoxin molecule. Conditions for the reconstitution of the Sepharose-immobilized Fe_2S_2 adrenodoxin cluster have been found. The results of the limited trypsin digestion of the 20S,22R-cholesterol hydroxylating cytochrome P-450 are presented. Proteolyzed cytochrome P-450 has been shown to retain its functional activity for a certain period of time both with a reconstituted in a solution 20S,22R-cholesterol hydroxylating system and with self-association of the system on a column with immobilized adrenodoxin.

The basic steps of biosynthesis of both corticosteroids and sex hormones involves processes of enzymic hydroxylation.A particular role in the total scheme of biosynthesis of corticosteroid hormones is played by the located in mitochondria membranes hydroxylating systems catalazing such key reactions as i) a siccessive cholesterol transformation to pregnenolone, ii) final steps of corticosterone and cortisol synthesis, iii) biosynthesis of aldosterone, the main representative of mineralocorticoids (Refs. 1-3). The study of these systems will, on the one hand, give a better view on the causes of a number of serious endocrine diseases and, on the other, outline perspectives to their use in the preparative synthesis of steroid hormones. Mitochondrial hydroxylating systems involve three major protein components: flavoproteid (adrenodoxin reductase), non-heme iron-containing protein (adrenodoxin) and hemoproteid (cytochrome P-450). These three proteins form a chain of electron transport to the active site of cytochrome P-450, indispensible for the molecular oxygen activation, whereas specific configuration of active sites of cytochromes P-450, differing in substrate specifisity, ensure selective stereospecific incorporation of the activated oxygen into a substrate molecule.

We have recently reported the isolation of individual components of 20S,22R-cholesterol hydroxylating system, their physico-chemical properties and the electron transport regulation, with some structural characteristics for the homogeneous adrenodoxin reductase and cytochrome P-450 preparations presented for the first time (Ref.4). It remained unclear, though, in what way the hydroxylating systems were arranged? We assume that the individual proteins are capable of self-association into a stable multienzyme complex. The reconstitution of the cholesterol hydroxylating system was carried out on a column with adrenodoxin immobilized on CNBr activated Sepharose 4B (330 nmol of adrenodoxin/ml of precipitated gel). A sequence of stages in the self-association of the 20S,22R-cholesterol hydroxylating system is furnished in Fig. 1. First, adrenodoxin reductase was passed through the column. Then cytochrome P-450, free of detergent (sodium sholate) was applied on the column. Adrenodoxin reductase and cytochrome P-450 were used in quantities exceeding necessary for the saturation of the column. It was established that the sequence of stages is inaffective for the self-association process. The process would occur both with independent and simultaneous applications of the proteins on the column. This fact testifies that the process of self-association of ternary complexes involves independent formation of binary complexes of adrenodoxin reductase and cytochrome P-450 with adrenodoxin.

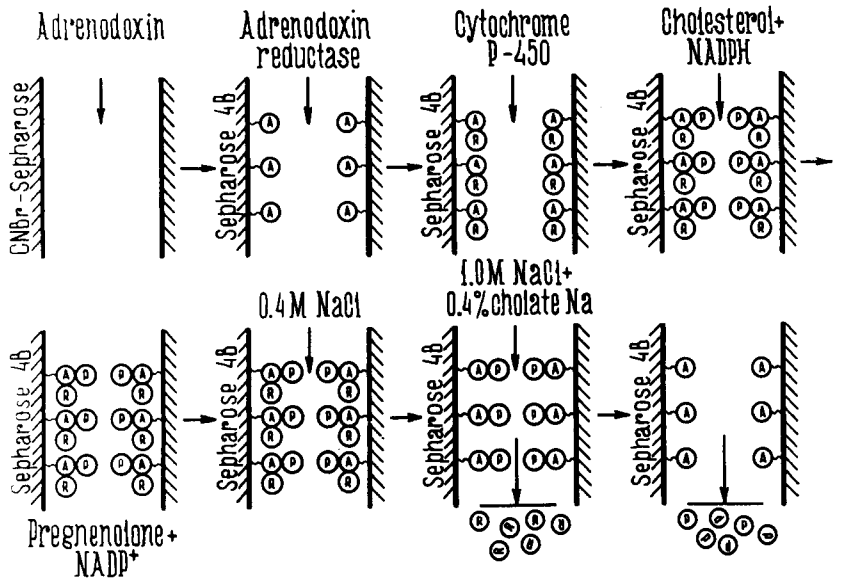

Fig. 1. Self-association of the 20S,22R-cholesterol hydroxylating system on a column with adrenodoxin-Sepharose, transformation reaction of cholesterol to pregnenolone and difference desorption of the proteins of the system. The initial stage shows immobilization of adrenodoxin on CNBr activated Sepharose 4B.

To prove functional activity thus reconstituted, NADPH and [4-C^{14}]-cholesterol were passed through the column. The examination of the eluate proved cholesterol transformation to pregnenolone in 30-40% yield. Using the different conditions for the adrenodoxin reductase and cytochrome P-450 desorption from immobilized adrenodoxin, we have succeeded in "disassembling" the system and eluting individual proteins. The proteins obtained did not differ from the initial ones in activity and after a short dialysis (or 10-fold dilution) could be repeatedly used for the self-association on adrenodoxin-Sepharose (Ref.5.). It is essential to note that the cytochrome P-450 complexing with immobilized adrenodoxin shown by us together with conditions found for the cytochrome P-450 desorption, permitted us to propose a principally new method for preparative isolation of the 20S,22R-cholesterol hydroxylating cytochrome P-450 in a homogenious state (Ref.6). The same approach has been used for the isolation of the 11β-hydroxylating cytochrome P-450. A specific self-association of the 20S,22R-cholesterol hydroxylating system has been verified by the experiment described below. Adrenodoxin was passed through the column with the reconstituted system according to lineary increasing gradient of concentration, which led to a selective desorption of adrenodoxin reductase and cytochrome P-450 from the column, i.e., to a selective "disassembling" of the system. An analogous approach with the same preparations of adrenodoxin and adrenodoxin reductase, but with 11β-hydroxylating cytochrome P-450, has been employed to reconstitute the system of corticosterone and cortisol biosynthesis. The steroid transformation in that case amounted to 60%.

Does the self-association occur in a solution? Using several independent methods we have succeeded in giving a positive answer to this question (Ref. 4). Thus the effect of the increasing adrenodoxin concentrations on the activity of the 20S,22R-cholesterol hydroxylating system in a solution with fixed amount of adrenodoxin reductase and cytochrome P-450 has been investigated. At concentrations comparable to those of other proteins of the system adrenodoxin stimulates cholesterol hydroxylation. However, with great excesses of adrenodoxin an inhibiting effect has been observed. This is likely to be interpreted as follows. Initially, adrenodoxin stimulates the process of hydroxylation by involving adrenodoxin reductase and cytochrome P-450 into forming ternary active complexes. With further increase in adrenodoxin concentration (above 40-fold) the excess amounts of it compete for the binding sites with adrenodoxin reductase and cytochrome P-450 and destroy the ternary complexes to form binary inactive ones. This effect

couldn't have been observed in the absence of adrenodoxin complexing with other proteins of the system, that is, in the absence of self-association of the system. When performing a process of hydroxylation of different steroid substrates on the column with reconstituted steroid hydroxylating systems a decrease in general activity of the hydroxylating systems has been marked. The adrenodoxin reductase and cytochrome P-450 eluted after the experiment did not differ in activity from the authentic preparation. So, this loss in the enzymic activity of immobilized systems could be due to an inactivation of adrenodoxin only. We have found that in the process of electron transport a decomposition of Fe_2S_2 cluster occurs. It was of great interest to elucidate the role of the cluster in the self-association of a multienzyme complex, and also to determine conditions for its recurrent incorporation. To achive a complete removal of the Fe_2S_2 cluster adrenodoxin-Sepharose after anaerobic reduction with dithionite was treated with large excesses of mercaptoethanol. Under these conditions a complete disappearence of the adrenodoxin-Sepharose colowring observed, though the immobilized adrenodoxin preserved its ability to complex with adrenodoxin reductase and cytochrome P-450. Treatment of the immobilized adrenodoxin with 4M urea and 8M urea in the presence of mercaptoethanol did not affect the complexing of individual protein systems. Even after a 56 h incubation of adrenodoxin-Sepharose in an 8M urea solution in the presence of mercaptoethanol, a self-association of the ternary complexes of the type: adrenodoxin reductase - free of Fe_2S_2 cluster adrenodoxin - cytochrome P-450 did occur when adrenodoxin reductase and cytochrome P-450 were applied on the column with adrenodoxin-Sepharose washed from urea. Thus, after the destruction of the Fe_2S_2 cluster in the process of hydroxylation, the proteins of the steroid hydroxylating system, adrenodoxin reductase and cytochrome P-450 do not dissociate from the column with adrenodoxin-Sepharose, but the cluster destruction results in an electron transport stop in the reconstituted on the column hydroxylating system thus leading to the mentioned above enzymic activity decrease. We have determined conditions for the reconstitution of the Fe_2S_2 cluster in immobilized adrenodoxin. To that purpose, adrenodoxin-Sepharose (immobilized adrenodoxin did not contain a cluster) in an 8M urea solution in the presence of mercaptoethanol was placed in an ultrafiltration cell (Whatman 3MM paper disk served as a filter), kept under anaerobic conditions, and then a mixture of Na_2S and Fe_2SO_4 was introduced into the cell. To remove urea and mercaptoethanol, 0,05M sodium phosphate buffer (pH 7,4) was passed through the incubation cell using the streame of nitrogen. The adrenodoxin-Sepharose was bubbled with oxygen for the oxidation of an incorporated cluster. After that a column was packed with reconstituted adrenodoxin-Sepharose. Adrenodoxin reductase and cytochrome P-450 were passed through for the self-association of the 20S,22R-cholesterol hydroxylating system. A subsequent hyddroxylation of steroids showed a 85% reconstitution of the enzymic activity of the hydroxylating system. Thus in the case of destruction of the hydroxylating process *in vitro* it can be done follow: i) to "disassemble" the reconstituted systems, ii) to repare or to substitute a non-operative component and iii) to perform a self-association of the systems on a column with adrenodoxin-Sepharose once again for their further use.

From the data presented above follows that on a cytochrome P-450 molecule, apart from the catalytic centre, there exists a "site" for binding with adrenodoxin. Using limited proteolysis we attempted to separate the catalytic site (an active centre containing protoheme IX) from that responsible for self-association of the enzyme with adrenodoxin. The experiment aimed at elucidating the ability of different in substrate specificity cytochromes to complex with one and the same protein of the electron transport chain, namelly, adrenodoxin. Presented here are the data on a limited proteolysis of the 20S,22R-cholesterol hydroxylating cytochrome P-450. In the hydrolysis of the 20S,22R-cholesterol hydroxylating cytochrome P-450 with trypsin ($20^{o}C$, trypsin/cytochrome ratio 1:50) under conditions of its disaggregation (0,05M sodium phosphate buffer, pH 7,2 , containing 1M NaCl and 0,3% sodium cholate (Ref. 4)) it was found that during the first couple of minutes there appear two fragments, F_1 (M=27000) and F_2 (M=21000) apart from the native cytochrome P-450 (30%). On the extending the hydrolysis time up to 20 min the cytochrome band disappear completely to leave fragments F_1 and F_2, only. Further prolongation of the proteolysis to 8 h results in a sertain decrease in the amount of the F_1 and F_2 fragments and in the appearance of two other fragments, F_3 (M=14000) and F_4 (M<10000). Time extention up to 24 h practically results in a retention of fragments F_1 and F_3. In enhancing a trypsin/cytochrome P-450 ratio (1:10) the same set of fragments was observed upon 3 h proteolysis.

Upon applying the 20 min hydrolysate (trypsin/cytochrome P-450 ratio 1:50) or 3 h hydrolysate (trypsin/cytochrome P-450 ratio 1:10) on a column with adrenodoxin-Sepharose, the fragments were sorbed in a way similar to the native cytochrome P-450, so that they were successfully eluted as a single fraction under conditions proposed by us for the desorption of cytochrome P-450 from adrenodoxin-Sepharose (Ref. 5.). This led us to assume that the fragments F_1 and F_2 or F_1 and F_3 formed in the hydrolysis of cytochrome P-450 remain firmly associated, so that the proteolyzed cytochrome P-450 both in the form of F_1 and F_2 fragments and F_1 and F_3 fragments could retain its activity for a sertain period of time both with reconstituted in a solution 20S,22R-cholesterol hydroxylating system and in the case of self-assembling of the system on a column with immobilized adrenodoxin. The stability to trypsin the fragments produced supports a biglobular (triglobular) structure for the native cytochrome P-450 monomer.

Upon the dissociation of the fragments in 0,05M phosphate buffer, containing 1M NaCl, 0,3% sodium cholate and 0,3% Tween 80 the fragments were separated on the column with Sephadex G-200. It was established that the heme is attached to the fragment F_1 globule; the same fragment may contain a specific adrenodoxin binding site.

Functional assignment of fragments F_2 and F_3 and a location of the F_1, F_2 and F_3 fragments in a polypeptide chain of cytochrome P-450 are in progress in our laboratory.

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ANALYSIS OF LOCAL ENVIRONMENT OF HISTIDINE RESIDUES IN PROTEINS BY HYDROGEN-TRITIUM EXCHANGE REACTION

K. Narita

Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan

Abstract - A general procedure to analyze local environment of individual histidine residues in proteins is presented. This procedure is based on the hydrogen-tritium exchange reaction at the $C^{\varepsilon 1}$ position of the imidazole ring of histidine. Since the exchange reaction is pH-dependent, dissociation constant (pKa) of the imidazole of a histidine residue can be measured from the plot of the rate constant versus pH of the reaction medium. Deviation of pKa of the histidine residue in question from its intrinsic pKa suggests that the neighboring charged group is interacting with the histidine. The magnitude of the exchange rate constant corresponds to solvent accessibility of the imidazole ring of the histidine in the three-dimensional structure of the protein. Using these correlations local environment of individual histidine residues in ribonuclease T_1 and a serine protease produced by Streptomyces erythreus, whose three-dimensional structures are unknown, is discussed.

INTRODUCTION

Histidine is known as one of active site residues in a number of enzymes. It is important to analyze local environment of the histidines to understand molecular mechanisms of their catalytic reactions. Kagamiyama et al. (Ref. 1) pointed out that non-terminal histidines were sometimes labelled with tritium when the classical C-terminal tritium labelling method of Matsuo et al. (Ref. 2) was applied to aspartate aminotransferase from pig heart. This phenomnon is of interest since we can label histidine residues in proteins selectively if we could find out suitable conditions. We looked for the best conditions for the labelling reaction with acetylhistidine (Ac-His) and found that the reaction was simply pH-dependent. We could then prove by ^1H NMR spectoscopy that the hydrogen atom attached to the $C^{\varepsilon 1}$ position of the imidazole ring was exchanged with deuterium during incubation of Ac-His in D_2O. Vaughan et al. (Ref. 3) proposed a mechanism of the deuteration of imidazole based on the kinetic measurements as shown in Fig. 1 as the major reaction course.

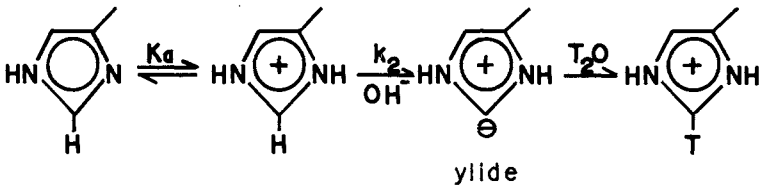


Fig. 1. Reaction mechanism for hydrogen-tritium exchange at the $C^{\varepsilon 1}$ position of the imidazole ring of histidine (according to Vaughan et al. (Ref. 3)).

The overall reaction is the pseudo-first-order for total concentration of histidine (rate constant, k_ψ) and the rate determining step is the formation of an intermediate ylide which is the second-order (k_2) in respect to the concentrations of OH^- anion and the imidazolium cation. Thus

$$\text{overall rate} = k_\psi[His_{total}] = k_2[His^+]\,[OH^-] \quad (1)$$

therefore

$$k_\psi = k_2 Kw/(Ka + [H^+]) \quad (2)$$

where Kw is the ion product of water. The k_ψ value can be measured experimentally by the following equation (3).

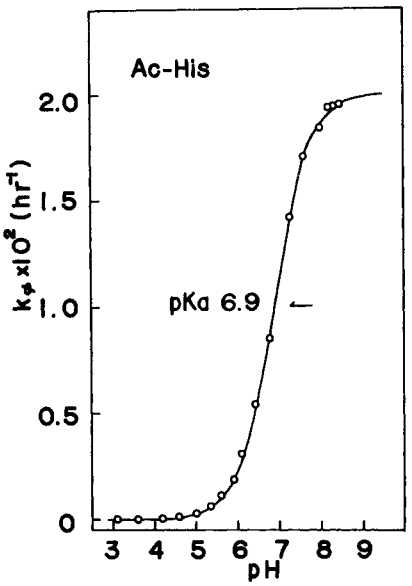


Fig. 2. Tritium exchange titration curve for Ac-His at 37°C.

$$\ln\{(\underline{a} - \underline{x})/\underline{a}\} = -k_\psi t \quad (3)$$

where $\underline{a}$ and $\underline{x}$ are the specific radioactivities incorporated at time ∞ and t, respectively. Figure 2 shows the pH-dependency of the hydrogen-tritium exchange reaction rate constant (k_ψ) at the $C^{\varepsilon 1}$ position of the imidazole ring of Ac-His at 37°C (Ref. 4). At lower pHs no exchange takes place and at higher pHs k_ψ values become constant (k_ψ^{max}).

$$k_\psi^{max} = k_2 Kw/Ka \quad (4)$$

thus $$k_2 = Ka\ k_\psi^{max}/Kw \quad (5)$$

Between these two extreme pHs k_ψ changes with pH of the incubation medium. When the relation of eq. (5) is used, eq.(2) can be rearranged into the following eq. (6).

$$\log \frac{k_\psi/k_\psi^{max}}{1 - k_\psi/k_\psi^{max}} = pH - pKa \quad (6)$$

Equation (6) corresponds to the Henderson-Hasselbalch equation and the left side term of the equation implys the ratio of mole fraction of imidazole to that of imidazolium cation which is reactive species in the exchange reaction as shown in Fig. 1. Thus the midpoint of the sigmoid shown in Fig. 2 corresponds to pKa of Ac-His. We can therefore estimate pKa values of histidine derivatives by hydrogen-tritium exchange reaction.

EFFECT OF NEIGHBORING CHARGED GROUPS ON pKa OF HISTIDINE

We applied this procedure to guanylic acid specific ribonuclease (RNase) St. prepared from the cultured broth of Streptomyces erythreus. It contains two histidines in 101 amino acid residues in a single polypeptide chain (Ref. 5). The results obtained at ionic strength 0.2 for the two histidines are shown in Fig. 3 (procedure for the tritium exchange titration for proteins is described later).
Values for k_ψ^{max} are similar to each other but pKa values are largely different for the two histidines (Ref. 6). To search for factors affecting pKa value of the imidazole ring, neutral acetylhistidine methylamide (Ac-His-NHMe), acidic Ac-His and basic histamine were subjected to the tritium exchange reaction as model compounds. As listed pKa values measured in Table 1, pKa of Ac-His-NHMe is 6.30, which seems to be an intrinsic pKa of histidine residues in proteins, and neighboring negative charge raises pKa (Ac-His) and positive charge lowers (histamine) (Ref. 7). These results suggest that strongly interacting negative charge exists around His-60 (pKa 7.95) in RNase St. in contrast to His-91 (pKa 6.30) in its three-dimensional structre (Ref. 6).

TABLE 1. Effects of charged groups on pKa of the imidazole ring of histidine derivatives in hydrogen-tritium exchange reaction at 37°C.

Compound	Charged group	pKa	ΔpKa
Ac-His-His-NHMe	----	6.30	0
Ac-His	$-COO^-$	6.90	+ 0.6
Histamine	$-NH_3^+$	5.90	- 0.4

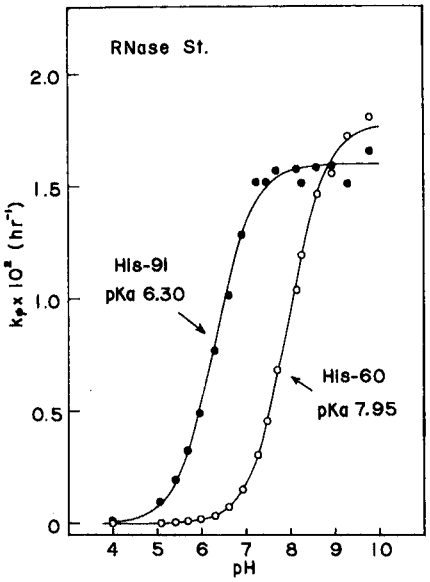


Fig. 3. Tritium exchange titration curves for His 60 and His-91 in RNase St. (Streptomyces erythreus) at 37°C and ionic strength 0.2 (Ref. 6).

FACTORS AFFECTING REACTIVITY OF THE IMIDAZOLE RING OF HISTIDINE IN PROTEINS

When the tritium exchange titration was applied at ionic strength 0.1 to bovine pancreatic RNase A containing four histidines, similar pKa values were measured but their k_ψ^{max} values were different from each other as shown in Fig. 4 (Refs. 8 & 9). We thus used several imidazole derivatives having different pKa values as model compounds to look for factors affecting reactivity of the imidazole ring in the tritium exchange reaction. As shown some typical examples in Table 2, pKa is clearly proportional to k_ψ^{max} linearly. The k_ψ changes as a function of pH, but k_2 is a constant for respective compounds (see eqs. (2) and (5)). When values of log k_2 for the four model compounds listed in Table 2 are plotted against their pKa values, a straight line can be obtained as shown in Fig. 5, which is similar to the Brønsted plot for the general base-catalyzed ester hydrolysis reaction, but the direction

TABLE 2. pKa and k_ψ^{max} of imidazole derivatives in hydrogrn-tritium exchange reaction at 37°C.

Compound	pKa	k_ψ^{max} (x 10^{-2}/h)
trans-Urocanic acid	5.65	0.95
Ac-His-NHMe	6.30	1.39
Ac-His	6.90	2.00
Imidazole propionic acid	7.30	2.86

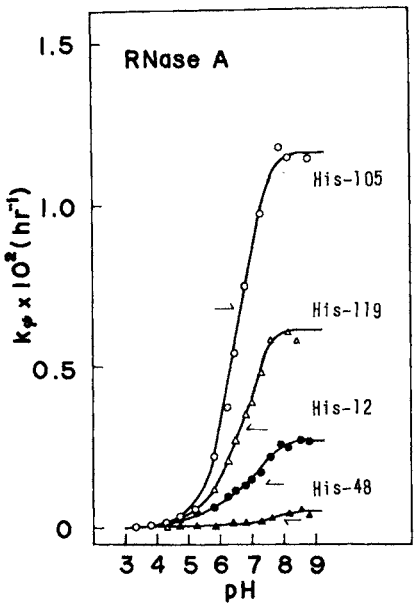


Fig. 4. Tritium exchange titration curves for the four histidines in RNase A at 37°C and ionic strength 0.1 (Refs. 8 & 9).

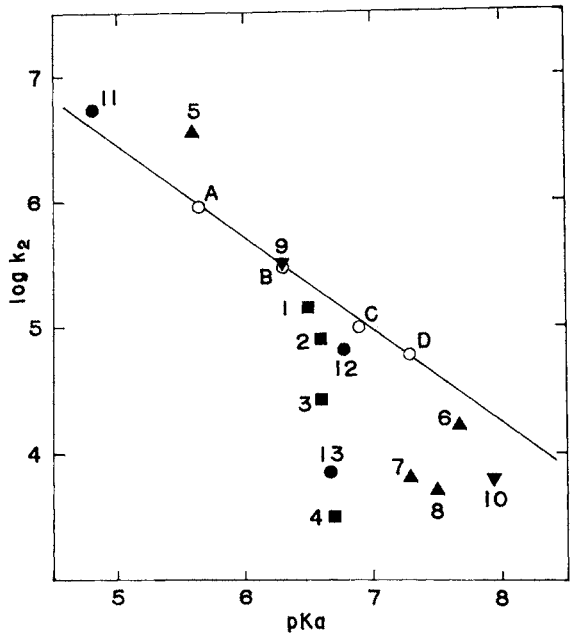


Fig. 5. The Brønsted plot for imidazole derivatives and histidine residues in several proteins (Ref. 7).
Model compounds : A, trans-urocanic acid; B, Ac-His-NHMe; C, Ac-His; D, imidazole propionic acid. RNase A : 1, His-105; 2, His-119; 3, His-12; 4, His-48. Lysozyme : 5, His-15. RNase T_1 : 6, His-40; 7, His-27; 8, His-92. RNase St.: 9, His-91; 10, His-60. St. eryth. trypsin : 11, His-27; 12, His 154; 13, His-57.

of the straight line is reverse. We call this relationship in the tritium exchange reaction as Brønsted plot hereafter. In Fig. 5, log k_2 versus pKa relations for the four histidines in RNase A are also plotted together with for histidines in several proteins. The perpendicular distances between the measured values for the four histidines in RNase A and the straight line in the Brønsted plot correspond clearly to solvent inaccessibilities of the individual histidines which can be deduced from the three-dimensional structure (Ref. 10) based on the X-ray crystallographic analysis, of which schematic drawing is shown in Fig. 6.

When the tritium exchange reaction of any protein is carried out in the presence of 6 M guanidine hydrochloride, pKa values of every histidines fall into 6.3 ∿ 6.5 and their relative values of k_{ψ}^{max} to that of Ac-His are about 0.55 (Ref. 9). Therefore it can be concluded that the differences in pKa and the reactivity of individual histidines in native proteins are controlled by their environmental factors in their three-dimensional structures. Using the information of the two parameters for individual histidines measured in the tritium exchange titration, we can predict local environment of each histidine residue even if their three-dimensional structures are unknown.

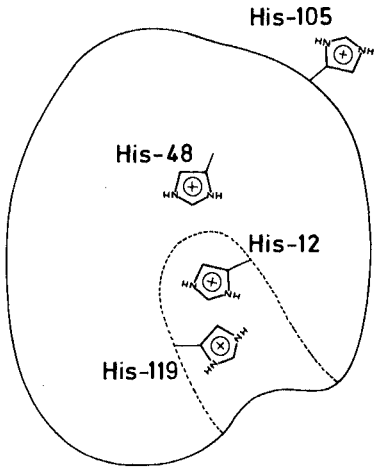


Fig. 6. Schematic drawing of locations of the four histidine residues in RNase A. Dotted line indicates region of the active site crevice.

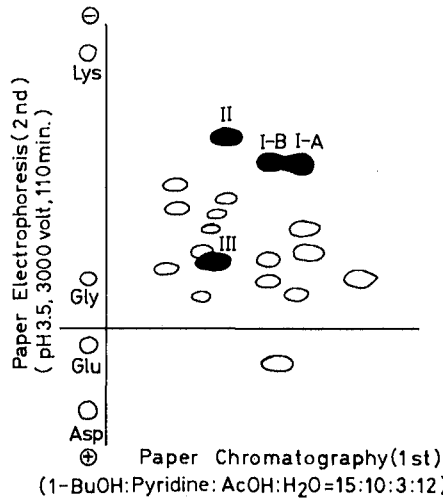
PROCEDURE OF TRITIUM EXCHANGE TITRATION FOR PROTEINS

In applying the tritium exchange titration to proteins containing more than two histidines, procedure is not simple. Protein samples (0.3 μmole) are incubated with buffered tritiated water (0.2 ml, 4 mCi) at a definite ionic strength at 37°C and various pHs in sealed tubes separately for 24 ∿ 48 h. After the incubation, the reaction is terminated by adding 0.5 ml of formic acid. The excess tritiated water is removed in vacuo and the removal of the exchangeable tritium is repeated several times by dissolving the dried sample in 30 % acetic acid. The radioactive protein in 0.5 M formic acid is subjected to gel filtration to remove salts. The lyophilized radioactive protein is oxidized with performic acid followed by digestion with proteolytic enzymes for 2 ∿ 4 h. When trypsin or chymotrypsin is used at pH 8, back exchange of the incorporated tritium takes place in about 10 % at 37°C for 4 h. The radioactive histidine-containing peptides are separated from each other by two-dimensional peptide map and are extracted with 30 % acetic acid from the map. We have to prepare about 30 peptide maps from the labelled protein samples at 30 different pHs to prepare sigmoid curve illustrating the k_{ψ}- pH correlation. The extracted peptides are hydrolyzed with 6 M HCl at 110°C for 24 h as usual. The specific radioactivity of histidine is calculated by counting radioactivity and by analyzing histidine content in the hydrolyzate. During the acid hydrolysis no back exchange of the incorporated tritium is proved to be taken place (Ref. 4). From the specific radioactivity $\underline{x}$ at time t and $\underline{a}$ at ∞, which is measured by use of Ac-His with the same lot of tritiated water, k_{ψ} can be calculated by using eq. (3). A sigmoid curve is then prepared by plotting k_{ψ} against the reaction pH for each histidine.

ANALYSIS OF LOCAL ENVIRONMENT AROUND INDIVIDUAL HISTIDINE RESIDUES IN PROTEINS

Ribonuclease T_1

RNase T_1 of unknown three-dimensional structure was subjected to the tritium exchange titration at ionic strength 0.2. RNase T_1 contains three histidines at the position of 27, 40 and 92 (Ref. 11). Figure 7 shows the peptide map of the tryptic-thermolytic digest (each for 2 h at 37°C) of the performic acid oxidized RNase T_1. The amino acid sequences of the four histidine-peptides, which were deduced from the results of amino acid and N-terminal analyses, are also shown in Fig. 7. Peptide I-A and I-B were combined since the two contained the same His-92. The k_{ψ} values measured at various pHs for the three peptides are plotted against pH as shown in Fig. 8. The three sigmoids in Fig. 8 are broken above pH 9 and k_{ψ}^{max} values of the three histidines are converging to about 0.01/h, suggesting denaturation of the enzyme. In the presence of a competitive inhibitor, 3'-GMP, sigmoids for His-40 and His-92 shifted to alkaline pH as seen in Fig. 9, suggesting that the two are participated in the active site of the enzyme (Ref. 12). The Brønsted plot for the three histidines shown in Fig. 5 suggests that His-40 is located near the molecular surface while His-27 and His-92 are



I-A : Val-Ile-Thr-His^{92}-Thr

I-B : Val-Ile-Thr-His^{92}-Thr-Gly

II : Val-Gly-Ser-Asn-Ser-Tyr-Pro^{40}-His-Lys

III : Leu-His^{27}-Glu-Asp-Gly-Glu-Thr

Fig. 7. Peptide map of the tryptic-thermolytic digest of the performic acid oxidized RNase T_1 and amino acid sequences of the four histidine-peptides. Paper chromatography was first carried out on Whatman 3MM paper with 1-butanol-pyridine-acetic acid-water (15 : 10 : 3 : 12, by vol.) followed by electrophoresis at pH 3.5 (pyridine-acetic acid-water = 1 : 10 : 289, by vol.) and at 50 volts/cm (Ref. 12).

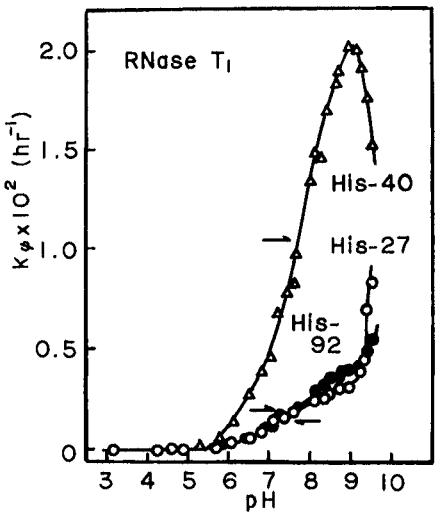


Fig. 8. Tritium exchange titration curves for His-27 (o), His-40 (Δ), and His-92 (•) in RNase T_1 at 37°C and ionic strength 0.2. Above pH 9 the enzyme starts to denaturation and their local environment is becoming a similar state (Ref. 12).

imbedded in the molecule and their solvent accessibilities are similar to that of His-12 in RNase A. The values of pKa of the three histidines are around 7.5, suggesting that strongly interacting carboxyl groups exist near respective histidine residues in the three-dimensional structure of the enzyme. In the tritium exchange titration the effects of these carboxyls could not be visualized, but ^{1}H NMR titration (Ref. 13) revealed the interaction with carboxyls in the three histidines as shown in Fig. 10; His-40 interacts with a carboxyl with pKa 4.1, probably active site Glu-58 (Ref. 14), His-27 with a pKa about 5, and His-92 with a pKa around 4.

Streptomyces erythreus trypsin

Next, the tritium exchange titration was applied to a protease possessing trypsin specificity produced by St. erythreus (St. eryth. trypsin) (Ref. 15) to estimate pKa of the active site histidine, since several investigators (Ref. 16-19) assigned acidic pKa (3.5 ∿ 4.5) and others (Refs. 20-22) neutral (6.5 ∿ 7.5). We found that St. eryth. trypsin containing four histidines in 227 amino acid residues is stable between pH 4 and 9 without autodigestion

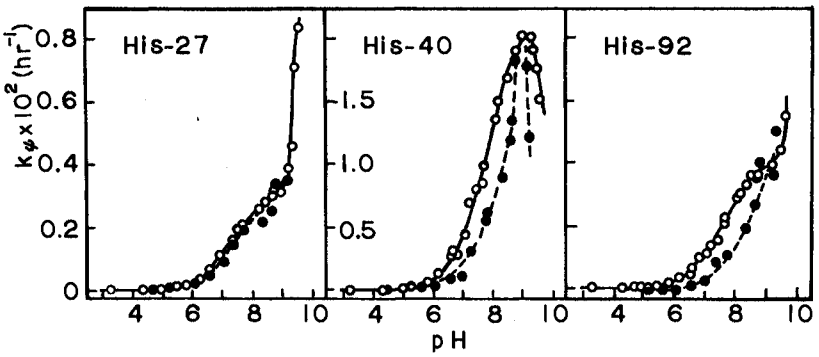


Fig. 9. Tritium exchange titration curves for the three histidines in RNase T_1 in the presence of 3'-GMP. No difference in the titration curve for His-27 between in the presence (broken line) and in the absence (full line) of the competitive inhibitor was observed, suggesting that His-27 is located in a region apart from the active site (Ref. 12).

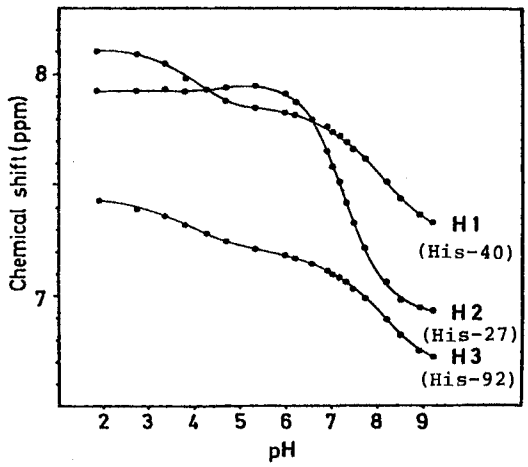
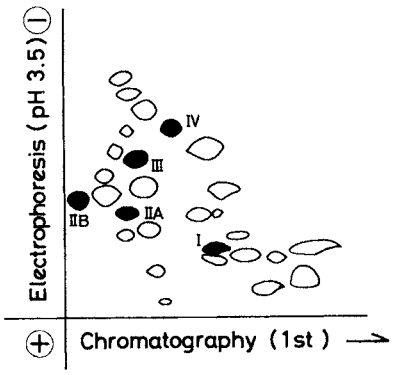


Fig. 10. Proton NMR titration curves for the three histidines in RNase T_1 at 32°C. Peak assignment was carried out by comparing rate constants of hydrogen-deuterium exchange with those of hydrogen-tritium exchange for the three histidines (Ref. 13).

under the conditions of the tritium exchange titration (Ref. 23). The tritiated protease was purified by affinity chromatography on a column of Sepharose 4B coupled with soybean trypsin inhibitor. The purified radioactive protease was reduced and aminoethylated with ethyleneimine instead of performic acid oxidation, since the primary structure has not been elucidated yet and the histidine-peptides extracted from the peptide map must be sequenced. The peptide map of the tryptic-chymotryptic digest of the aminoethylated protease and amino acid sequences of the four histidine-peptides are shown in Fig. 11. The active site histidine (His-57, chymtrypsinogen numbering system) was concluded to be involved in peptide IV in Fig. 11, because this peptide disappeared on the map upon treatment of the native protease with tosyllysine chloromethylketone and sequence similarity with those of other serine proteases. The tritium exchange titration curves for the four histidines are shown in Fig. 12. His-91 could not be labelled with tritium, suggesting that it is imbedded in the molecule or located in a solvent inaccessible region. The present method gave pKa 6.7 for the active site His-57, suggesting that it is proyonated below pH 6 (optimum for enzyme is pH 8). The Brønsted plot for the three histidines is shown in Fig. 5. His-57 seems to be imbedded in a manner similar to His=48 in RNase A. His-27 appears exposed on the molecular surface and some unknown factors seem to accerelate the tritium exchange reaction. Similar accerelation was observed for His-15 in hen's egg white lysozyme (Ref. 4). A cationic group must be interacting strongly with His-27 since its pKa is very low (pKa 4.8). It is well established that the active site His-57 is hydrogen bonded to Asp-1o2 in serine proteases. Therefore, pKa of His-57 must be higher than the intrinsic pKa of histidine residue (pKa 6.3 $\sim$ 6.5) in proteins due to interaction with the neighboring carboxyl group of Asp-102. This



I : Ile-Val-Gly-Gly-Glu-Asp-Ala-Asn-Val-
^{27}
Gln-Asp-His-Pro-Phe

IIA : Gly-Trp-Gly-Asn-Thr-Ser-Glu-Gly-Gly-
^{154}
Gln-Gln-Ala-Asp-His-Leu-Gln-Lys

IIB : Gly-Asn-Thr-Ser-Glu-Gly-Gly-Gln-Gln-
^{154}
Ala-Asp-His-Leu-Gln-Lys

III : Val-His-Pro-Glu-Tyr-Gln-Asp-Ala-Ala-Lys
^{91}

IV : Val-Val-Thr-Ala-Ala-^{57}His-Cys

Fig. 11. Peptide map of the tryptic-chymotryptic digest of the reduced and aminoethylated St. eryth. trypsin and amino acid sequences of the histidine-peptides (Ref. 23).

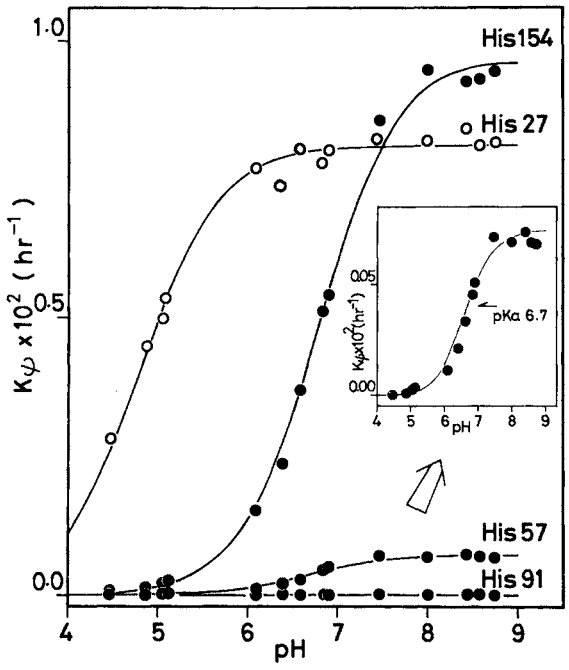


Fig. 12. Tritium exchange titration curves for the four histidine residues in St. eryth. trypsin at 37°C and ionic strength 0.2. The inset in this figure shows the sigmoid for His-57 with an enlarged ordinate (Ref. 23).

is not the case. There exists, therefore, some factors around His-57 to reduce acidity of Asp-102. One of the candidates is induced nucleophilicity of the O^{γ} atom of the active site Ser-195 through hydrogen bonding system (charge relay system) originally proposed by Blow et al. (Ref. 24), although recent refined X-ray crystallographic data suggested that no hydrogen bond may exist between $N^{\epsilon 2}$ of His-57 and O^{γ} of Ser-195 (Ref. 25). In order to prove the above speculation further studies must be performed.

CONCLUDING REMARKS

1. The hydrogen-tritium exchange reaction at the $C^{\epsilon 1}$ position of the imidazole ring of histidine residues in proteins can be used to analyze local environment of individual histidines in proteins even though their three-dimensional structures are unknown.
2. The neighboring cationic group lowers pKa of the histidine residue, while the neighboring anionic group raises.
3. The magnitude of the second-order rate constant in the exchange reaction seems to correspond to solvent accessibility of the histidine residue in the protein.
4. The present reaction can be used for assignment of the active site histidines by carrying

out the exchange reaction in the presence of a competitive inhibitor, if they are participated in the formation of the active site of the enzyme.
5. The present reaction may be utilized for analysis of the interaction between a protein and other biological macromolecules, if histidines are concerned.
6. The present reaction can be used for the assignment of histidine peaks in ^{1}H NMR titration of proteins.

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THE CONFORMATIONS OBSERVED IN THE N TERMINAL A CHAIN RESIDUES OF INSULIN

E. Dodson, G. G. Dodson and D. Crowfoot Hodgkin*

Department of Chemistry, University of York, Heslington, York, UK
**Chemical Crystallography, University of Oxford, UK*

Abstract - The two independent molecules in the asymmetric unit of the 2 zinc insulin crystal assume distinctly different conformations at the A chain N terminus. The variation seems to arise from the interactions made to the two B_5 histidines which are packed closely together by the crystal organisation of 2 zinc insulin. In 4 zinc insulin where only one B_5 histidine interacts with the initial A chain residues the packing is less constricted, and a similar preferred conformation in the initial A chain residues is seen for both molecules.

INTRODUCTION

Professor Shemyakin's name is in my mind intimately connected with problems of peptide chemistry, particularly rings including peptide groups, although I know he has made contributions in many other fields of organic chemistry. I therefore thought it worth recording in his memory, the present state of our observations on the internal peptide disulphide rings of the A chains of insulin.

I first came across work in the Soviet Union in the field of peptide chemistry during the war, in 1944, when Professor Florey visited Moscow and brought the news and later some actual crystals of the antibiotic peptide, gramicidin S. Its discovery and use in treating wounds was published by Gauze and Brazhnikova (1) in Nature in 1944; it seemed a potentially very useful subject for exact study in its own right and also possibly for the light it might throw on the structure of proteins. The crystals passed into the hands of R.L.M. Synge who gave some of these to me. We planned a parallel chemical and crystallographic study. At that moment, the X-ray analysis of even the smallest protein molecule we knew, insulin, seemed far out of our reach. As the years went on, other small biologically active peptides appeared and, particularly, oxytocin and vasopressin. In developing chromatographic methods of amino acid sequence determination, the order of events we had imagined for X-ray analysis actually occurred. Synge tried these techniques first on gramicidin S and his success was followed by others on oxytocin and vasopressin. In 1956 Sanger (3) was able to show that within the A chain of insulin, the cystine disulphide bond closed a peptide ring identical in size with that of oxytocin. It was tempting, in the absence of exact knowledge, to suggest that the structures were geometrically similar and I succumbed to the temptation. The most plausible model for gramicidin S was based on β-pleated sheets which could easily, by β bends, bring residues carrying cysteine groups at the correct interval to react with one another, to form the disulphide ring of either oxytocin or vasopressin.

Now that many peptide and protein structures have been solved by X-ray analysis we realise that a number of variations are possible in peptide chain geometry, even controlled as it must be by conformational restrictions, as Ramachandran and others have calculated. It is all the same surprising to find that the initial residues of the insulin A chains, 1-11, adopt markedly different conformations in the two insulin molecules which constitute a dimer in the 2-zinc insulin (pig) crystals. These differences were first observed in the structure analysis at 2.8Å resolution reported in 1969, (4) 1971. (5) They have now been checked in detail through various processes of refinement of the original crystal structure analysis at Oxford, currently based on data extending to 1.5Å. In one of the operations used, 1/8 of the atomic distribution at a time was omitted in the calculations of phase constants required for the evaluation of new electron density distributions: several residue rearrangements followed from the revised calculations While many

of the small adjustments which resulted from the refinement decreased the differences originally observed between the B chains of the two insulin molecules, the differences between the A chains persisted. They are confirmed by a totally independent solution of the structure of 2-zinc insulin carried out by the Peking insulin group (6).
In both insulin molecules the B chain consists of three distinct regions : an extended chain between 1 and 10, an α helix between 10 and 19 and a short loop, followed by an extended chain, 23-30. In both molecules the A chain rests on the B chain helix and makes contacts with the extended regions. The two individual molecules may be described as follows:

Molecule I (Oxford)
The initial eight residues follow the general course of a rather irregular α-helix. There was some initial obscurity about glycine A1: the first maps suggested the carbonyl group was directed away from the line of the helix, associated possibly with some disorder. The refined structure shows predominantly the helical form. The oxygen to nitrogen hydrogen bonded distances within the helical region vary from 2.80-2.96Å with the exception of the distance O3 - NH7 which is 3.3Å. Here the carbonyl oxygen makes a close contact, 2.85Å, with a very well-defined water molecule. The glycine terminal N^+H_3 group is in contact at 2.8Å with glutamic acid CO_2^- at A4: it is also hydrogen bonded to two well defined water molecules. There is no hydrogen bond directly across the peptide 6-11 disulphide ring. Approximately the CO and NH groups are directed at angles approaching 90° to the ring making external contacts. Two, 11NH and 11CO, hydrogen bond with 4CO and 4NH as in a β sheet; CO 7, 8 and 9 all interact with histidine B5 either of molecule I or II. They also make contact with water molecules.

Molecule II (Oxford)
The initial residues again follow a helical course but only the first three α helical hydrogen bonds are made, CO1-NH5, CO2-NH6, CO3-NH7. From this point the helix widens to a π helix CO4 contacts NH9 and CO5 makes a rather long contact, 3.3Å with NH10. Again the glycine A1 NH_3^+ is salt bridged to glutamic acid A4 CO_2^-. It is also salt bridged in a beautifully defined arrangement, found on structure refinement, with the B chain terminal carboxyl group. Again there is no hydrogen bond across the peptide disulphide ring and the CO and NH groups make external contacts, 11CO and NH with 4NH and CO as before and CO7 and CO9 with histidine B5 (molecule II).

Figures 1,2 and 3 illustrate the different chain conformations and the contacts made within the chains and by the chains and the crystal. Details

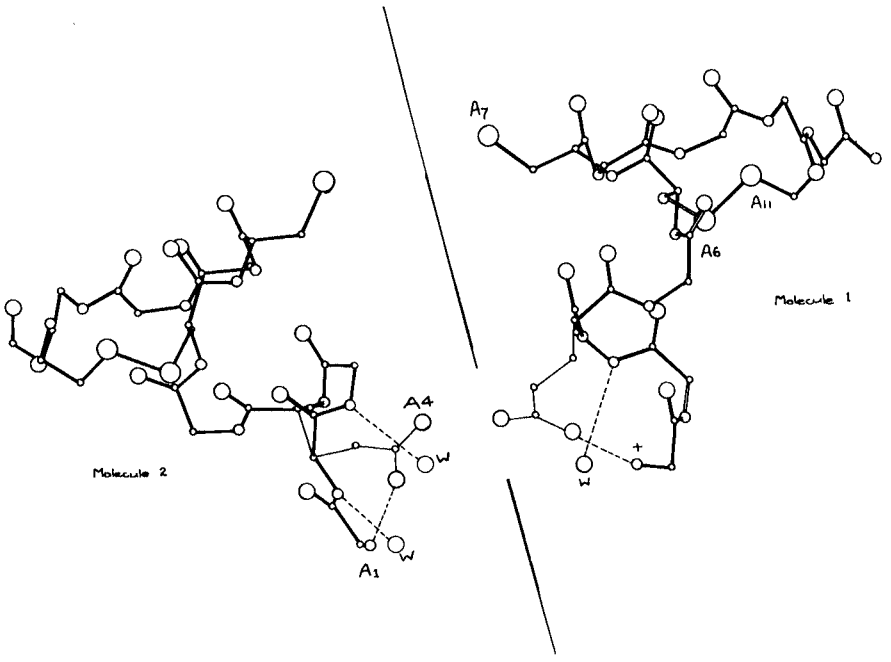


Fig. 1. Projections of the A chain residues 1-11 in 2-zinc insulin of molecule I and molecule II seen parallel with the c axis. The line of the approximate 2-fold axis is shown.

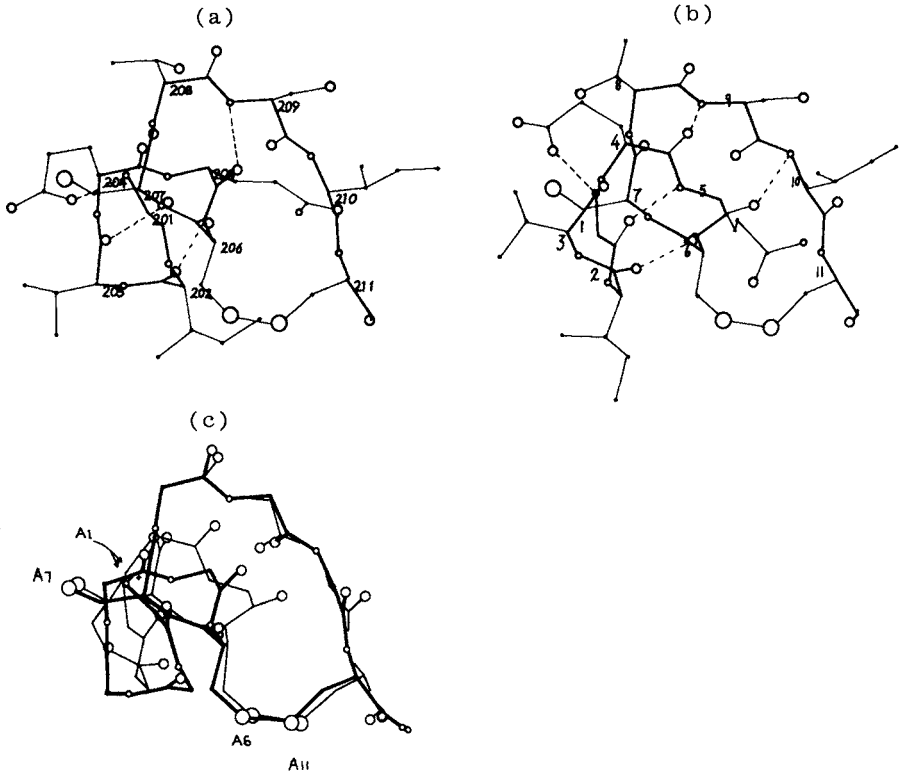


Fig. 2. Projection of the A chain residues 1-11 in 2 zinc insulin seen roughly normal to the plane of the ring. (a) molecule I, (b) molecule II, (c) molecule I (thick line) and molecule II superimposed.

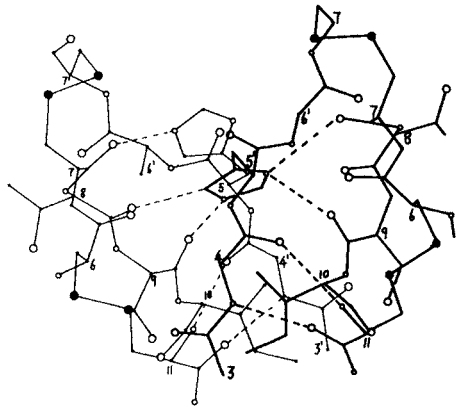


Fig. 3. Contacts between the A & B chains of molecules I and II, 2 zinc insulin, seen in the direction of the c axis. Molecule II above, thick line; molecule I below, thin line.

of the ϕ and ψ angles which define them are shown in the Ramachandran plot in Figure 4. These indicate that the principle difference is due to a relative rotation about the bond 6Cα-NH which appears as a change in ϕ of A6. Many of the residue conformations are unchanged relative to the α helices.

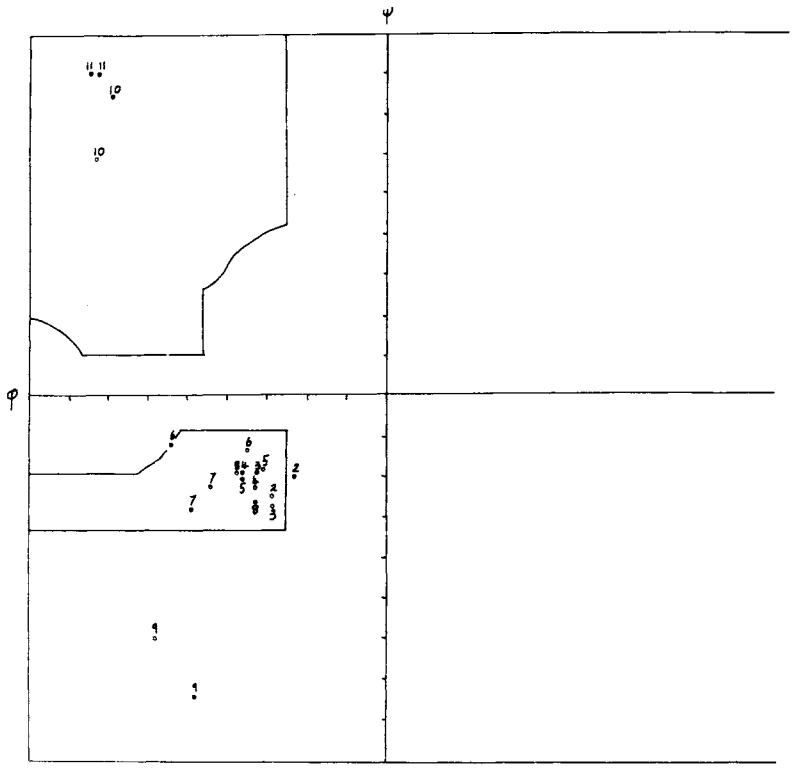


Fig. 4. Ramachandran plot; A chain residues 1-11, 2 zinc insulin.

We have now examined a number of other insulin crystal structures, none in such detail as 2-zinc insulin, but three to a sufficient degree of accuracy to indicate the probable A chain conformation. Both in hagfish insulin and in cubic insulin (8) there is no zinc in the crystals and the insulin dimers are symmetrical; in both, molecule II fits the observed electron density better than molecule I. In 4-zinc insulin, the two insulin molecules differ from one another in the initial B chain residues (9) : as seen in Figure 5

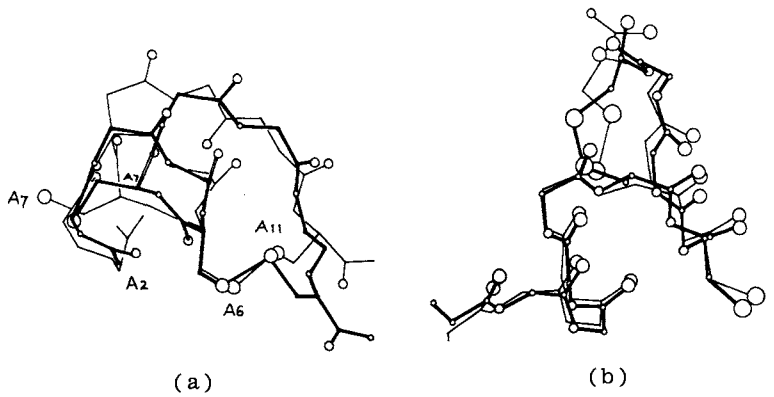


Fig. 5. Projection of the initial A chain resdues in 4-zinc insulin,
a) molecule I (thick line) and molecule II seen roughly normal to the plane of the ring.
b) 4-zinc insulin molecule II (thick line) and 2-zinc insulin molecule II seen along the C axis.
(The 4-zinc insulin coordinates are taken from the 1.5Å refinement being carried out by David Vallely at York).

the A chains are rather more like one another than in 2-zinc insulin in the initial helical residues. They differ in a geometry of the residues of the peptide ring from A7 to 11 both from one another and from the 2 zinc A chains.

The relations suggest that the main reason for the change in the A chains in 2-zinc insulin is crystallographic and due principally to the contact illustrated in Figure 3 between the two molecules parallel with the threefold axis where the histidine B5 groups are in contact with one another in 2-zinc insulin and are far apart in 4-zinc insulin. In 4-zinc insulin further changes arise from the geometrical differences at A7 B7 in molecule I; the reason for additional changes at molecule II is not yet clear.

It seems likely from the observations that the preferred conformation of histidine B5 is that found in molecule II. If the two molecules were identical, the histidine group of molecule I would project directly above that of molecule II and the molecules as a whole would be forced further apart. Close packing can be achieved by turning histidine B5, molecule I, which forces CO 7 further away and perhaps initiates the changes we observe. So many other subtle differences result we cannot be certain yet that we have found the primary cause of the changes we observe.
It is perhaps worth adding a post script on the other peptide ring containing crystals mentioned earlier: oxytocin, vasopressin and gramicidin S have all proved more resistant to X-ray analysis than many proteins. Oxytocin and vasopressin themselves have not so far crystallised well enough: recently good crystals of desamido oxytocin have been obtained by T. L. Blundell, the crystal structure is a little complicated, 2 molecules in the unit cell, but it seems likely it will soon yield. N.m.r. measurements taken in dimethyl sulphoxide suggest a conformation shown in Figure 6 for oxytocin similar to the old one suggested long ago but in water it appears very different. There has been a great deal of work, theoretical and spectroscopic, on gramicidin S and many proposed conformations. X-ray analysis was complicated

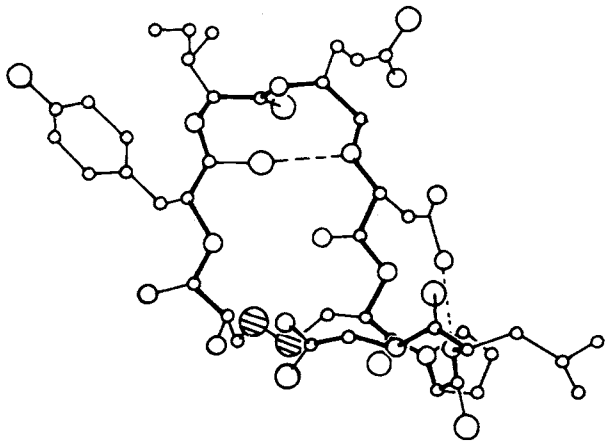


Fig. 6. Conformation of oxytocin proposed by Walter.

owing to crystals with asymmetric units containing many molecules. Some six years ago crystals were grown from solutions containing urea and hydrochloric acid and found to have a single molecule in the asymmetric unit. Very recently the structure of these crystals was solved at York (11). The form of the molecule shown in figure 8, looks, at first sight, very like the old proposed β pleated sheet structure of Figure 7 proposed by Schwyzer and by ourselves (12). But there are complications, which all of us who have seen β sheets in proteins would now expect. The molecule is very much twisted and, no doubt, as in other peptide rings, there are variants of the twists observed here which complicate the molecular structure in other crystals of this, apparently, so simple peptide.

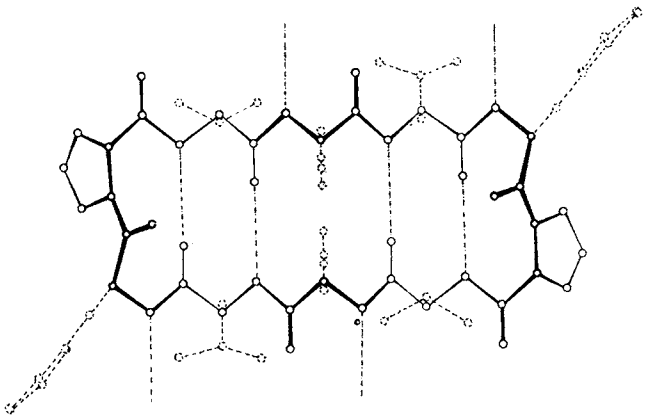
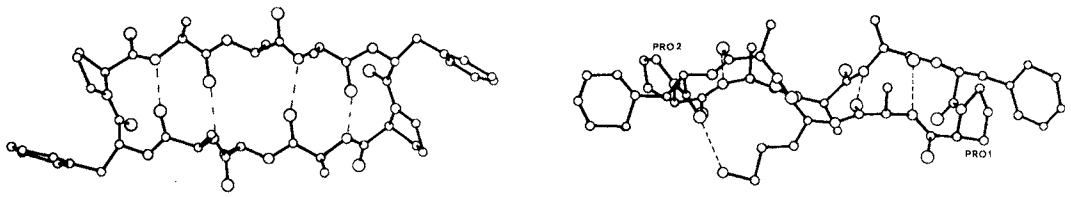


Fig. 7. Conformation first proposed for gramicidin S.



(a) (b)

Fig. 8. Projections of the molecular structure found for gramicidin S.
a) down the approximate 2 fold axis
b) perpendicular to the approximate 2 fold axis.

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GLOBULAR PROTEINS AND THEIR ASSOCIATIONS

B. K. Vainshtein

Institute of Crystallography, Academy of Sciences of the USSR, Leninsky prospekt 59, Moscow 117333, USSR

Abstract - The possibilities of modern X-ray structure analysis and electron microscopy in the investigation of the structure of molecules of globular proteins and their associations are considered. X-ray studies of leghaemoglobin at 2,0 Å resolution and its complex with nicotinic acid allowed explanation of the high affinity of this protein for oxygen. In the course of X-ray investigation of aspartate transaminase at 3,5Å resolution the folding of the protein chain and the domain structure of protein molecules were established. Electron microscope studies of tubular crystals of proteins and a number of bacteriophages made it possible to determine the parameters of the helical packing of molecules, their dimensions and shape.

INTRODUCTION

During recent years the investigation of the molecules of biological origin has become one of the most important applications of the method of crystal structure analysis and other diffraction methods as well as of electron microscopy. At present, the method of X-ray structure analysis allows one to investigate, with very good accuracy, the structure of globular proteins if one succeeds in crystallizing these molecules, i.e. in obtaining a protein crystal. The best works have been made at the resolution of 1,5-2 Å, the accuracy of atomic coordinates being of 0,1-0,2 Å. Electron microscopy of biomolecules gives resolution of about 20 Å which makes it possible to study the subunit structure of protein molecules and their packing in associations of various kind. In this communication new data on the tertiary and quaternary structures of some globular proteins will be presented as well as the results of the investigation of the structure of associations of protein molecules, such as viruses, plane, tubular and three-dimensional crystals. At present, about one hundred protein structures have been solved, and we know the structure of a series of families of globular proteins which are close in function, such as globins, cytochromes, proteases, dehydrogenases, immunoglobins and some others. Homology of protein structures of a given family is very extensive; it appears to be defined by the origin from one common protoprotein. It is possible to illustrate this on the example of leghaemoglobin which belongs to the globin family.

X-RAY STUDY OF LEGHAEMOGLOBIN

The best known common feature of all myoglobins and haemoglobins studied by X-ray crystallography is so-called myoglobin fold firstly revealed in sperm whale myoglobin and then found in other investigated haemoglobins, both monomeric and tetrameric. Leghaemoglobin was found in the root nodules of leguminous plants (soya, kidney beans, lupin, pea etc.) which have remarkable and practically important property: in symbiosis with effective strains of bacteria Rhizobium they have ability to fix atmospheric nitrogen. It seems that leghaemoglobin, as many other haemoglobins, concerns with the oxygen transport and supplies oxygen to bacteroids. The value of oxygen affinity of leghaemoglobin is much higher than that of myoglobin and other known haemoglobins. Two main fractions of lefhaemoglobin with different primary structure were isolated. The good crystals suitable for X-ray work were firstly obtained with the second fraction (in order of elution). The parameters of the unit cell of fraction II are a=92.95, b=38.31, c=52,15 Å, γ =98°45', space group B2. The unit cell contains one molecule per asymmetric unit. X-ray analysis showed that leghaemoglobin possesses a myoglobin fold (Refs. 1 & 2). Now this structure is refined at 2 Å resolution (Ref. 3). In the

interpretation of the electron density map (Fig.1) we could use the primary structure of the protein (Ref.4). Fig.2 shows the α-carbon chain. A number of deviations of helices from the classical α-helix was observed, The first turns in C and G helices are close to a 3/10-helix, the last turn in G being close to a π-helix. Fig.2 gives as well the comparison of the structure of leghaemoglobin with another protein of this family - erythrocruorin - globin from insects (Ref.5). The differences in primary structures for some proteins of this type are shown in Table 1. The differences between

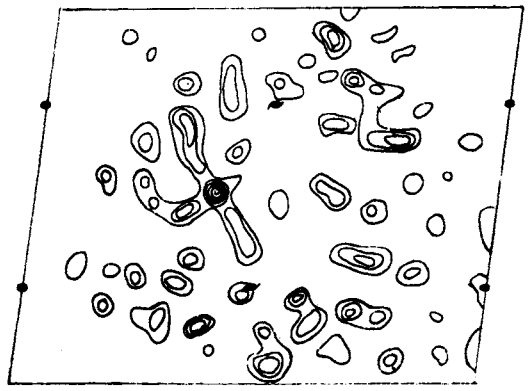


Fig.1

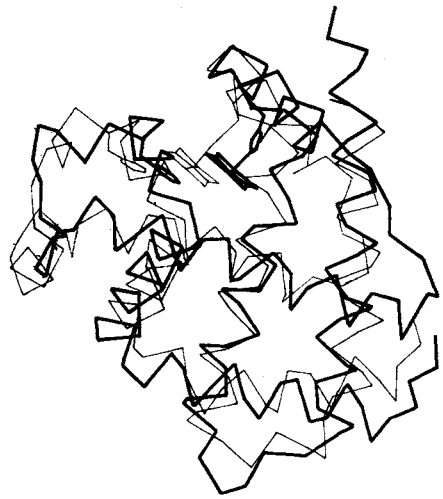


Fig.2

Fig.1. Fourier synthesis of the electron density of leghaemoglobin, 2.0 Å resolution. Section at z=0,56 through the haem group.

Fig.2. Folding of the polypeptide chain in leghaemoglobin (solid line) and erythrocruorin (thin line).

plant and animal haemoglobins reach 80-90%. Inside the group of leghaemoglobins they are naturally less - about 20-50%.

TABLE 1. The differences in primary structure in some globins (%).

1. Sperm whale MB	88					
2. Human HB chain	84	78				
3. Lamprey HB	87	75	75			
4. Erythrocruorin	83	79	88	79		
5. Soya bean Lg	55	85	83	86	86	
Lupinus luteus Lg I comp.	↑	1	2	3	4	

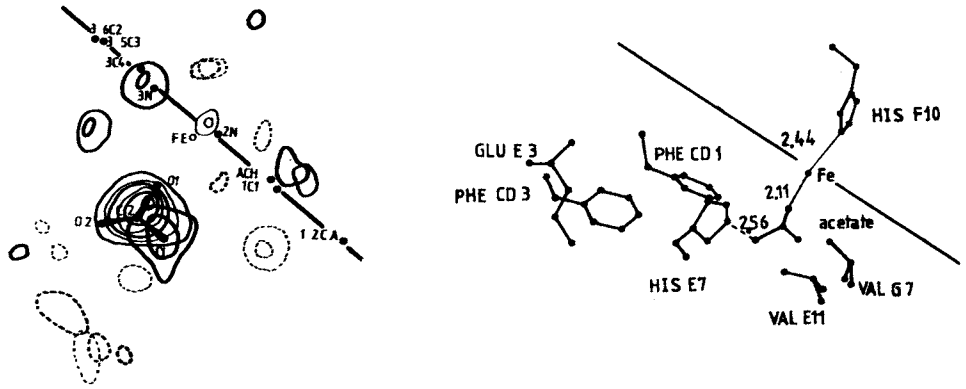
1. Soya Bean Lg	55		
2. Kidney bean Lg	51	20	
3. Broad bean Lg	57	38	35
	↑	1	2
	Lupinus luteus Lg I comp.		

Table II gives the number of residues in helices in leghaemoglobin and myoglobin. In this respect leghaemoglobin is similar to other globins, the only special feature being the absence of D-helix and longer E and F helices. The similarity of animal and plant haemoglobins is considered to be an example of the evolutionary divergence. In that case we must accept the existence of a protohaemoglobin in organisms which lived about 1.5 billion years ago - the time of divergence of plants and animals. The structural

TABLE II. The number of residues in helical and irregular segments in leghaemoglobin and myoglobin.

	NA	A	AB	B	C	CD	D	E	EF	F	FG	G	GH	H	HC
Lg	3	7	-	16	7	13	-	26	5	14	2	20	3	26	1
Mb	2	16	1	16	7	8	7	20	8	10	4	19	5	26	4

features of leghaemoglobin provide an explanation of its high affinity for oxygen as compared with that of most other haemoglobins. In the main, these features are in the structure of the functional part of the molecule, i.e. of the haem pocket (Fig.2). On the side of distal histidine the size of the haem pocket is larger than in other globins. There is a displacement of distal histidine towards the edge of the porphyrin system. During the refinement of the structure at 2 Å resolution it was found that in crystals of leghaemoglobin investigated the acetic group is the sixth ligand of the Fe atom, but not the water molecule as usual. The acetic group is linked with the Fe atom ($Fe-O_1=2.1$Å)via one oxygen atom, whereas the other O atom



(a) (b)

Fig.3a. Difference Fourier synthesis of the electron density showing the position of acetic group. Section of the haem plane is shown in solid line. b/ Positions of some residues in the haem pocket.

is hydrogen bonded to N of distal histidine. Replacement of the water molecule by acetic acid appears to take place during crystallization from protein solutions in acetic buffer. Stabilization of the acetate group in the haem pocket is due to additional non-valence interactions with the atoms of the haem group and side groups of neighbouring amino-acid residues: phe CDI, his E7, Val EII and Val 7 (Fig.3b). It has also been found that proximal histidine is linked with the Fe atom much more weakly than in other haemoglobins, and than the acetic group in leghaemoglobin ($Fe-N_{\varepsilon\ prox}$ =2.44 Å). The displacement of the Fe atom from the plane of the haem group in the direction of distal histidine by 0,1 Å was observed. On the basis of data obtained one may assume that the strong binding of the oxygen molecule requires the weakening of the link of proximal histidine with the Fe atom. The essential changes in structure take place when it combines with nicotinic acid (Ref.6). It is known that nicotinic acid (NA) is a natural ligand of leghaemoglobin with especially high affinity for met-form of the protein. The analysis of the structure complex of leghaemoglobin+NA was carried out using the method of heavy-atom derivatives at 2.8 Å resolution (Fig.4). It was found that the residues His E7, Phe CD3 interact directly withNA. The NA molecule occupies the site of distal histidine which is

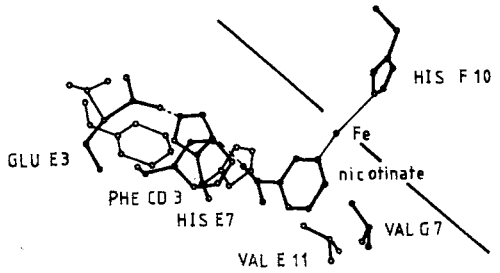


Fig.4. Scheme of the arrangement of nicotinate and some residues in the haem pocket of leghaemoglobin (solid lines). Positions of his E7, phe D_3 in acetic form, when nicotinate is not present, are shown in thin lines.

moved away and is attached to haem through the formation of linkage of the Fe atom to the N atom of the pyridine ring. In such a way, in leghaemoglobin the haem pocket proved to be so large that it could accomodate such a bulky ligand as NA.

X-RAY STUDY OF ASPARTATE TRANSAMINASE

While leghaemoglobin is a new member of well known protein family, another protein investigated, aspartate transaminase, may serve as an example of polypeptide chain folding, unknown before. Aspartate transaminase (aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) catalyses the transfer of amino group from aspartic acid on α -ketoglutarate, according to the equilibrium: L-aspartate + α -ketoglutarate $\rightleftharpoons$ oxalacetate + L-glutamate. The molecule of aspartate transaminase is a dimer composed of two chemically identical subunits. Up to now no cooperativity between subunits in dimeric state has been found. The molecular weight of the dimer is about 94000. The enzyme has a cofactor, pyridoxal-5'-phosphate. According to the general theory suggested by Braunstein and Shemyakin (Refs.7 & 8) there is an intermediate transfer of amino group on the coenzyme, which is converted to the form of pyridoxamine-5'-phosphate. The subsequent transfer of the amino group on keto acid provides the regeneration of pyridoxal-5'-phosphate. We investigated the enzyme from chicken heart cytosol . The crystals were grown in the presence of α -methyl-aspartate; they contain the enzyme in the form of the cofactor-substrate aldimine (Refs.9 & 10). The crystals are orthorhombic, space group $P2_12_12_1$, a=62.7 Å, b=118.1 Å, c=124.5 Å, the asymmetric unit contains one molecule of the protein, that is one dimer. The heavy-atom derivatives were prepared using mercury-organic compounds reacting with SH groups and two platinum derivatives. Average m is 0.81. The 5 Å resolution electron density map (Ref.10) and recent 3.5 Å map showed that the molecule of aspartate-transaminase consists of two compact subunits which may be approximated by the ellipsoids of 40x48x60 Å (Fig.5). The contact area between subunits of the enzyme is dense, with some elements inter-locking. The subunits are related by the non-crystallographic two-fold axis. In each subunit the α -helical segments of the molecule are clearly seen. The relative positions of these segments are very similar in two subunits revealing quite clearly the structural equivalence of the subunits. There are 9 helical segments of different length per one subunit. This accounts for about 40% of the structure. The special attention is paid to a long helix running along the surface of the molecule, its length being about 48 Å. The β -structure segments are also distinctly seen. In attempts of localizing the active center of the enzyme a number of experiments has been carried out with the protein in different functional states. The active site of the enzyme (coenzyme position) has been established from the difference map between two complexes of Asp-transaminase with quasi-substrates, one of them being alpha-methyl-aspartate, and the

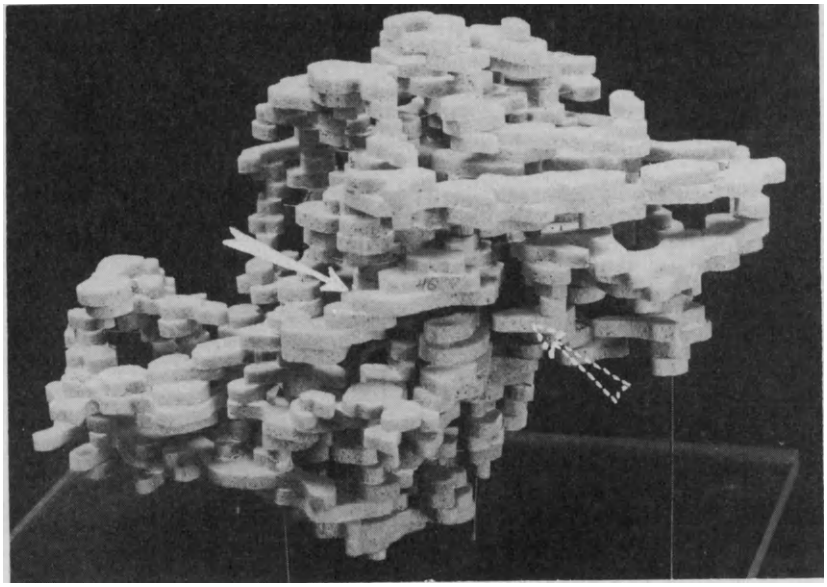


Fig.5. Structure of a dimer of Asp-transaminase. Model is constructed on the basis of 5 Å resolution electron density map. Positions of coenzymes are indicated.

other erythro-oxy-aspartate. The coenzyme is placed between the ends of two alpha-helices one of which forms the binding site for the phosphate group and the other seems to interact with hydroxyl and methyl groups of the coenzyme. The coenzyme is situated about 8-10 Å apart from the contact area between subunits. The active site of the enzyme has been seen to be placed between three domains of the protein. One domain is closely similar to so-called nucleotide-binding domain firstly found in the structure of lactate dehydrogenase (Ref.11). The substrate molecules are bound to another domain which shows easy change in conformation.

ELECTRON MICROSCOPY OF PROTEIN MOLECULES ASSOCIATIONS

The simplest (from the structural, but not functional point of view) case is the association of several protein subunits into a molecule with the quaternary structure. In the process of association the protein molecules interact with each other, mainly by electrostatic forces, i.e. between charged groups on the surface. Mutual-complimentary shape of protein subunits or molecules is also very important in this process. Another type of associations of protein molecules - when their number is extremely large- is the formation of one-, two- or three-dimensional periodic structure, i.e. chain or cylindrical structure, layers and three-dimensional crystals. The most effective method for investigation of the packing of protein molecules in the associations as well as the quaternary (subunit) structure of molecules is electron microscopy in combination with the mathematical methods of three-dimensional reconstruction of the structure of objects from its two-dimensional projections (Refs.12 & 13). The first work carried out as early as 1968 by help of algebraic three-dimensional reconstruction was the determination of the packing of molecules and their quaternary structure for enzyme catalase, m.w. 250000 (Ref.14). The quaternary structure of the enzyme leucine aminopeptidase has been recently established (Ref.15). It is a protein of molecular weight 328000, composed of six subunits. The images of the molecule in one projection are triangles of symmetry 3m with sides of 90 Å (Fig.6a). The side projections (Fig.6b) and the images of crystals were also obtained. The structure is shown in Fig.6c,d. Six subunits are arranged with the symmetry 32 at the vertices of a distorted three-faced prism. Each of the subunits has an "outgrowth", the ends of the subunits being joined in pairs. Of great interest are the structures with helical symmetry G_3^2 - rod-like viruses, tubular crystals, bacteriophage tails. The characteristic value which allows division of the structures

TABLE 3.

Object	Group of symmetry */	Object	Group of symmetry
Protein molecules	G_0^3	Rod-like viruses	G_1^3
Protein crystals	G_3^3	Tubular crystals	
Ribosomes	G_0^3 =1	Muscle proteins	
Spherical viruses	G_0^3 =532	Bacteriophage tails	
		Crystalline layers and membranes	G_2^2 , G_2^3

*/ G_n^m - symmetry group, m - dimensionality of object, n - number of space measurements along which the object is periodical.

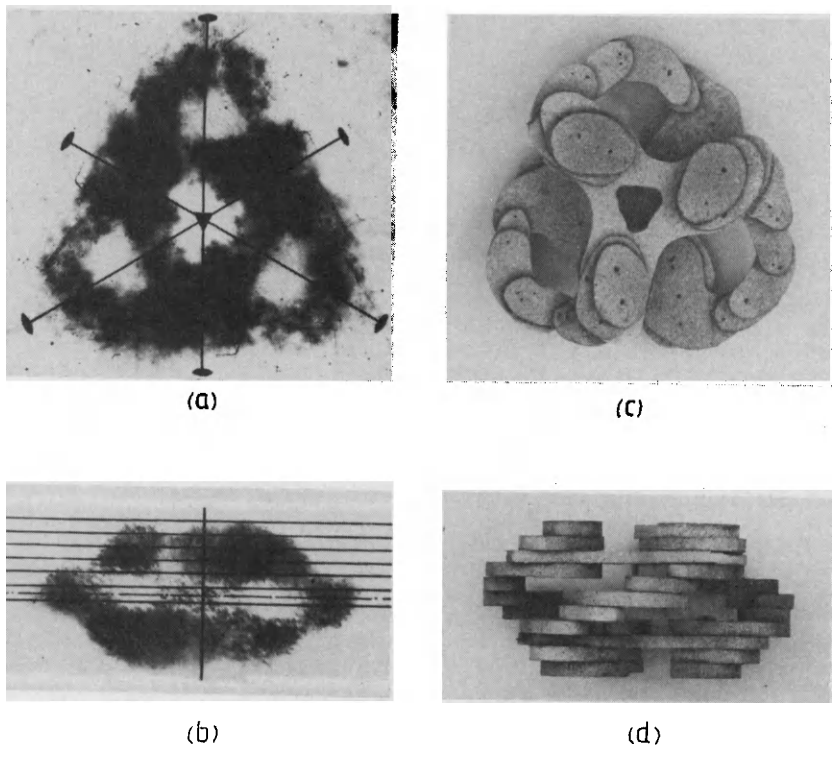


Fig.6. Leucine aminopeptidase. a,b - Averaged electron microscope images of the molecule along axes 3 (a) and 2 (b). c,d - Corresponding views of model obtained by three-dimensional reconstruction.

with G_1^3 symmetry into three classes in the ratio of the external radius to the size of the molecule (Ref.16). In this way we may distinguish "chain", "rod" and "tubular" helical structures. The well-known TMV and other similar viruses are the classical example of the rod-like structure. Among helical G_2^3 structures of special interest are the tubular crystals of globular proteins. Such crystals were firstly discovered in electron-microscopic studies of catalase (Ref.17). Later on, the tubular crystals were found for phosphorylase b and phosphorylase a as well as for glucoseoxydase. The tubular crystals in vivo and in vitro are formed by protein tubulin. The length of tubes for all these proteins reaches several thousands Angstroms. For catalase from ox-liver period c=840 Å, helical parameters p/q=142/17. The diameter of tubular crystals of this protein is 280-320 Å.

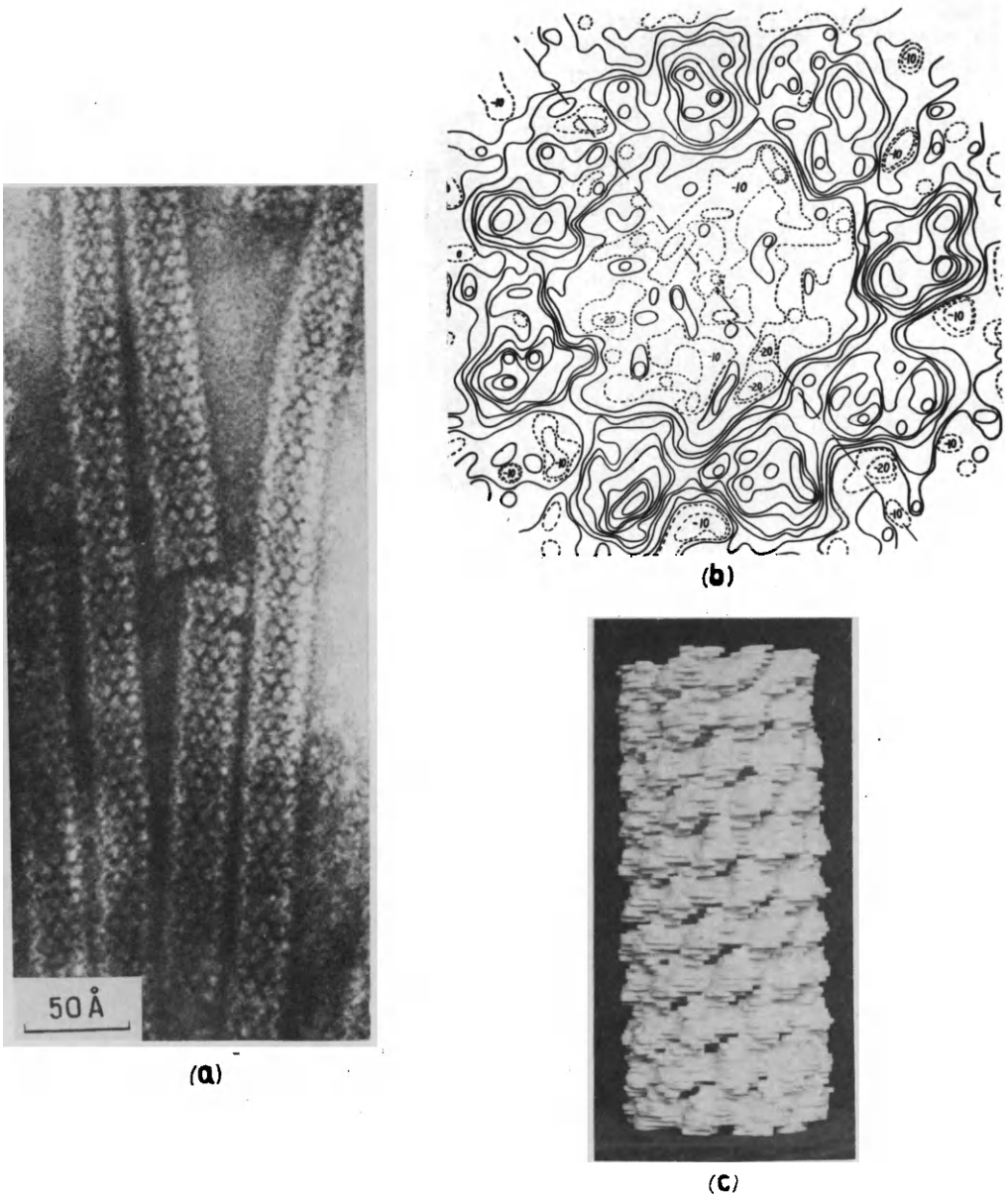


Fig. 7. a. Electron micrograph of tubular crystals of catalase from ox-liver. b. Reconstruction of a tube section. c. Model of the packing of molecules.

Reconstruction of one horizontal section of the tube (Fig.7b) gives cross-sections of the helically packed molecules at different levels, with the difference in height being c/p=5.9 Å. The result of three-dimensional reconstruction is given in Fig.7c. The catalase molecule has tetrahedral symmetry 222 and consists of four subunits. Its volume equals 300000 Å^3, the dimensions are 79x87x93 Å. Of much interest is the structure of the phage tail possessing helical symmetry and consisting of several kinds of protein molecules. The tail is an organ which injects the nucleic acid into the host bacterium. Some phages have the non-contractile tail which is built of protein molecules of one sort. The other phages have the contractile tail consisting of a narrow tubular core with a central channel surrounded by a sheath. In both cases the structure can be described as a pile of stacking discs with sixfold symmetry (N=6) with a rotation determined by the helical parameters p and q. The phage Buturicum (Ref.19) may serve as an example of phages with the long non-contractile tail. The diameter of the tail is 160 Å. The protein molecules have dimensions of about 70x30x40 Å, they are arranged with their long axis along the radii. The size of the axial channel is

50-60 Å (Fig.8a,b). The T-even phages T2,T4,T6 and DD6 similar in structure may serve as phages with contractile tails (Refs.20,21 & 22). For phage DD6 (Fig.9a,b) the diameter is 170 Å; N=6. The two families of helical

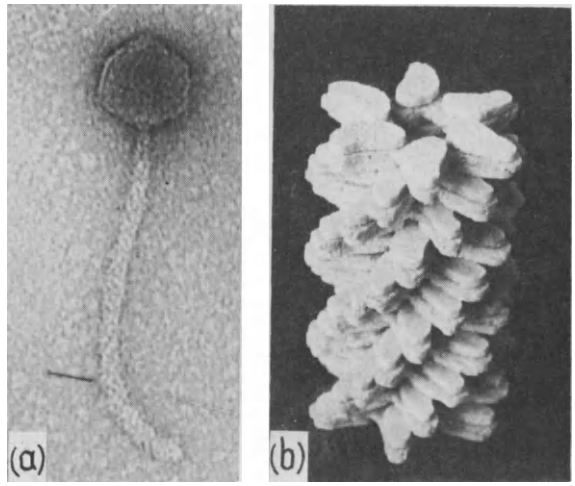


Fig.8. Bacteriophage Buturicum. a. Electron micrograph. b. Reconstruction of the structure of the tail.

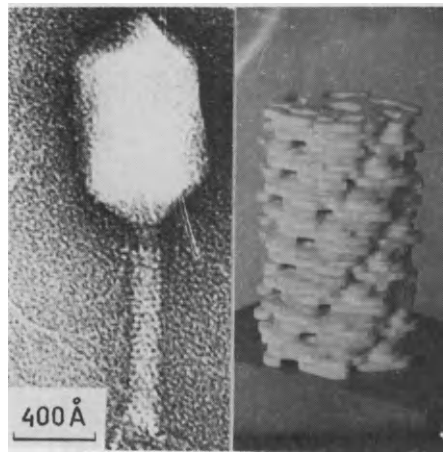
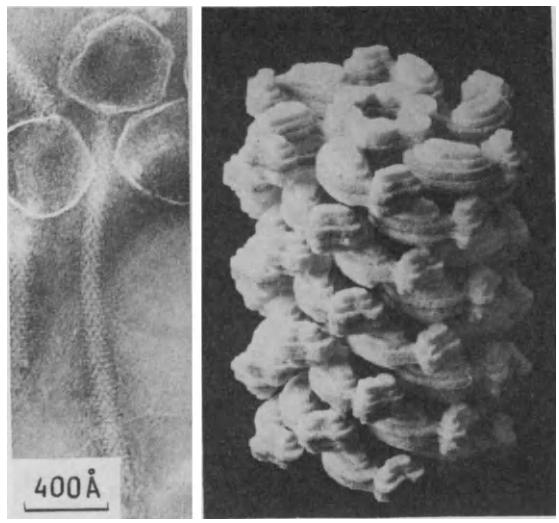


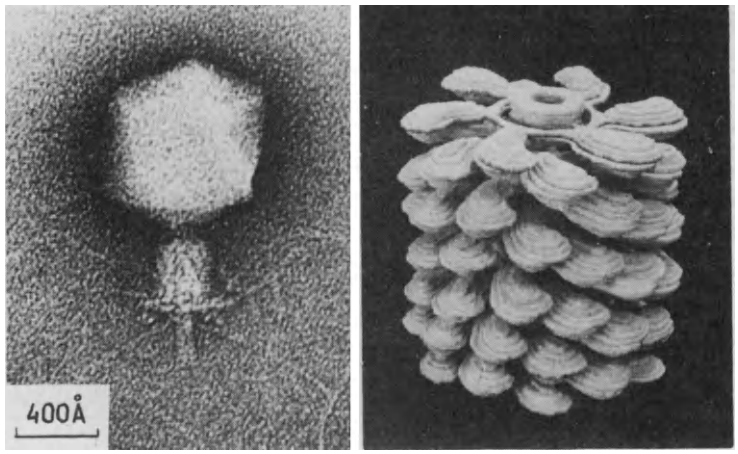
Fig.9. Bacteriophage DD6 in the intact state. a. Electron micrograph. b. Reconstruction of the structure of the tail.

grooves are observed on the outer surface of the tail that seem to form "parallelograms" on the surface of a cylinder. There is a central cylindrical channel along the axis (d = 30-35 Å) and, additionally, six helical channels run between the subunits at some distance from the axis of the tail. The near-axis maxima corresponding to the protein molecules (m.w. 20000) that form the phage core, and the peripheral ones correspond to the protein molecules (m.w. 80000) that form the sheath. For bacteriophage Phy-5*K-12 E.coli (Ref.24) the core diameter is 220-230 Å. As one can see from reconstruction (Fig.10a,b), the sheath molecules are certainly dimers. The biological function of the tail is the injection of DNA into the bacterium. Thus, during the process of contraction of the tail its length becomes shorter and diameter increases, apparently due to the rearrangement of protein subunits. Fig.11a,b shows the phage DD6 in the contracted state, and the result of reconstruction (cf. Fig.9a,b (Ref.25)). The sheath diameter increases from 210 Å to 300 Å, with the simultaneous decrease in its height from 1000 Å down to 360 Å. In the intact structure the sheath subunits are arranged with their long axis being approximately tangential to the circumference, whereas in the contracted state they are rotated in the



(a) (b)

Fig.10. Bacteriophage Phy 5*K-12 E.coli. a. Electron micrograph. b. Reconstruction of the structure of the tail.



(a) (b)

Fig.11. Bacteriophage DD6 with the tail in the contracted state. a. Electron micrograph. b. Reconstruction of the structure of the tail.

horizontal plane at an angle of 45° and are found to be close to the radial direction.

CONCLUSION

These investigations were carried out in the Institute of Crystallography in Moscow, partly in collaboration with some other institutes. They illustrate the possibilities of physical methods in the investigation of structure and function of the molecules of biopolymers.

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COMPLEMENTATION OF PROTEIN CRYSTAL STRUCTURES BY NMR STUDIES IN SOLUTION

K. Wüthrich

Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, 8093 Zürich-Hönggerberg, Switzerland

Abstract - A description of new high resolution nuclear magnetic resonance (NMR) experiments for studies of spatial protein structures in solution is presented. The potential of modern NMR techniques to provide a many-parameter characterization of static and dynamic aspects of protein conformation is illustrated with recent investigations of a group of small globular proteins related to the basic pancreatic trypsin inhibitor.

INTRODUCTION

Over the last two decades high standards of detailed description of spatial protein structures were established by X-ray crystallography (1). Limitations of the crystallographic approach are that suitable single crystals are required and that the X-ray data can only characterize an average structure and possibly the spatial distribution of the atom positions related by internal thermal motions or static disorder in the protein molecule (2). Recent developments in the investigations of structure-function relations in proteins emphasize the need to complement the X-ray data with information obtained from experimental observations in solution (2-6). Among the techniques suitable for such studies high resolution nuclear magnetic resonance (NMR) is quite unique in that it can provide a truly many-parameter description of static and dynamic aspects of protein conformations (7-9).

NMR techniques may be used to investigate proteins which have for technical reasons not been amenable to crystallographic studies as well as for complementation of X-ray structures with data on the corresponding average molecular structures in solution and on internal mobility in proteins (7-9). The present biological applications of high resolution NMR depend largely on very recent developments of instrumentation and methods. In the first part of this paper some new experiments are surveyed. The second part describes structural data for a group of globular proteins which were obtained with the use of these new techniques.

SPECTRAL RESOLUTION

^{1}H NMR spectra of the basic pancreatic trypsin inhibitor (BPTI) are shown in Fig. 1. BPTI will be used as an illustration throughout this paper. It is a small globular protein with molecular weight 6500, which contains 324 non-labile and 114 labile protons per molecule. Fig. 1 A shows the spectrum computed for the random coil form of the polypeptide chain, which corresponds closely to that observed for denatured BPTI (9), and Fig. 1 B corresponds to the high resolution ^{1}H NMR spectrum observed for native globular BPTI. The spatial folding of the polypeptide chain is responsible for the differences between the two spectra, which are thus the primary source of information for studies of the protein conformation.

At the high magnetic field of 84 kilogauss, the spectrum of Fig. 1 B contains a considerable number of resolved resonance lines. Between 0 and 2 ppm these correspond mainly to the methyl groups of aliphatic amino acid side chains, from 2 to 4 ppm to side chain methylene and methine protons, from 4 to 5.5 ppm to the α-protons of the backbone, between 5.5 and 7.5 ppm to the aromatic rings of phenylalanine and tyrosine, and between 7.5 and 11 ppm to slowly exchanging amide protons located in the interior of the globular protein (9). The potential of high resolution NMR to provide a many-parameter characterization of protein conformation depends primarily on the ability to resolve and assign a maximum number of resonance lines in the NMR spectra. While important progress was achieved with the use of very high fields, the spectral resolution is still a critical limitation for studies of biological macromolecules. In the following two techniques for further resolution enhancement are described.

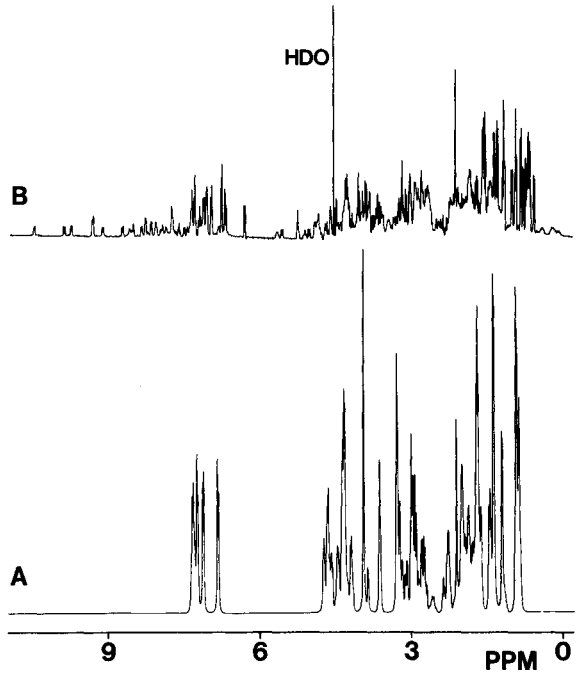

Fig. 1. 360 MHz ^{1}H NMR spectra of BPTI. A: Hypothetical "random coil spectrum" for the polypeptide chain of BPTI computed as the normalized sum of the resonances of the constituent amino acid residues (9). B: Experimental spectrum recorded shortly after dissolving the protein in $^{2}H_2O$, pD = 4.5, T = 45° C.

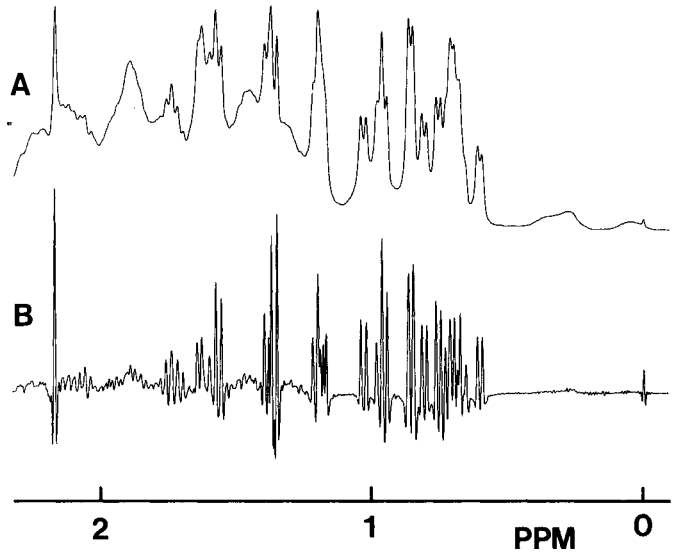

Fig. 2. High field region from 0 to 2 ppm of the 360 MHz ^{1}H NMR spectrum of BPTI, T = 45° C. A: Normal Fourier transform spectrum. B: Spectrum obtained after multiplication of the free induction decay with a phase-shifted sine bell, $\sin(\pi t/t_s + 0.04\,\pi)$, where t_s is the experimental acquisition time (12,13).

Modern NMR equipment works almost exclusively with the method of Fourier transform spectroscopy (9). The computer facilities included in the spectrometers are usually capable to perform data manipulations which may greatly improve the spectral resolution (7-13). As an illustration, Fig. 2 shows the improvement of the resolution in the high field region of the spectrum of Fig. 1 B obtained by multiplication of the free induction decay with a phase shifted sine bell (12,13). Even though quite remarkable resolution enhancement can thus be obtained, mutual overlap of resonances in crowded regions of the spectrum of a protein may still make it difficult to identify individual spin multiplets. This may also limit the use of multiple irradiation techniques for studies of connectivities between different individual resonances (7-9).

Two-dimensional (2D) NMR is a different concept for resolution enhancement in complex spectra (14,15). By the introduction of a second frequency variable the resonances are spread in a frequency plane rather than on a frequency axis, so that each peak is characterized by two frequency coordinates instead of only a single one. A particular 2D NMR technique, i.e. J-resolved 2D ^{1}H NMR (16) has been adapted for studies of biological macromolecules (17-21). The principle of J-resolved 2D NMR is illustrated by Fig. 3. The top trace shows the high field region of the conventional one-dimensional ^{1}H NMR spectrum of BPTI (Fig. 1 B). In the J-resolved 2D NMR spectrum shown in Fig. 3 the individual multiplets of the one-dimensional spectrum are simply rotated by 90° about their center frequencies, and hence the spin-spin coupling produces a spread of the spectrum in a second dimension. Thereby, all the components of a spin multiplet are arranged on a straight line which forms an angle of 90° with the chemical shift axis and intersects the J = 0 line at the chemical shift of the proton considered. As a consequence the components of each multiplet in a weakly coupled spin system (9) are readily assigned from inspection of the 2D NMR spectrum. Furthermore it is evident that multiplets overlapping in a one-dimensional spectrum will be separated in a 2D spectrum whenever they possess different chemical shifts.

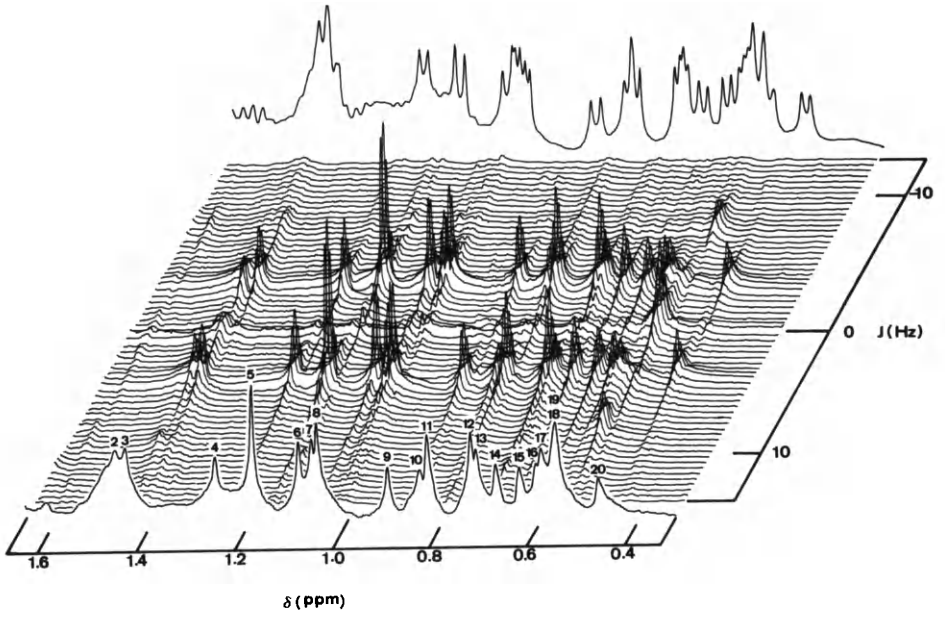

Fig. 3. High field region from 0.4 to 1.6 ppm of the 360 MHz ^{1}H NMR spectrum of BPTI, T = 60°. The top trace shows the conventional one-dimensional spectrum. The J-resolved 2D spectrum is shown in the (J,δ) presentation (19). The bottom trace shows the projection of the 2D spectrum along the direction of the J-axis onto the chemical shift axis. The numbers identify the resonances of the 19 methyl groups seen in this spectral region, which have all been individually assigned (22) (reproduced from ref. 19).

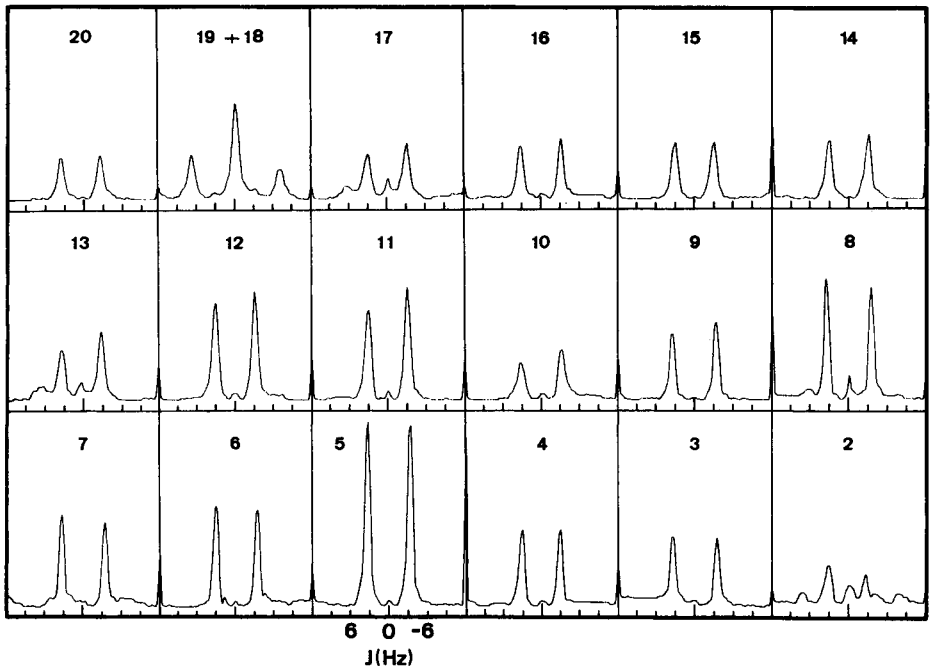

Fig. 4. Cross section representation (see text) of the multiplets in the J-resolved 2D ^{1}H NMR spectrum of BPTI in Fig. 3. The numbers identifying the individual resonances correspond to those in the projection of the same spectrum in Fig. 3 (reproduced from ref. 19).

BPTI contains a total of 20 methyl groups, of which 19 are contained in the spectral region shown in Fig. 3. 17 doublets can readily be distinguished in the 2D spectrum, whereas the two methyl triplet resonances of isoleucine (9) have exactly identical chemical shifts and are therefore overlapped also in the 2D spectrum. The information content of a J-resolved 2D NMR spectrum can also be presented in more easily accessible forms (19). A drastic simplification is obtained by projection along the J-axis onto the chemical shift axis. In the projection (Fig. 3, bottom trace) each multiplet collapses into a single line so that, effectively, a homonuclear spin decoupled spectrum is obtained (16,19). Obviously, in the projected spectrum, all information on spin-spin coupling is lost. However, since all the multiplet components lie on a straight line, a small number of cross sections perpendicular to the chemical shift axis and through the peaks in the projection will provide all the information on spin-spin coupling in weakly coupled spin systems (19). Cross sections through the 2D spectrum of Fig. 3 are shown in Fig. 4. Well resolved multiplets can be observed for all the methyl resonances. Because of the increased digital resolution along the J-axis in the 2 D spectra, as compared to one-dimensional spectra, spin-spin couplings, which are an important source of structural information in proteins, can thus be measured with an accuracy of ± 0.2 Hz.

THROUGH-SPACE CONNECTIVITIES BETWEEN NEARBY PROTONS

The potential of nuclear Overhauser effects (NOE) for studies of non-bonding nearest neighbor interactions between protons in biological macromolecules have for some time been recognized (23). However, it was also pointed out that spin diffusion in proteins (24) may cause the conventional steady-state NOE's (25) to be less specific and hence less useful. In contrast, the initial build-up rates of NOE's are simply related to the inverse sixth power of the proton-proton distance (24,26,27). It was hence of great practical interest to develop techniques for the observation of spectral features which are in a simple way related to the initial build-up rates of the NOE's. Two different experiments have recently been used with proteins, i.e. transient NOE's (27) and truncated driven NOE's (TOE) (28,29). Experience so far has indicated that TOE difference spectra (30) are the more useful technique in practice (28,31,32); therefore the following discussion concentrates on this experiment.

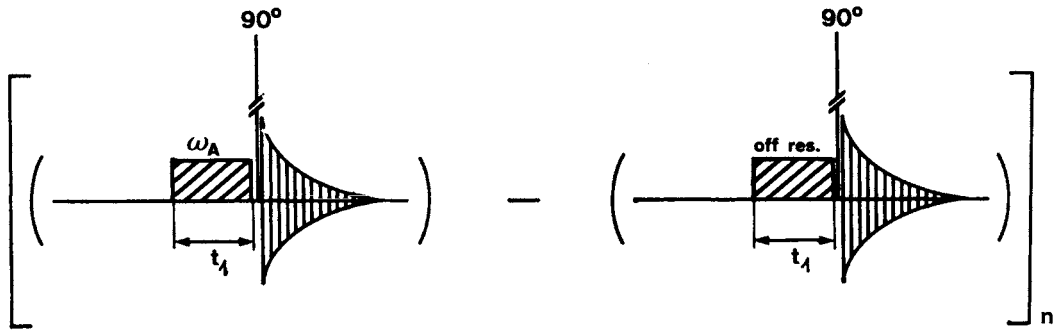

Fig. 5. Pulse sequence used to record driven NOE difference spectra (see text).

TOE difference spectra can be recorded with the pulse sequence shown in Fig. 5. A selective low power radio frequency field is applied to resonance A for a period of time t_1, which is followed immediately by the observation pulse. After a waiting time t_2, a reference spectrum without NOE is recorded. n free induction decays with and without NOE are accumulated in different parts of the memory and the difference spectrum is obtained by subtracting the free induction decay with NOE from that without NOE (30). Since in this experiment the radio frequency field ω_A is applied over the entire time span t_1 during which NOE's in the spin system are built up, we proposed the term "radio frequency driven NOE's" to distinguish this type of measurements from transient NOE's (28,29). If the irradiation period t_1 in a driven NOE experiment is sufficiently long, a conventional steady state NOE difference spectrum is obtained. The limited selectivity of steady state NOE's in macromolecules is illustrated by the experiment in Fig. 6, where the NOE difference spectrum of BPTI contains a large number

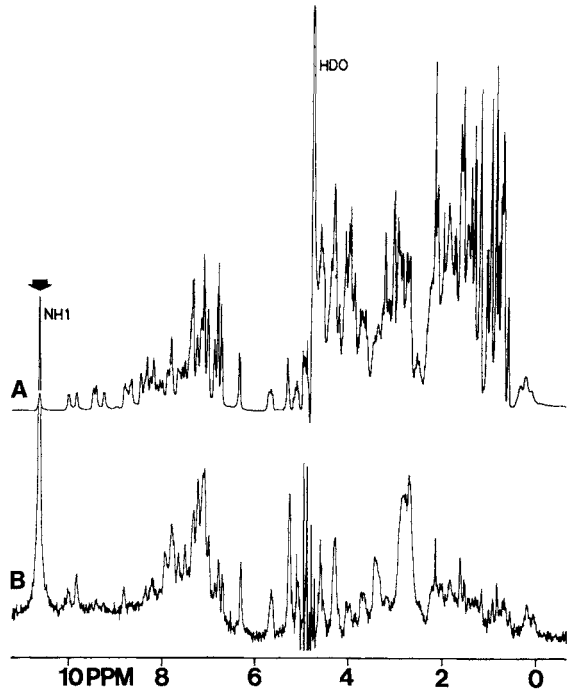

Fig. 6. 360 MHz ^1H NMR spectra recorded in a 0.02 M solution of BPTI in ^2H_2O, p^2H = 4.5, T = 15° C. A: Normal Fourier transform spectrum. B: Steady-state NOE difference spectrum obtained with presaturation of the amide proton line at 10.6 ppm (arrow).

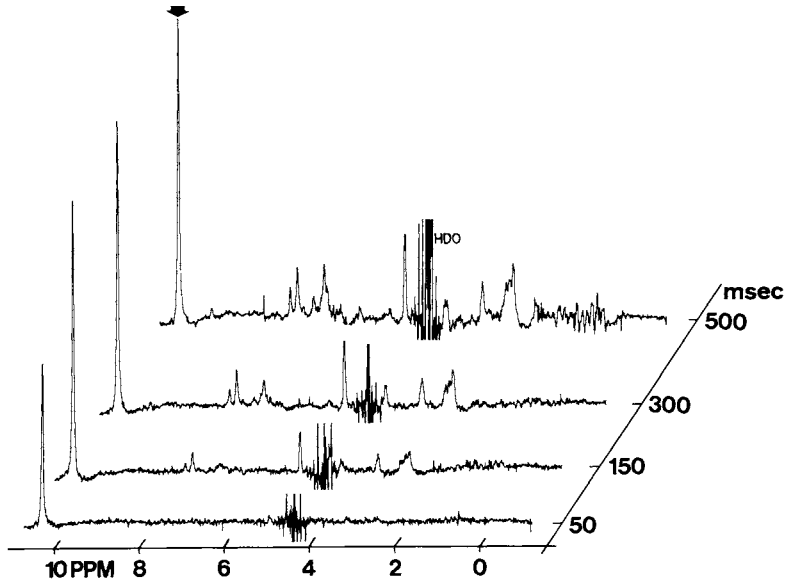

Fig. 7. TOE difference spectra of the BPTI solution in Fig. 6 obtained with irradiation of the amide proton resonance at 10.6 ppm (arrow) during different periods of time t_1 (Fig. 5), as indicated on the right.

of lines between 0 and 10 ppm. In contrast, highly selective TOE difference spectra may be obtained when the build-up of the driven NOE's is truncated after a relatively short time span t_1 (Fig. 7). By measurement of a series of TOE's with different t_1's which are all short compared to the irradiation time required for a steady state NOE, the build-up of the driven NOE's for individual resonance lines can be followed (Fig. 7). It was shown that with a suitable choice of t_1, the line intensities in a single TOE difference spectrum can be simply related to the initial build-up rates of the NOE's and hence to the distances between irradiated and observed nuclei (29).

Figs. 8 and 9 illustrate the use of TOE experiments for comparative studies of protein conformations in single crystals and in solution. In the crystal structure of BPTI the side

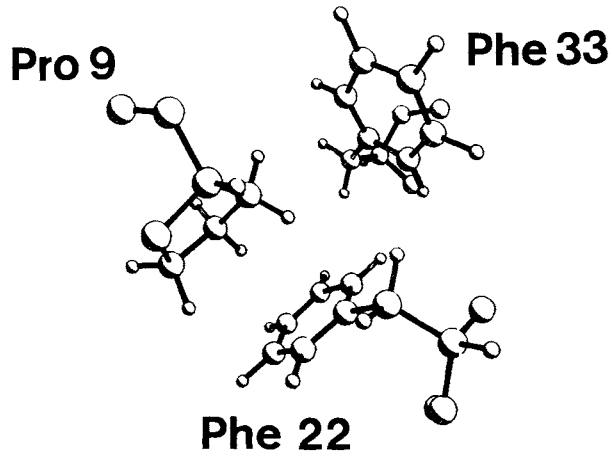

Fig. 8. Computer drawing of the local structure formed by Pro 9, Phe 22 and Phe 33 in BPTI based on the refined atomic coordinates from X-ray studies (33).

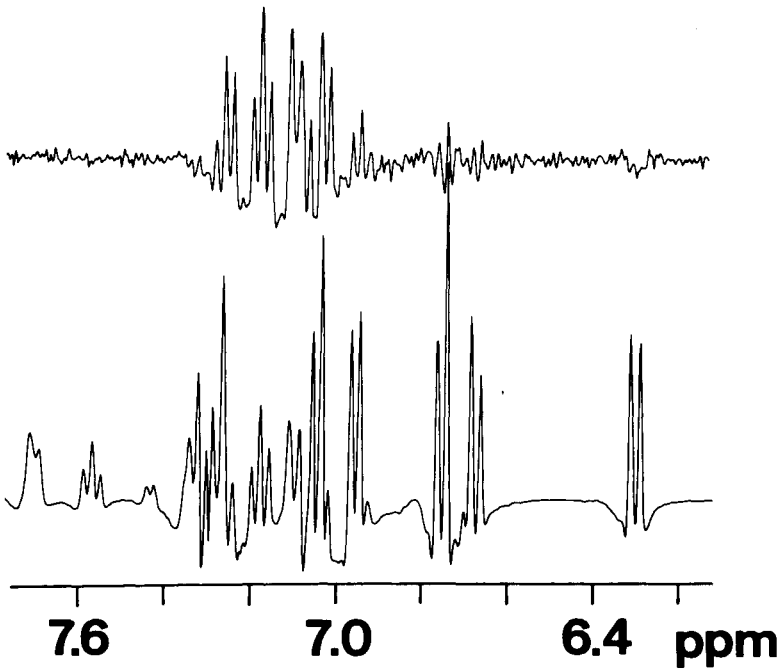

Fig. 9. 360 MHz ^{1}H NMR spectra of the aromatic resonances in BPTI, $p^{2}H$ 6.0, T = 35° C. The bottom trace shows the normal Fourier transform spectrum after multiplication of the free induction decay with a sine bell (12). The top trace shows the TOE difference spectrum obtained with irradiation of the high field lines at ca. 0.2 ppm (Fig. 1 B).

chains of Pro 9, Phe 22 and Phe 33 are located in close proximity from each other (Fig. 8). Because of the interactions with the ring current fields of the two phenylalanines (9), the resonances of three of the four β- and γ-methylene protons of Pro 9 are shifted to the extreme high field end of the spectrum (Fig. 1 B). In a TOE difference spectrum recorded with preirradiation of the high field shifted resonances of Pro 9 during 1 sec, only the spin systems of Phe 22 and Phe 33 can be observed in the aromatic region (Fig. 9), showing that the local structure seen in the BPTI crystals is preserved in the solution conformation.

INTERNAL FLEXIBILITY IN GLOBULAR PROTEINS

High resolution ^{1}H NMR can provide quantitative information on protein denaturation, on the exchange rates of interior labile protons with solvent protons and on the internal mobility of the aromatic rings of phenylalanine and tyrosine (9). To investigate correlations between these three structural features, which all manifest concerted mobility of a large number of atoms in the protein, a group of ten small globular proteins related through homology or chemical modification with the basic pancreatic trypsin inhibitor was studied. With the use of the experiments in Figs. 2-9 and other NMR techniques it could be shown that the average molecular structure seen for BPTI in single crystals is preserved in solutions of all the proteins used in this project (13,22,34-37). With the use of these proteins the influence of local variations of covalent bonds on the different conformational parameters could thus be investigated in a given type of spatial protein structure.

The thermal stability and the amide proton exchange rates in the different BPTI-related proteins were found to be correlated, i.e. the proton exchange was faster in the proteins with lower denaturation temperature. In contrast, the rotational motions of the aromatic rings are not related with the denaturation temperatures or the amide proton exchange rates, and were found to be nearly identical in all the different proteins. From these and additional observations it is suggested that globular proteins are best described by a dynamic ensemble of rapidly interconverting molecular structures. The structures contained in this ensemble can be characterized on the basis of the NMR data. Salient features of a model description for solution conformations of proteins based on these data are that at

least two different types of internal fluctuation modes must be distinguished and that the overall stability of the spatial structure seems to depend primarily on the presence of hydrophobic "stability domains". Detailed discussions of these results are presented in several recent publications (38-40).

Acknowledgements - The research projects described in this paper were financed by grants of the Swiss National Science Foundation (project 3.0040.76) and the E.T.H. Zürich. Warm thanks go to the colleagues and students cited in the references whose abilities and enthusiasm made these investigations possible.

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STUDIES OF ACID PROTEASE STRUCTURES BY MOLECULAR REPLACEMENT METHOD

M. G. Safro and N. S. Andreeva

Institute of Molecular Biology, Academy of Sciences of the USSR, Moscow, USSR

Abstract - The internal symmetry of acid protease molecule is described and the convenient representation of their three dimensional structure is proposed. The paper contains the description of the development of chymosin crystal structure studies by the molecular replacement method.

Two problems are the subject of this communication. The first one concerns the internal symmetry of porcine pepsin molecule and the description of the same symmetry in other acid proteases. The second dealts with the problem of the development of chymosin structural studies by means of molecular replacement method.

The conformation of a porcine pepsin polypeptide chain was established before (Refs. 1,2). Electron density maps of the monoclinic pepsin crystals corresponding to 3 Å - 2.7 Å resolution were gradually improved by the including additional derivative and improvement of the phasing procedure. During these studies (Ref. 2) a good correlation was found between pepsin chain conformation and the structure of acid proteases isolated from primitive organizms (Refs. 2,3,4,5). The interpretation of pepsin maps has been additionally confirmed by a good positioning of side chains in a mirror comparator especially those with the aromatic side chains.

The existence of the two domains is the first prominent feature which become obvious during the inspection of chain tracing of any acid protease (Refs. 1,3,4,5). The topological similarity of the two domains and the relation of this similarity with the evolution of acid proteases was the subject of publication by J.Tang et al.(Ref. 6).

In process of comparing in detail a pepsin chain conformation to those of acid proteases isolated from Rhizopus chinensis we have found that every domain of acid proteases consists also of two topologically similar "units". Each "unit" contains four structural elements following after each other: β-hairpin (A), wide loop (B), helix (C) and the second β-hairpin (D). There are some small irregular "spacers" between these elements some of which are of different length in different units. Neverthless the topological similarities of "units" can be well illustrated by their superposition. N-Terminal segments of both domains are not included in these "units". The arrangement of the "units" within each domain is symmetrical. It provides very interesting packing of structural elements and the formation of the extensive β-sheet, described in works of other authors (Ref. 7). This sheet and other structural properties of acid proteases including symmetrical packing of the "units" within domains are well represented on the drawing, corresponding to the view at the molecule from the entrance into the active site cleft (Fig. 1). The position of any amino acid in the three dimensional structure of acid proteases can be simply denoted by the letters corresponding to the notation of the structural element (see Table 1). For example active Asp 32 belongs to B_1 loop, active Asp 215 - to B_3 loop, Tyr 75 is in D_1 hairpin, Asp 304 - in C_4 helix, Met 290 - in B_4 loop, C-terminal hairpin is D_4. The discription of the positions of different amino acids in the three dimensional structure of the enzymes by means of notation of different loops is widely used during stidues of serine proteases.

We think that this description of acid proteases is convenient for the future studies, at the same time it is closely related to the internal symmetry of their molecular structure.

Chymosin (rennin) is the acid protease which has many features similar to those of pepsin. Their primary structures are almost identical for the most of segments of molecules. The homology is as high as 57 per cent. X-ray crystallographic studies of chymosin orthorombic crystals were star-

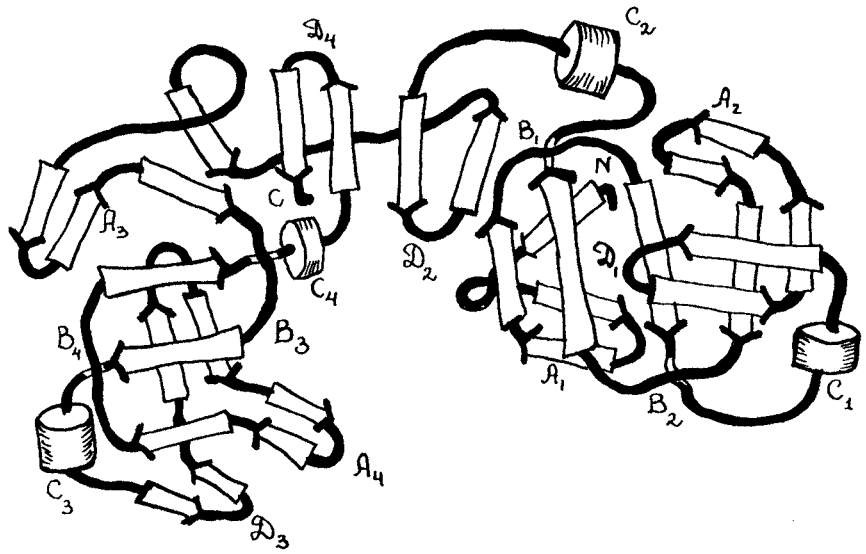

Fig. 1. The scheme of the arrangement of the main structural elements illustrating the important features of supersecondary structure of acid proteases

TABLE 1. The numbers of residues which belong to A, B, C, D elements of structural units

Domain	Structural unit	A	B	C	D
I	1	14-24	25-42	58-62	70-84
	2	88-96	104-122	138-143	152-165
II	3	194-206	211-224	225-235	237-248
	4	275-284	286-303	304-308	310-323

ted in 1965 by C.Bunn in the Royal Institution of London. It was found that the space group of crystals is I222 or $I2_12_12_1$. The unit cell has dimensions: a=79.7 A, b=113.8 A, c=73.8 A (Ref.8). The using of the isomorphous replacement method was unsuccessful because of the difficulties concerning the interpretation of the difference Patterson maps. The set of the experimental intensities of native chymosin crystals for 6 A resolution was sent to us by C.Bunn. The purpose was to use the molecular replacement method to overcame the difficulties on the base of pepsin structure. We received also T.Blandell's data for native chymosin crystals as well as for $PtCl_4$ derivative for the same purpose.

In 1976 we published the results of rotation function (RF) calculations (Ref. 9) pepsin versus chymosin on the base of M.G.Rossman-D.M.Blow conventional approach (Ref. 10). This function had one very prominent maximum which confirmed the high homology of the three dimensional structures of both proteins.

However this maximum had rather specific shape with two submaxima separated by the distance of about 25° in Eulerian space, as shown in Fig. 2a.

There are factors which may affect the result of the Rotation Function:

1. resolution limits (inner and outer) of the data;
2. radius of integration around the Patterson origin;
3. percentage of intensities used in the calculation.

In order to examine the influence of these factors on RF result, we needed a program of much more speed comparing to the conventional version (Ref.11). We used at this stage the Fast Rotation Function program by R.A.Crowther (Ref. 12). Crowther has shown that by expanding the Patterson function in terms of spherical harmonic functions, and expressing a rotated harmonic RY_l^m as a weighed sum of the (2l+1) unrotated harmonics Y_l^m, RF may be presented in the following form:

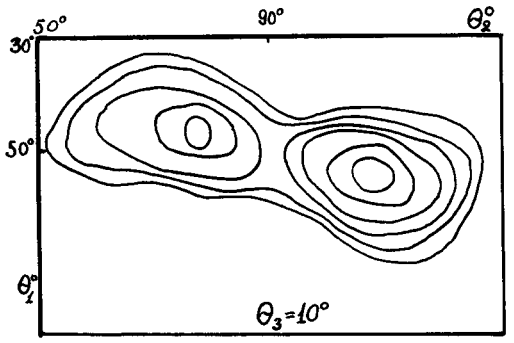

Fig. 2a. Section through $R(\theta_1\theta_2\theta_3)$ for the pepsin-chymosin comparison: the peak in R is at θ_1=53°, θ_2=106°, θ_3=10°. Radius of integration 25 Å

$$R(\Omega) = \sum_{lmm'} (\sum_n a^*_{lmn}\, b_{lm'n}) D^l_{mm'}(\Omega)$$

where $D^l_{mm'}(\Omega) = d^l_{mm'}(\beta)\, \exp i(m\gamma + m'\alpha)$ and (α, β, γ) are a triple of Eulerian angles.

Our calculation confirmed the previous results, and the corresponding matrix elements for a S matrix have the same value. Because of different initial orientations of systems and relation between triple Eulerian angles (α, β, γ) and $(\theta_1, \theta_2, \theta_3)$: $\theta_1 = \alpha + \pi/2$, $\theta_2 = \beta$, $\theta_3 = \gamma - \pi/2$ we found that in our case $(\theta_1, \theta_2, \theta_3)$ will correspond to $(-\alpha, \pi + \beta, \gamma)$. Appearing of the two submaxima observed at radius integration 25 Å, and resolution limits 6-15 Å

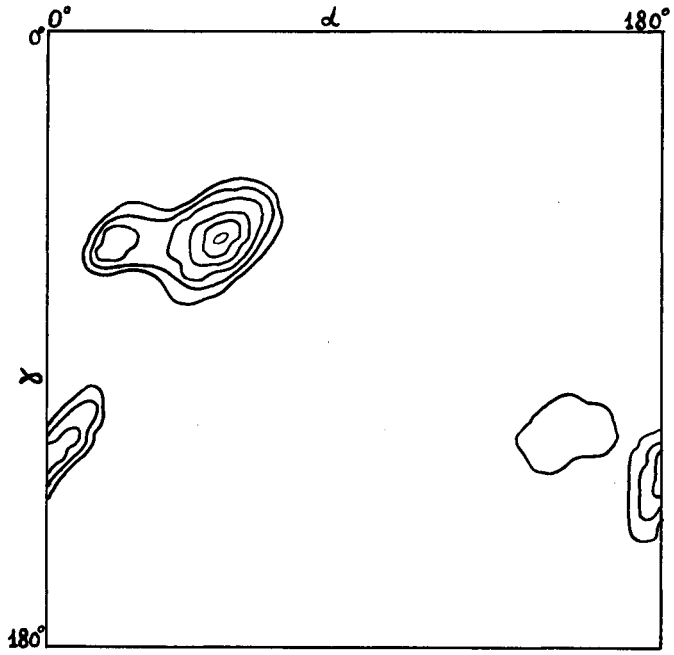

Fig. 2b. Fast Rotation function (6 Å resolution) for pepsin versus chymosin, section β =55°. Maximum is at α =45°, γ =65°. Countour levels from 120 to 170 in steps of 10.

(Fig. 2b). With decreasing of the radius integration the maximum becomes wider whithout substructure. This was found at radius 21 Å and at the same resolution (Fig. 2c).

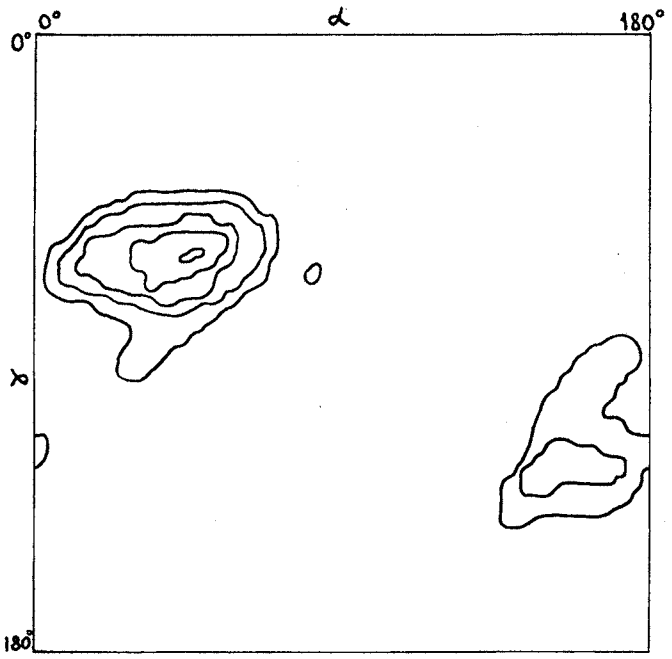

Fig. 2c. Fast Rotation function (6 Å resolution) for pepsin versus chymosin, section β_0=55°. Maximum is at α =45°, γ =65°. Radius of integration 21 Å. Coutour levels from 75 to 120 in steps of 10

The maximum with the same double peak feature for RF chymosin versus Endothia parasitica was described in 1977 by T.Blundell (Ref. 4). Becides that we calculated the RF for the system pepsin versus acid proteinase isolated from Rhizopus chinensis. In this case the strong single peak was obser ved. This fact showed that the complicated structure of the maximum is connected with the some specificity of chymosin crystals.

Two possible explanation for this feature of RF for chymosin were proposed by T.Blunndell. The first one implies slightly different angular separation of the two domains in different acid proteases. However this explanation contradicts the RF data for Rhyzopus chinensis enzyme. At the same time the angular separation of subpeaks seems to be rather large for the conservativity of the active site loops in the two domains. Another explanation concerned with the possible disorder of chymosin molecules in crystals. This possibility may prevent the development studies of this crystal form.

More recent publications showed that all acid proteinases have some kind of symmetry in their three dimensional structure (Refs. 2-7). Therefore there is another possibility to explain the specific shape of RF maximum for chymosin crystals. The chymosin molecule as well as any other acid proteinase must have the pseudo two-fold axis of the symmetry. If this axis forms the small angle with two-fold crystallographic axis of the unit cell, the asymmetric unit of the Eulerian space will have two maxima. One of them concerns the pseudosymmetry of the molecule and is visible because of the localization at the slope of the main peak. Using the data on C_α carbon coordinates of pepsin we have calculated the orientation of the two-fold axis of the pseudo symmetry of acid proteinase molecule in the unit cell of chymosin crystals. It was found that this axis forms rather small angle with the Z axis unit cell. Therefore RF must have double peak shape.

The next step of the molecular replacement method is the calculations of the translation function. It can reveal the position of a correctly oriented test molecule, relative to a chosen symmetry element. Following to T_1-function of R.A.Crowther-D.M.Blow (Ref. 13) the program in which the intramolecular vectors of the experimental Patterson function are approximately removed to improve the signal-to-noise ratio was written. The electron density of isolated pepsin molecule, correctly oriented and spanned on the grids of chymosin unit cell was used for the calculation conventional

$F_M(hA_o)$ structure factors. The delineating of the molecular boundary was done by means of polygons drawn on the map sections and using the data about C-α-coordinates of pepsin molecule (Ref. 2). In our case the translation function may provide a possibility to select alternative space group I222 or $I2_12_12_1$. This follows from the fact that the self-consistent solution in three Harker sections (for one atom in the asymmetric units) is different for the three intersecting and non-intersecting two-fold axes.

The translation function is very sensitive to the outer and inner resolution limits. Therefore during the interpretation of this function each possible solution has to be tested by the correctness of molecular packing. The large number of symmetry elements in the space groups I222 and $I2_12_12_1$ and the high asymmetry of a big acid protease molecule restricts rigorously every solution of translation function. On the sections XYO, OYZ and XOZ were found (Fig.3) three related peaks, corresponding to the space group I222. Only one of these maxima belongs to the strongest in the correspon-

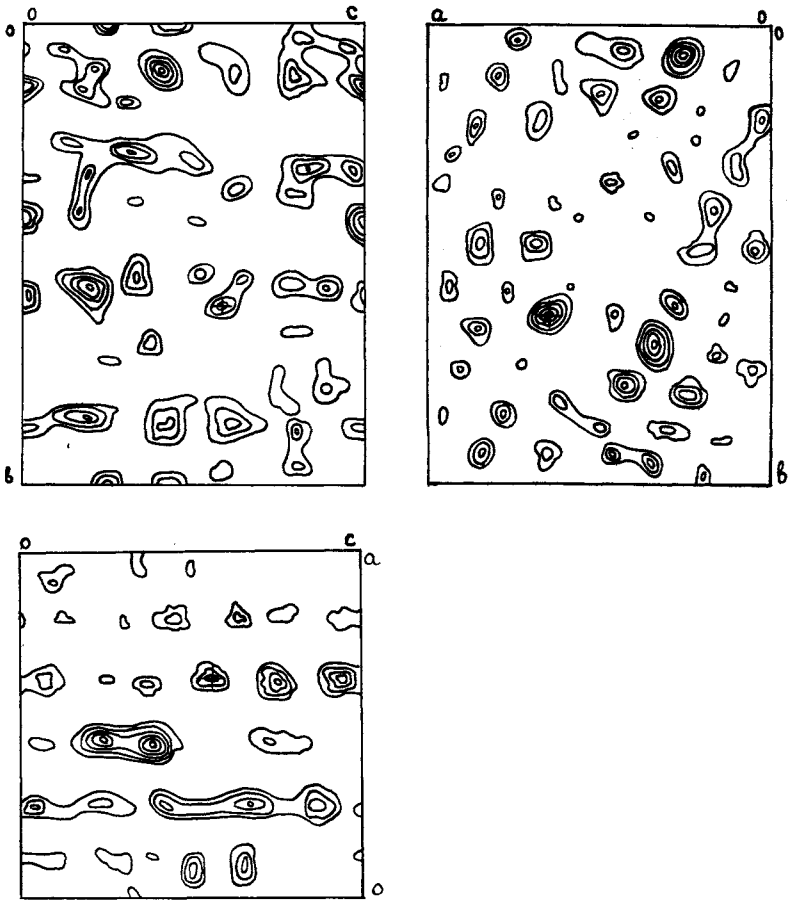

Fig. 3. Translation function results at XYO, XOZ, OYZ sections for pepsin molecule in chymosin unit cell. The data between 6.5 Å and 12.5 Å were used. The solution is marked by cross in each section

ding section, but the solution is in a good agreement with packing requirements. The scheme of arrangement of acid protease molecules in the unit cell of chymosin is presented on Fig. 4, as one electron density section.

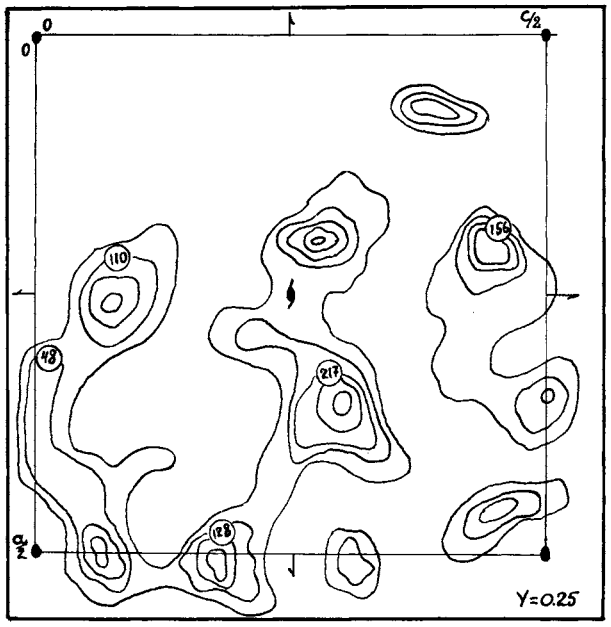

Fig. 4. Diagram to show of the molecular arrangement in space group I222 at some section Y =0.25. The positions of α-carbons of pepsin are shown

The longest distance (55 Å) in pepsin molecule is oriented approximately along the b axis of chymosin unit cell. These data gives the possibility of development of the chymosin crystal studies.

Acknowledgement - We are very grateful to Charles Bunn who initiated these studies; David Blow and Michael Rossman for the programs and very helpful discussion; Tom Blundell for the sending of his experimental data and R.Crowther for the Fast Rotation Function program.

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DRUG METABOLISM AND THE FUNCTION OF CYTOCHROME P-450

W. Scheler

Research Center of Molecular Biology and Medicine of the Academy of Sciences of GDR, 1115 Berlin-Buch, German Democratic Republic

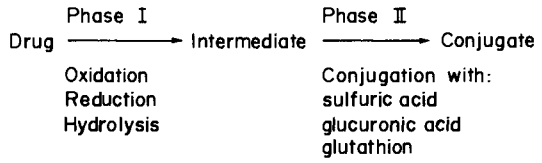
Abstract - Cytochrome P-450 (cyt P-450) is a constituent of monooxygenase systems in various procaryotic and eucaryotic cells. In liver cell microsomes this enzyme system also includes a NADPH dependent reductase of the flavoprotein type and membrane phospholipids. In the presence of NADPH and O_2 the oxidative conversion of several endogenous and exogenous chemical compounds is catalyzed by this system.

Analogous chemical conversions could be obtained by us in cyt P-450 preparations (in the absence of reductase and NADPH) by controlled cathodic reduction of oxygen by a mercury pool electrode. The long-term stability of cyt P-450 was increased by various immobilization procedures. In further experiments we could show that methemoglobin and even an iron protophorphyrin IX carrying imidazole-substituted poly-(hydroxy-ethylmethacrylate) can be substituted for cyt P-450 in the case of aniline hydroxylation or benzphetamine demethylation. The turnover numbers are in the same order of magnitude as for the intact liver microsomal monooxygenase system.

In the first part of my talk I want to deal with some results and problems in the field of the hepatic microsomal cytochrome P-450 system and than I want to show how we proceeded from these studies to some biotechnological questions. Because I am not sure how far all of you are familiar with the topic, I briefly want to outline some of our general knowledge in this field and than discuss some selected results of our cytochrome P-450 group in the Central Institute of Molecular Biology at Berlin-Buch.

Most of the lipid-soluble xenobiotics are eliminated from the organism only, after being converted to hydrophilic metabolites.

In many cases two steps of bioconversion can be observed. In a first step the drugs are enzymatically converted into more water-soluble intermediates and in a second step these polar derivatives are coupled to glucuronic acid, sulfuric acid, amino acids, and peptides such as glutathione. In this form they are easily excreted by the organism.



In the step I reactions the hepatic microsomal monooxygenase system plays an important role. The reactions are NADPH- and oxygen-dependent as it is shown in the stoichiometric equation.

$$R{\cdot}H + NADPH + H^+ + O_2 \rightsquigarrow R{\cdot}OH + NADP^+ + H_2O$$

During the last 10 to 15 years this membrane-bound enzyme system has attracted the interest of scientists of various fields, especially of pharmacologists and biochemists but also from biotechnologists and microbiologists because analogous systems are also found in microorganisms (1-3).

The hepatic system consists of only two proteins and the membrane phospholipids as essential constituents:

Cytochrome P-450	:	NADPH-Cytochrome P-450-Reductase	:	Phospholipids
35-40		1		~6000

Cytochrome P-450 acts as terminal oxidase. It is a b-type cytochrome and its CO-complex has a specific absorption band at 450 nm. In microsomes of rabbit liver cells 7 different fractions of P-450 are present, the main fractions being LM_2 and LM4 (4). Most of the investigations in the recent years have been performed with the phenobarbital-inducible LM_2 fraction.

The molecule contains a single heme group whose central iron atom may be linked to a sulfur group of the protein. The molecular weight is near 49000 (5). The amino acid sequence is under investigation.

The reductase is a flavoprotein containing one molecule each of FAD and FMN. Its molecular weight is about 79000. The reductase acts as an electron transmitter from NADPH to cytochrome P-450. There are two separate electron reductions steps performed by the reductase. The electrons are used for the reduction of the molecular oxygen (6).

The phospholipids provide the hydrophobic environment for both membrane enzymes. Therefore no monooxygenase activity is obtained in reconstitution experiments with cytochrome P-450 and reductase when the phospholipids are omitted (7).

The molar ratios of the constituents show that in the microsomal membrane only a small fraction of the P-450 molecules is in contact to the reductase, the major fraction moves freely. We can therefore speculate that during the enzymatic process a rapid exchange of substrate-bound freely moving P-450 molecules and reductase-connected P-450 molecules should take place (8).

Within the monooxygenase complex the binding of the substrates takes place at definite binding sites of cytochrome P-450. There are two types of substrates:

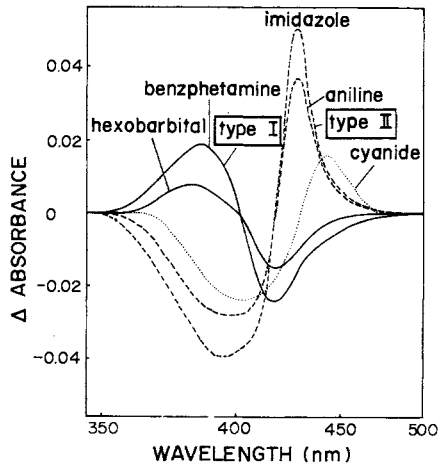
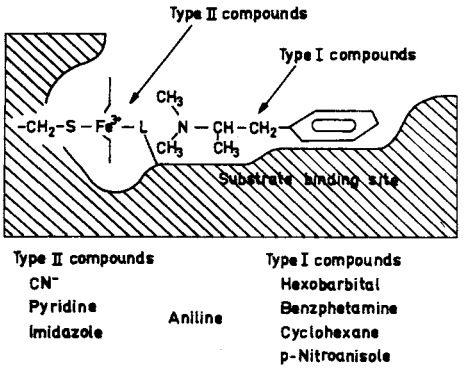


Fig. 1. Difference spectra of solubilized cytochrome P-450 ([cytochrome P-450] = 4,5 μM in 0.1 M phosphate buffer, pH 7.4, with 20% (v/v) glycerol) from phenobarbital pretreated rat liver microsomes in dependence on different substrates. [substrate] = 0.5 mM. (From (9)).

Type I substrates shift the Soret-maximum of ferric cytochrome P-450 from 416 nm to shorter wavelengths and type II compounds induce a red shift, respectively (9). Many years ago we could show together with F. Jung and P. Schoffa that spectral changes in several hemoprotein complexes are closely correlated with changes of the spin state of the hemin iron (10).

The same is true for cytochrome P-450. Dr. Rein in our department determined the change of the spin state in cytochrome P-450 substrate complexes. Type I substrates favour the high spin, type II compounds the low spin state. The shifts of the spin equilibrium amount to about 10-40 per cent (cyclohexane about 40 per cent, benzphetamine about 26 per cent, hexobarbital about 15 per cent) (11).

Evidently, type II compounds come into direct contact to the hemin iron, whereas type I substrates interact with a binding site in the neighbourhood of the heme group but without direct contact to hemin iron.



I must add that type II compounds generally act as inhibitors of the monooxygenatic reactions. On the one hand they inhibit the reduction of the hemin iron and on the other hand they interfer with the binding of oxygen to the reduced state (12). An important exception are the steroids. They also belong to the group of type II compounds but they are hydroxylated.

The binding site of type I substrates has strong apolar properties. This was shown by investigations in which we studied the thermodynamics of the binding equilibria both of type I and type II compounds.

TABLE 1. Changes of the binding enthalpy, the Gibbs energy and the binding entropy caused by the binding of type I and type II substances to solubilized cytochrome P-450 from rat liver pretreated with phenobarbital1/. (From (9))

	type	$\Delta H'$ (kcal·mol^{-1})		$\Delta G'$ (kcal·mol^{-1})		$\Delta S'$ (cal·mol^{-1}·K^{-1})	
		K_1'	K_2'	K_1'	K_2'	K_1'	K_2'
cyanide	II	-2.2	-7.0	-4.2	-3.6	-6.3	-12.0
aniline	II	-3.1	-2.5	-4.6	-4.2	-4.7	-5.4
imidazole	II	-1.6	-2.3	-5.8	-5.1	14.0	-9.4
benzphetamine	I	5.7	9.5	-6.1	-5.7	39.5	51.0
hexobarbital	I	7.3	7.3	-4.7	-4.2	40.2	38.6

1/ For the titration a cytochrome P-450 concentration of 3.0 µM was used (0.1 M phosphate buffer, pH 7.4 with 20% (v/v)glycerol). For the calculation of the thermodynamic values the association constants were used. $\Delta G'$ and $\Delta S'$ are given for 25°C.

The binding of type I substrates is entropy-controlled. The binding affinity increases both with increasing temperature and hydrophobicity of the molecules. Their basicity and stereochemical properties are of minor relevance. The binding of type II compounds, however, is predominantly enthalpy-driven, their interaction with the enzyme is weakened with rising temperature (9).

The binding sites of cytochrome P-450 are furthermore easily accessible also for relatively large substrates. This can be shown both by equilibrium and kinetic investigations with ligands of different size but also by measurements of the paramagnetic relaxation rates of solvent protons induced by the ferric iron of cytochrome P-450. These experiments were done in cooperation with the group of Dr. Maričić from Zagreb (13).

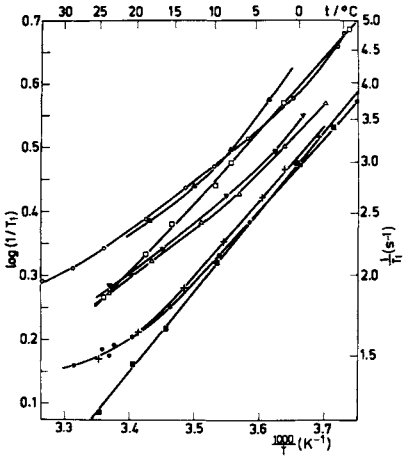


Fig. 2. Logarithms of the longitudinal proton magnetic relaxation rates in dependence on temperature. ○——○, original sample (472 µM in haem) in 20% glycerol, H_2O; △——△, original sample, diluted (429 µM in haem); +——+, reduced by dithionite (472 µM in haem); □——□; original dialyzed against 20% $C_3H_5(O^2H)_3$ in ^2H_2O (472 µM in haem); ▲——▲, original + aniline (429 µM in haem; 10 mM aniline); ▼——▼, original + methphenethamin HCl (Spasman®) (472 µM in haem, 5.1 mM Spasman); ●——●, original reduced + CO (472 µM in haem) in 20% glycerol, H_2O; ■——■, original reduced + CO dialyzed against 20% $C_3H_5(O^2H)_3$ in ^2H_2O (472 µM in haem).

Fig. 2 shows the temperature dependence of the relaxation rates of the solvent protons between 0°C and 37°C. Several preparations of microsomes have been used and compared both in the presence and absence of ligands. These experiments prove a fast exchange mechanism of the solvent molecules and a good accessibility of the heme group in cytochrome P-450.

Cytochrome P-450 dependent monooxygenatic systems are also localized in mitochondria of various organs. They play an important role in the steroid metabolism especially in such organs as the adrenal cortex or the gonads (14, 15). We have no own experience with these enzymes, therefore I do not want to discuss this topic.

Furthermore cytochrome P-450 systems are present also in plants and microorganisms. Microbial systems are of special interest with respect to biotechnical utilization. Cytochrome P-450 activity was found both in eukaryotic and prokaryotic microorganisms.

Cytochrome P-450 activity in microorganisms is predominantly observed in connection with biosynthetic potencies such as alkaloid biosynthesis or hydrocarbon hydroxylation.

In our institute the group of Dr. Müller could show that the content of cytochrome P-450 in Candida guilliermondii, a strain used for microbial protein biosynthesis, is strongly inducible in dependence on the nature of the substrates.

TABLE 2. Cytochrome P-450 containing enzyme systems in eukaryotic microorganisms. (From (16-26))

MICROORGANISM	CATALYZED REACTION	AUTHORS
Candida tropicalis	hydroxylation of n-alkanes, fatty acids and drugs	Coon et al. 1971 Azoulay et al. 1971
Candida guilliermondii	hydroxylation of n-alkanes	Müller et al. 1977 Weide et al. 1976
Endomycopsis lipolytica	hydroxylation of n-alkanes	Delaisse and Nyns 1974
Torulopsis spec.	hydroxylation of fatty acids	Tulloch et al. 1970
Rhizopus nigricans	hydroxylation of progesterone	Hudnik-Plevnik et al. 1977
Cunninghamella bainieri	hydroxylation of aryl hydrocarbons	Ferris et al. 1976
Claviceps purpurea	involvement in alkaloid biosynthesis	Ambike et al. 1970
Saccharomyces cerevisiae	unknown	Lindenmayer and Smith 1964 Yoshida et al. 1977

TABLE 3. Cytochrome P-450 containing enzyme systems in prokaryotic microorganisms. (From (27-33))

MICROORGANISM	CATALYZED REACTION	AUTHORS
Pseudomonas putida	hydroxylation of camphor	Gunsalus et al. 1968
Bacillus megaterium	15β-hydroxylation of 3-oxo-Δ^4-steroids	Gustafsson et al. 1975
Bacillus megaterium	hydroxylation of fatty acids	Fulco et al. 1975
Nocardia spec.	demethylation of phenolic ethers	Broadbent and Cartwright 1971
Rhizobium japonicum	involvement in a oxidative phosphorylation pathway	Appleby et al. 1976
Corynebacterium spec.	hydroxylation of n-alkanes	Cardini and Jurtschuk 1968
Acinetobacter spec.	involvement in alkane degradation	Müller et al. 1978

Growing on glucose, an oxygen-rich carbon source, the cytochrome P-450 content was nearly zero. Also in the presence of glycerol as carbon substrate the cytochrome P-450 content amounts only to 10 per cent of the values found with n-alkanes (C_{12} - C_{19}) as carbon source (33). Now we have started detailed investigations of this microbial P-450 system with respect to its specific molecular and functional properties.

The relatively wide range of chemical substances that can be oxidatively metabolized by the monooxygenase system raises the question as to how this enzyme system may be utilized in technological hydroxylation processes (35, 36). Isolated monooxygenase preparations or modified systems are of practical use only if they are superior to microbial systems and if they have properties which are essential for technical purposes. One of the critical properties is their instability under non-physiological conditions. For the stabilization several immobilization techniques have been used in the case of cytochrome P-450 preparations.

In most of these cases the enzymes were active after immobilization but the activity decreases within a few hours or days.

TABLE 4. Immobilization of cytochrome P-450 and properties of the immobilized enzyme system. (From (36))

Species/source	Matrix	Substrate/product
Rat/LM[1]	BrCN-activated	benzphetamine
Bovine/ adrenal glands	Sepharose 4B	deoxycorticosterone/ corticosterone
Rat/LM; rabbit/LM	poly-(hydroxyethyl-methacrylates), containing imidazole groups	benzphetamine aniline/p-amino-phenol
Rabbit/LM	hollow fibre system	hexobarbital
Purified Cyt. P-450 + reductase	Sepharose	toxins, drugs
Pig/LM	zirconia-clad glass beads	amines/aminooxides

1/ LM = liver microsomes

Another problem with respect to a continuous substrate conversion by cytochrome P-450 systems is the permanent regeneration of NADPH. It can be carried out by different methods:

- Chemically by reducing agents (f.e. $NaBH_4$; $Na_2S_2O_4$)
- Electrochemically by cathodic reduction
- Enzymatically by hexokinase, glucose-6-phosphate dehydrogenase and ATP

The investigations of Dr. Scheller in our department showed that only the enzymatic way does not disturb the cytochrome P-450 system. In the case of the chemical regeneration of NADPH the enzymes become inactivated by side products of the reaction.

During the electrochemical regeneration a gradual loss of coenzymatic activity was observed which is due to intermediately occurring radicals of the coenzyme which are dimerizing irreversibly under formation of inactive products (37 - 39).

To overcome these difficulties we tried a quite different way. Several years ago the group of Ernster, Gustafsson and other Swedish colleagues in the Karolinska Institutet in Stockholm was able to hydroxylate steroids in the presence of cytochrome P-450 by addition of cumene hydroperoxide. In the following years similar investigations were carried out in other laboratories with different kinds of substrates and with other oxidizing agents such as periodate (40) and iodosobenzene (41) but also with hydrogen peroxide itself (42). All these reactions take place in absence of NADPH and reductase and under exclusion of oxygen. From these investigations it was concluded the cytochrome P-450 forms an hydroperoxide complex as an intermediate which in turn hydroxylates the substrate molecule probably via an oxene complex.

From the technical point of view the use of such oxidants as iodosobenzene, hydroperoxides and hydrogen peroxide is not suitable, not at least because a more or less rapid inactivation of the enzymes occurs, especially an oxidative degradation of the porphyrin ring.

To avoid critical concentrations of hydrogen peroxide, its controlled electrochemical generation by cathodic reduction of oxygen by a mercury pool electrode was applied in Dr. Schellers laboratory (42). By this method the oxidative demethylation of benzphetamine, aminopyrine and p-nitroanisole was possible without any decrease of the enzyme activity during the experimental period. In recent experiments we could also achieve hydroxylations of testosterone, progesterone and aniline by this method.

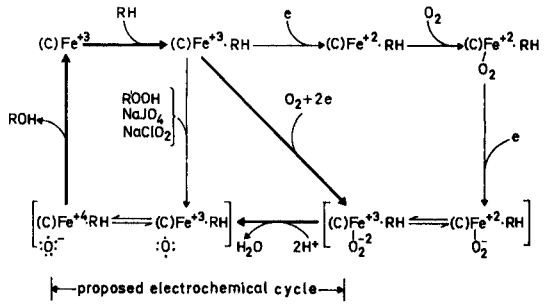


Fig. 3. Mechanism of oxygen activation in the hydroxylation reaction of cytochrome P-450_{LM}.

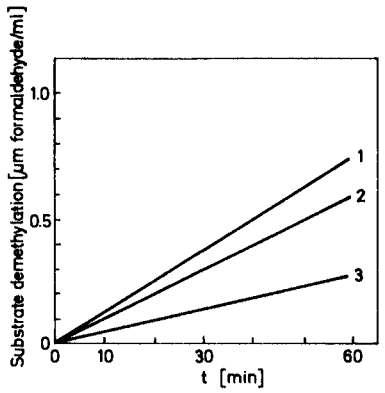


Fig. 4. Demethylation of substrates, phosphate buffer, pH 6.5, 15°C, 5 µM cytochrome P-450.
1: 1.8 mM benzphetamine I = 1 mA,
2: 2 mM p-nitroanisole I = 4 mA,
3: 2 mM aminopyrine I = 1 mA.

Catalase inhibits all these conversions, indicating that hydrogen peroxide occurs as an intermediate.

With regard to practical applications the cytochrome P-450 catalysis using cathodically reduced oxygen is undoubtedly a step forward because (1) eletron donating cofactors and their continuous regeneration are not necessary, (2) electron transferring proteins are not required and (3) the electrochemical reduction of oxygen is relatively inexpensive in comparison with enzymatically generated hydrogen peroxide.

The last part of my talk I want to begin with an observation made two years ago by J. Mieyal, R. S. Ackerman, J. L. Blumer and L. S. Freeman (44). In reconstitution experiments with the components of rat liver microsomes they could show that native hemoglobin can be substituted for cytochrome P-450. NADPH and the reductase were essential in these preparations whereas the phospholipids were not.

Now we tried in analogous experiments with methemoglobin to substitute the electrochemical reduction of oxygen for the enzymatic reduction by NADPH and reductase. These experiments were successful (45).

In the presence of methemoglobin the hydroxylation of aniline to p-aminophenol is easily possible by cathodic reduction of oxygen. We were surprised when we observed that the hydroxylation activity of methemoglobin is even enhanced after heat denaturation.

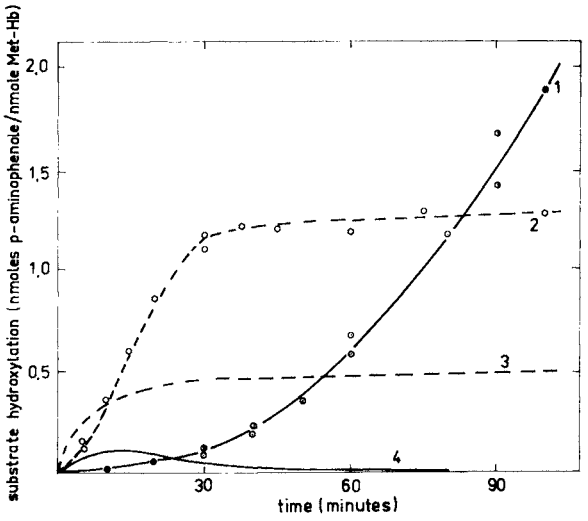
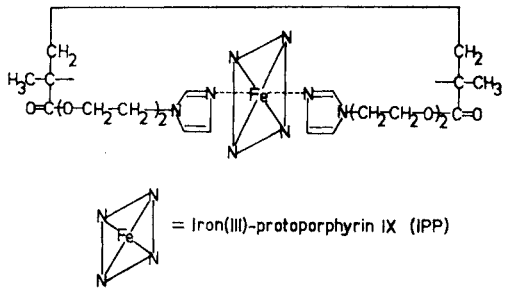


Fig. 5. Hydroxylation of aniline by human methemoglobin. ([MetHb] = 1 μM in 0.1 M phosphate buffer, pH 6.5; [aniline] = 200 μM; [NaN_3] = 1 mM; t = 20°C. MetHb was denatured by heating to 90-95°C). 1 and 2 native and denatured MetHb with cathodically reduced O_2; 3 and 4 denatured and native MetHb with H_2O_2 added.

It is known that denaturated methemoglobin has a parahematin structure of the hemin group at which the fifth and the sixth coordination positions of the iron atom are occupied by nitrogens from the neighbouring histidine imidazoles. Considering these facts, we used in further experiments imidazole substituted poly-(hydroxy-ethylmethacrylate)-derivatives as matrix for iron-protoporphyrin (46).



The iron-porphyrin interacts with the imidazole groups of the polymer as could be evidenced by EPR-measurements (47). Additionally there are unspecific interactions between polymer and porphyrin.

TABLE 5. Catalytic activities of rabbit liver microsomal cytochrome P-450 and of the synthetic polymer diimidazole iron-protoporphyrin complex ([H_2O_2] = 800 μM; pH 7.0)

Catalytic active system	Turnover number (nmol product/nmol Fe/min)	
	Aniline hydroxylation	Benzphetamine demethylation
Polymeric diimidazole-iron-protoporphyrin	0.29	0.5
Microsomal cytochrome P-450	0.10	1.0

The turnover numbers of such complexes prove a considerable catalytic activity in the aniline hydroxylation when the electrochemical reduction of oxygen was applied. The catalytic activity was in the same order as that of microsomes under identical conditions.

With this experiment I want to end my talk in which I tried to show how we connected the analysis of natural biological systems and principles with their use in biotechnology. Starting from the investigations of the natural system of the hepatic microsomal monooxygenase, we have now an artificial system that imitates the properties of the natural one and which has more favourable technological properties.

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CHANGES IN RAT LIVER GLYCOLYTIC REACTIONS DURING FLUOROACETATE POISONING

V. Bobyleva-Guarriero and P. Buffa

Istituto di Chimica Biologica, Istituto di Patologia Generale, Università di Modena, Italy

Abstract - Fluoroacetate poisoning induces in rat liver rapid glycogenolysis and concentration changes of the allosteric effectors of phosphofructokinase, citrate, ATP, ADP, AMP and inorganic orthophosphate. The break-down of liver glycogen induced by fluoroacetate was studied in rats following bilateral adrenal enucleation. The results suggested that apparently it is under the influence of the adrenal cortex. Adrenal medulla did not seem to play a part in the phenomenon. As judged by the concentrations of glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate in the liver tissue, the overall changes in concentrations of the phosphofructokinase effectors caused a stimulation of the activity of this enzyme.

INTRODUCTION

Monofluoroacetate, the main toxic principle of the South African plant Dichapetalum cymosum, shows peculiar biological properties. It has proved lethal for all the animal species so far tested, although the toxicity of the compound varies greatly from one species to another. The pathological effects of fluoroacetate vary in different animals: in some it affects mainly the central nervous system, in others it causes heart failure (1).
In the animal organism fluoroacetate is transformed into fluorocitrate (2) and this compound inhibits the mitochondrial enzyme aconitate hydratase thus producing a slowing down of the tricarboxylic acid cycle activity. This biochemical lesion is followed by large increase of citrate concentrations in organs and tissues (3,4), and lowering of the energy production shown by a decrease of ATP concentration (5-8).
So far, the lethal effect of fluoroacetate in animals has resisted all therapeutic attempts (9) and the mitochondrial biochemical lesion seems to be irreversible (10). Acute fluoroacetate poisoning causes mitochondrial swelling (8-11), but only irrilevant histological changes (unpublished observation). It is lethal for adult and embryonic organisms; however, it is not lethal for cells cultured in vitro (12-14).
It is likely that the inhibition of the tricarboxylic acid cycle and the consequent metabolic changes provoke alterations of some specific functions vital for the whole organism; however, the biochemical events leading to the death of the organism have not yet been clarified. Some metabolic alterations have been recorded in fluoroacetate poisoned animals: rapid decrease of liver glycogen and increase of glucose, lactate and ketone bodies concentrations in the blood. Further, it has been shown that the accumulation of citrate in the heart of the rat poisoned with fluoroacetate is paralleled by an inhibition of the glycolytic enzyme phosphofructokinase. This effect has been interpreted as due to citrate, a negative effector of that allosteric enzyme (7,15). Glycogenolysis does not take place in liver slices poisoned with fluoroacetate in vitro (16)and this fact indicates that the glycogen breakdown induced by fluoroacetate in vivo depends on extra hepatic factors, either hormonal, or nervous or both.

Parallel to glycogen degradation, in the liver of the rat poisoned with fluoroacetate significant changes take place in citrate, ATP, ADP, AMP and Pi concentrations (8, 16). These compounds are known allosteric effectors of phosphofructokinase *in vitro*; hence the questions arises whether these effectors influence the activity of the enzyme *in vivo* in the liver of the poisoned rat. Studies are in progress on these problems and in the present report the results are presented of some experiments carried out to determine a) if the adrenals play a part in the very rapid glycogenolysis induced in rat liver by acute fluoroacetate poisoning; and b) if the changes in concentrations of phosphofructokinase effectors are accompanied by changes in the activity of the enzyme.

METHODS

Young adult Wistar albino rats of both sexes were used. They were fed on a standard balanced diet and water *ad libitum*.
Bilateral enucleation of the adrenals was performed according to Hartman et al. (17), in a single stage under light ether anaesthesia. The operated animals were given 1% sodium chloride solution in place of water.
The rats were intraperitoneally injected with a freshly prepared solution containing 10 mg of sodium fluoroacetate/ml. The animals were killed by decapitation and the livers were rapidly excised and frozen between two blocks of dry ice. A perchloric acid extract of the tissue was made (18) and used as such for Pi determination (19,20). Neutralized perchloric acid extracts were enzymically assayed for glucose (21); G-6-P, F-6-P and F-1,6-DP (22) lactate (23); citrate (24); ATP (18); ADP and AMP (25). The glycogen was determined on separate samples of frozen tissue (26). Enzymes for analysis were obtained from Boehringer Biochemia S.R.L., Milan, Italy. A DK-24 Beckman spectrophotometer was used for measuring extinction changes. Pure fluoride-free sodium fluoroacetate was kindly given by Sir Rudolph Peters, F.R.S.

RESULTS

To study the influence of the adrenals on the degradation of liver glycogen in fluoroacetate poisoning rats were used following bilateral enucleation of the glands. This operation seems to be a near equivalent of adrenalectomy; in fact the whole medulla and the greater part of the cortex are removed, with the exception of a rim of cells of the zona glomerulosa adhering to the capsule. Enucleation is followed by an interval of marked adrenal insufficiency which lasts about one week, then the zona glomerulosa cells proliferate and give rise to a new cortex, whereas the medulla does not regenerate. Hence by using animals at intervals after adrenals enucleation it is possible to discriminate between the influence of the cortex and the medulla.
The effects of fluoroacetate poisoning on the concentrations of glycogen and citrate were studied in rats 8, 16 and 24 days after the operation.
The results, summarized in Fig. 1, show that 8 days after enucleation fluoroacetate caused the lysis of approximately 14% of the liver glycogen and did not provoke citrate accumulation. After 16 days the glycogen lysed was 41% and the accumulated citrate 3.12 µmol / g liver (910% over control); after 24 days 87% of the glycogen was lysed and the accumulated citrate was 3.69 umol / g liver (700% over control).
Phosphofructokinase activity was judged by the relative concentrations of G-6-P, F-6-P and F-1,6-DP. The results given in Table 1 show that with respect to control the concentrations of G-6-P and F-6-P were 50% and 43% reduced respectively, whereas the concentration of F-1,6-DP was 108% increased. This decrease of G-6-P and F-6-P and the concomitant increase of F-1,6-DP were taken as indicating a stimulation of PFK activity.
The fall of glycogen concentration together with the apparent increased activity of PFK and the marked increment of lactate concentration were consistent

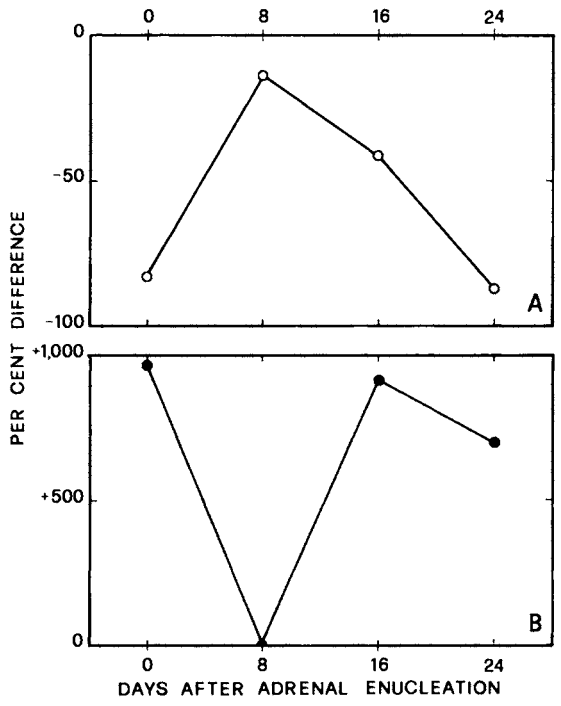

Fig. 1. Effects of fluoroacetate poisoning on glycogen (A) and citrate (B) concentrations in the liver of the rat following bilateral enucleation of the adrenals. Treatment: 10 mg sodium fluoroacetate / kg body weight / 2 h.

with an increase of glycolysis accompanied by high accumulations of citrate (Table 2). Glucose concentration resulted increased in male rats and decreased in females.

FAC poisoning causes hyperglycaemia (27-29). In the present work blood glucose was not measured; thus it is impossible to distinguish between intra-cellular and extra-cellular glucose because in the poisoned rat the liver is very congested as a consequence of cardiac failure. Moreover, it is to be noted that the concentrations of glycolysis intermediates in the tissue may show a wide range of variability (18). The differences in glucose concentrations between the two sexes did not seem to depend on differences of ATP availability in males and females.

The results reported in Table 2 show that in FAC poisoned rats the concentrations of the negative effectors of PFK, citrate and ATP, as well as the concentrations of the positive effectors, ADP, AMP, and Pi, were significantly changed. It is to be noted that ATP decreased with respect to control, thus its effect on PFK was actually positive.

In order to have comparable parameters of the actual changes occurred in the tissue concentrations of PFK effectors, the results were given in μmol per g liver. It can be seen that in spite of an increment of over 2145% the actual mean concentration of citrate in FAC poisoned rats was 2.47μmol / g liver; the same order of magnitude was found for AMP concentration, 2.14μmol / g liver.

TABLE 1. Effect of fluoroacetate poisoning on the concentrations of glycogen and glycolytic intermediates in the rat liver (Sodium fluoroacetate 10 mg / kg body weight / 2 h)

	GLYCOGEN	GLUCOSE	G-6-P	F-6-P	F-1,6-DP	LACTATE
			(µmol / g wet weight ± S.E.M.)			
CONTROL MALES	188.33±21.1 (6)	11.90±0.40 (6)	0.306±0.040 (6)	0.059±0.010 (6)	0.032±0.002 (6)	2.59±0.54 (6)
CONTROL FEMALES	151.72±21.1 (6)	12.94±0.62 (6)	0.334±0.035 (6)	0.074±0.008 (6)	0.028±0.008 (6)	2.92±0.64 (6)
FAC MALES	55.06±21.7 (6)	22.93±3.05 (6)	0.211±0.064 (6)	0.047±0.013 (6)	0.043±0.016 (6)	9.41±3.38 (6)
PER CENT Δ	-71 %	+92 %	-31 %	-20 %	+34 %	+263 %
FAC FEMALES	30.39±20.5 (7)	9.40±2.04 (6)	0.116±0.039 (7)	0.031±0.00 (7)	0.079±0.022 (7)	13.64±1.40 (6)
PER CENT Δ	-80 %	-27 %	-65 %	-58 %	+182 %	+367 %
PER CENT Δ (M + F)	-75 %	+30 %	-50 %	-43 %	+108 %	+319 %

In parentheses the number of observations in each group.

TABLE 2. Concentration changes of phosphofructokinase effectors in rat liver following fluoroacetate poisoning (Sodium fluoroacetate 10 mg / kg body weight / 2 h)

	Negative effectors		Positive effectors		
	CITRATE	ATP	ADP	AMP	Pi
	(µmol / g wet weight ± S.E.M.)				
CONTROL MALES	0.10±0.02 (6)	1.41±0.02 (6)	1.69±0.15 (6)	1.26±0.25 (6)	7.51±1.17 (6)
CONTROL FEMALES	0.12±0.01 (6)	1.66±0.10 (6)	1.28±0.17 (5)	1.25±0.22 (5)	8.87±1.51 (6)
CONTROL M. + F.	0.11±0.01 (12)	1.53±0.12 (12)	1.50±0.11 (11)	1.26±0.16 (11)	8.19±0.93 (12)
FAC MALES	3.37±1.15 (6)	1.05±0.17 (5)	1.72±0.26 (6)	2.04±0.34 (6)	10.09±1.92 (6)
FAC FEMALES	1.38±0.46 (5)	0.70±0.11 (7)	1.49±0.17 (7)	2.22±0.41 (7)	11.23±2.01 (7)
FAC M. + F.	2.47±0.81 (11)	0.85±0.10 (12)	1.60±0.14 (13)	2.14±0.26 (13)	10.70±1.34 (13)
µmol Δ	+2.36	−0.68	+0.10	+0.88	+2.51

In parentheses the number of observations in each group.

DISCUSSION

The present results seem to indicate an involvment of the adrenal cortex in the glycogenolysis induced by FAC poisoning. Since the medulla does not regenerate the present observations demonstrate that it does not play a part in FAC induced glycogen degradation in liver. They show also that citrate accumulation in the liver of the FAC poisoned rat depends on the glycogenolysis and this in turn implies an active glycolysis to generate pyruvate for citrate synthesis.

So far a direct influence of adrenal cortical hormones on glycogenolysis has not been shown. The lysis of glycogen occurs very early in FAC poisoning and this implies an earlier involvment of the adrenal cortex.

No explanations are at present available for this effect. However, some reports have appeared showing an influence of glucocorticoid hormones on the secretion of pancreatic glucagon in man (30, 31) and the hypophysectomized duck (32). If in the rat glucagon secretion during FAC poisoning would be under the control of the adrenal cortex, then the reported observations could be explained. The validity of this suggestion will be decided by further studies. However, the present results seem to rule out an influence of adrenaline on the FAC induced glycogen breakdown in rat liver.

The determinations of the glycolytic intermediates have shown that in the liver of the FAC poisoned rat the degradation of glycogen is followed by active glycolysis demonstrated by the high concertration of lactate and, indirectly, by the accumulation of citrate. The lowering of the concentrations of the hexose phosphates G-6-P and F-6-P and the increase of F-1,6-DP concentration are interpreted as showing a stimulation of the phosphofructokinase activity.

In the liver tissue presumably the negative action of citrate on PFK was counteracted by the combined actions of the positive effectors ADP, AMP, and Pi and also by the deinhibitory effect caused by the lowered concentration of ATP. Further, citrate accumulated in the FAC poisoned liver is approximately 70% localized in the mitochondria and 30% in the cytosol (33,8), where the PFK enzyme in known to be situated. Then, it is likely that only the citrate localized in the cytosol is active on PFK. Further, at least in the isolated PFK obtained from rabbit skeletal muscle, 1 mol of AMP counteracted the inhibitory effect of 4 mol of citrate (34). If the liver enzyme *in situ* has the same sensitivity towards the two effectors as the rabbit muscle enzyme *in vitro*, then an inhibition of PFK by citrate *in situ* is very unlikely. On a more general ground if one takes into account the actual concentrations of the allosteric effectors of PFK in the cell, the distribution of the effectors and of the enzyme in the cellular compartments, as well as the relative activities of the regulatory molecules, then it does not seem very probable that citrate in tissues under physiological conditions might play a role as an effector of PFK.

In conclusion, the present experiments suggested that the adrenal cortex plays a role in the degradation of liver glycogen induced by fluoroacetate poisoning and that the activity of phosphofructokinase is stimulated by the overall changes in concentrations of the allosteric effectors in the poisoned liver tissue.

Abbreviations - ADP, adenosine 5'-diphosphate; AMP, adenosine 5'-phosphate; ATP, adenosine 5'-triphosphate; FAC, fluoroacetate; FCIT, fluorocitrate; F-6-P, fructose-6-phosphate F-1,6-DP, fructose-1,6-diphosphate; G-6-P, glucose-6-phosphate; PFK, phosphofructokinase; Pi, inorganic orthophosphate.

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CHEMISTRY AND BIOCHEMISTRY OF THURINGIENSIN, A NATURAL ANALOGUE OF ATP

K. Šebesta

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, 166 10 Praha 6, Flemingovo náměstí 2, Czechoslovakia

Abstract - Thuringiensin, a low molecular substance excreted by Bacillus thuringiensis was discovered in 1958 due to its insecticidal properties. In further work thuringiensin was found to be a natural analogue of ATP competing with this vital metabolite in the transcription of genetic information. In this respect thuringiensin was shown to be a specific tool for the study of RNA biosynthesis by the DNA-dependent RNA polymerase in vitro and in vivo.

The study of the chemical structure of thuringiensin by means of chemical and physicochemical methods led to the proposition of its structure which was confirmed by synthesis. This synthetic procedure was extended to the preparation of some of thuringiensin analogues. On the basis of this work it was possible to formulate the mechanism of the competition with ATP in the transcription of DNA as well as to define in more detail structural requirements of the elongation binding site for ATP on the DNA-dependent RNA polymerase.

The study of individual biochemical reactions catalyzed by single enzymes operating along metabolic pathways proved to be very rewarding. Since the discovery of sulfanilamide, antimetabolites, mainly analogues of normal substrates, played a key role in the specific inhibition of enzymatic reactions thus enabling their detailed study. Consequently substrate analogues were sometimes regarded as artificial tools designed to interfere with specific reactions. However, it turned out that analogues of current metabolites were often constituents of normal cells mainly in prokaryots. It is hardly necessary to cite examples. Regarding nucleic acid constituents Suhadolnik´s book "Nucleoside antibiotics" lists a great number of these cases. Very interesting among them are compounds analogous to ATP which compete with this vital metabolite in different enzymic reactions.

It is the aim of my presentation to report on a similar analogue with a rather peculiar structure. The case history of this compound is a good example of the close relationship between bioorganic chemistry and molecular biology.

For some time it has been known that Bacillus thuringiensis is a microorganism with highly insecticidal properties which are caused by a protein deposited in a crystalline form in the bacterial spores. In 1958 an additional insecticidal factor was found: this time a low molecular thermostable substance secreted into the culture medium (Ref. 1). When we started to investigate the structure and function of this substance in 1966, three of its constituents were described by French authors, namely adenine, ribose and phosphoric acid (Ref. 2). As already described by Šorm at the IUPAC Meeting in Riga in 1971 (Ref. 3), the real structure turned out to be more complicated (Fig. 1). In addition to adenosine and phosphoric acid it includes also glucose and allaric acid (Refs. 4,5,6). This structure was at first accepted with some doubts (Ref. 1). The reason for this was mainly the unusual type of linkage between ribose and glucose which is not glycosidic but ethereal. By the time of the IUPAC presentation we have also found that the toxicity of the substance was due to the inhibition of RNA biosynthesis at the level of transcription (Refs. 7,8). This finding also showed that the action of this compound which was believed to be limited to insects is in fact of general character.

I should like to concentrate on three main lines of investigation pursued at our Institute since the IUPAC presentation I mentioned above. They are: (1) total synthesis of the substance - named thuringiensin - and of some of its analogues; (2) mechanism of inhibition of DNA -dependent RNA polymerase by thuringiensin, and (3) the use of thuringiensin as a tool in investigating the properties of the active site for elongation of this enzyme.

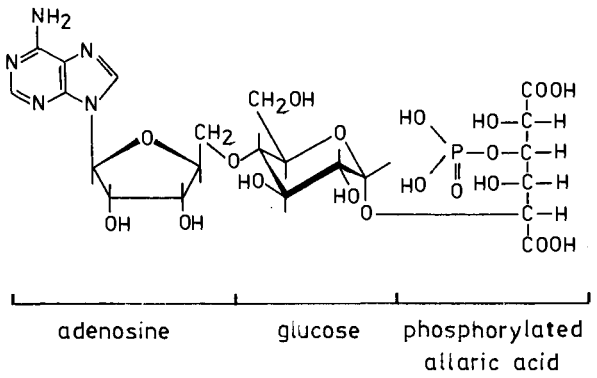
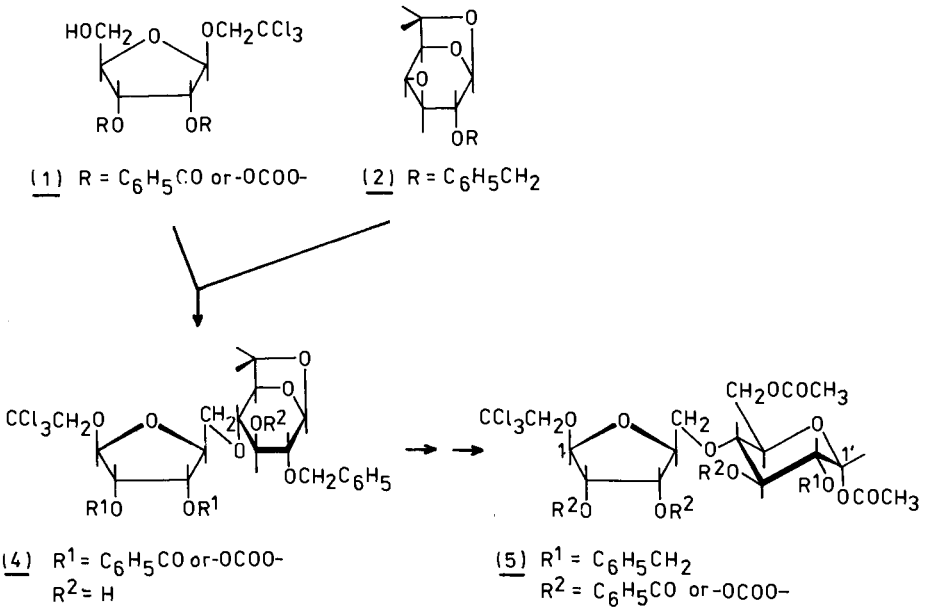
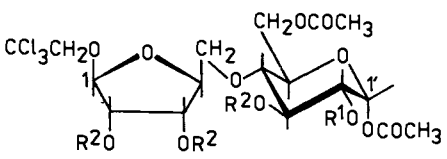


Fig. 1. Structure of thuringiensin

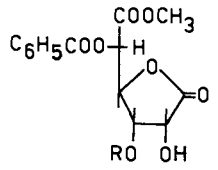
The strategy of the synthesis of thuringiensin performed by Drs Prystasz and Kalvoda consisted in the following key steps: (1) formation of the nucleosidic bond, (2) formation of the anomalous type of the ethereal bond, (3) formation of the α-glucosidic bond, and (4) selective phosphorylation. Twenty four combinations were possible but, as shown by preliminary experiments, only two routes appeared promising. They were based on the formation of the ethereal bond during the first step, and on the phosphorylation during the last step. One single route based on the formation of the whole sugar moiety of thuringiensin, the subsequent selective nucleosidation, and the final phosphorylation was successful. This route was performed (Refs. 9, 10, 11) using two alternatives that differed in the kind of blocking groups and in the transformation of the whole sugar moiety before the nucleosidation step.

For the synthesis of thuringiensin the sugar intermediates 1 - 3 were used. The unusual ethereal bond was formed by trans-doaxial epoxide ring opening of the substance 2 to afford diglycoside ether 4, the 1,6-anhydro ring of which was acetolyzed. The intermediate 5 was treated with the lactone ester of allaric acid 3 under strong acidic condition with predominant formation of the α-glucoside 6 that represents the whole sugar moiety. This intermediate was then transformed stepwise into halogenose 8 that, in turn, was used to sterically controlled nucleosidation. The lactone ring of the nucleoside 9 was opened under very mild conditions to give alcohol 10 which was further phosphorylated using phosphorus oxychloride as a reagent. The same approach was used for the synthesis of thuringiensin analogues with modified either basic or acidic moiety (Ref. 12).

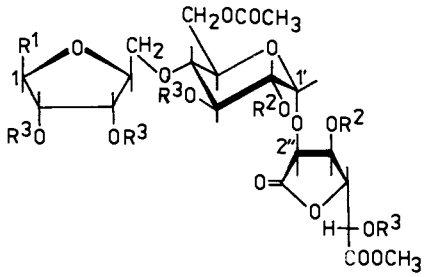




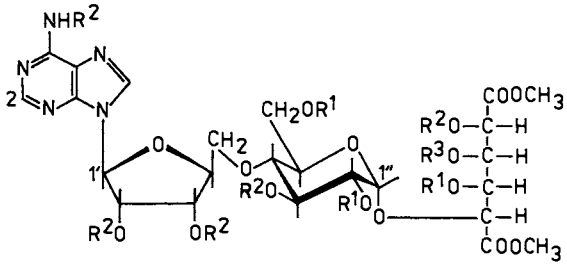
(5) $R^1 = C_6H_5CH_2$
$R^2 = C_6H_5CO$ or
$-OCOO-$



(3) $R = C_6H_5CH_2$ or
C_6H_5CO



(6) $R^1 = CCl_3CH_2O$; $R^2 = C_6H_5CH_2$
(7) $R^1 = CCl_3CH_2O$; $R^2 = CH_3CO$
in formulae (6), (7) $R^3 = C_6H_5CO$ or
$-OCOO-$
(8) $R^1 = Br$, $R^2 = CH_3CO$
(9) $R^1 = N^6$-benzoyladenine /
$R^2 = CH_3CO$
in formulae (8), (9) $R^3 = C_6H_5CO$ or
CH_3CO



(10) $R^1 = CH_3CO$; $R^3 = H$
(11) $R^1 = CH_3CO$; $R^3 = Cl_2PO$
in formulae (10), (11) $R^2 = C_6H_5CO$ or CH_3CO

The second line of our interest dealt with the mechanism of the inhibition of transcription. This reaction catalyzed by DNA-dependent RNA polymerase comprises four major steps: association, initiation, elongation and termination. Our studies were focused on the elongation step as the remaining ones seemed to be unaffected by the inhibitor.

The first clue to the mechanism of inhibition of the RNA polymerase reaction was furnished by the finding that there was a very close relationship between thuringiensin and ATP. Of all four nucleoside triphosphates which are the substrates of the polymerase, only ATP was able to revert the inhibition by thuringiensin (Ref. 7) (Fig. 2) with the implication that thuringiensin may function as an analogue of ATP.

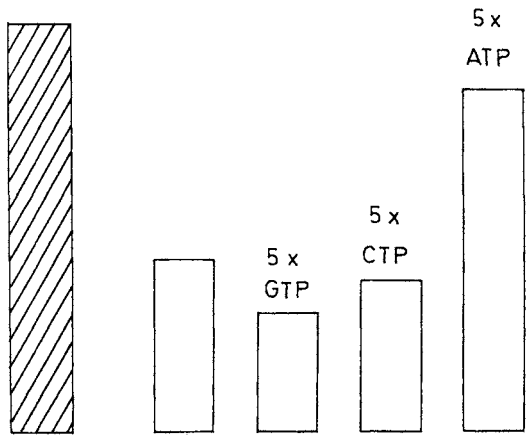


Fig. 2. Inhibition of DNA-dependent RNA polymerase from Escherichia coli by thuringiensin and its reversion by elevated concentration of the single nucleoside triphosphates (Ref. 7). Full column - control, empty columns - in presence of thuringiensin.

This proposition was further strengthened in experiments where competitive inhibition was shown to occur between ATP and thuringiensin (Ref. 13) (Fig. 3).Final confirmation of thuringiensin functioning as an analogue of ATP was obtained in using synthetic templates. With

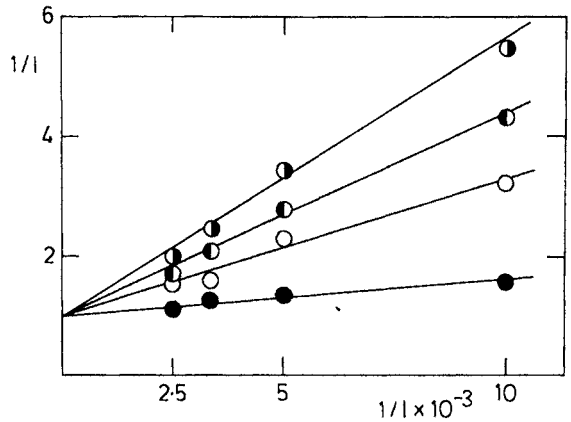


Fig. 3. Double reciprocal plot of DNA-dependent RNA polymerase activity at different ratios of thuringiensin/ATP (Ref. 13). Values for 20 (●), 60 (○), 100 (◐), and 140 (◑) nmoles of ATP in the reaction mixture.

poly dT instead of DNA as a template the inhibition by thuringiensin remained unchanged. However, with poly dIdC which supports the incorporation of ITP but not that of ATP, practically no inhibition occurred (Ref. 14). It was therefore assumed that the adenosine moiety forms hydrogen bonds with thymine of the template at the same active site of the enzyme as does ATP.

The finding that the mechanism of inhibition is based on the displacement of ATP by thuringiensin at the elongation site enabled us to study the structural requirements of this site more in detail. This was all the more attractive since in distinction to other ATP analogues thuringiensin differs from ATP by the structure of its acidic moiety very considerably. In our further work of much assistance were the findings of Mildvan and coworkers (Ref. 15) concerning the conformation of ATP at the active site for elongation (Fig. 4). Using NMR spectra Mildvan has shown that the acidic part of the triphosphate (i.e. α, β and γ phosphate) forms three bonds to Mn^{2+} which is located at the active site and established the conformation of the ATP molecule attached to the active site. By model building we tried to achieve a similar conformation with thuringiensin (Fig. 5). It turned out that thuringiensin could easily form a similar conformation as ATP, and that three bonds to Mn^{2+} were formed by one phosphate group and by two carboxylic group of the inhibition (Ref. 16).

The fact that thuringiensin binds to the elongation site of the enzyme indicates that this site is not specific for the triphosphate group which may thus be replaced by other groups forming bonds to Mn^{2+}. The nonspecificity with respect to the character of the acidic groups

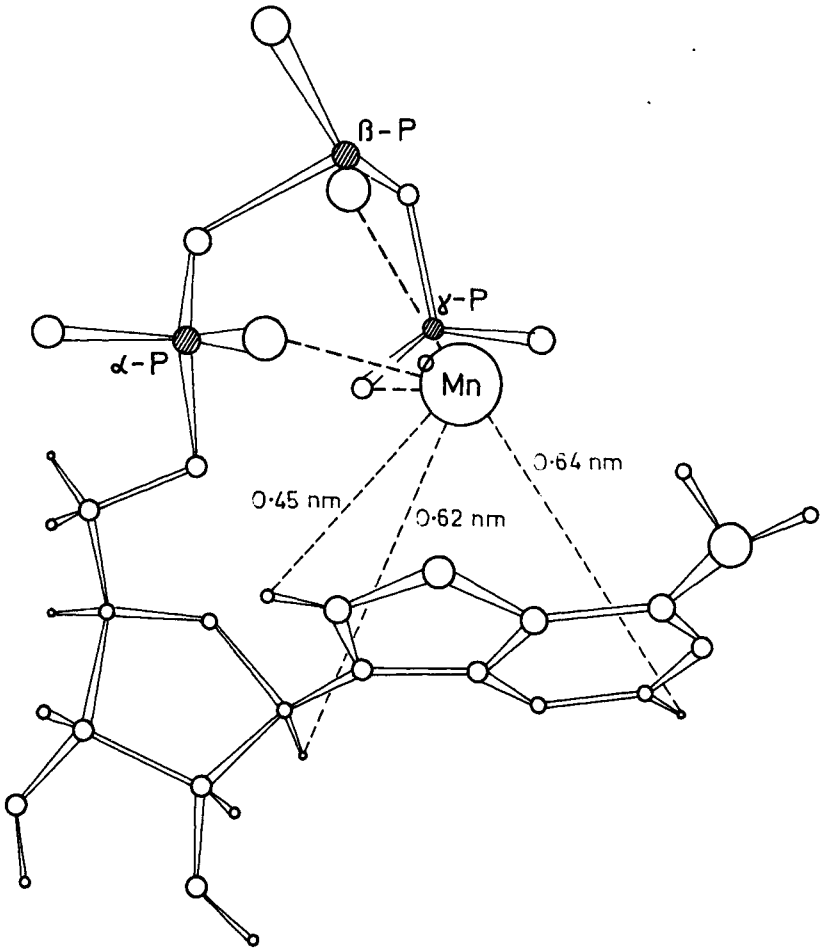


Fig. 4. Conformation of ATP in the active site for elongation of DNA-dependent RNA polymerase (Ref. 16).

raised a further question, namely how strict are the spatial requirements of the active site. For this purpose we tested the inhibitory activity of several thuringiensin analogues (Ref. 12). In three of them allaric acid was replaced by glucaric acid, ribaric acid, and allonic acid respectively (Fig. 6). We tested also a natural derivative of thuringiensin, namely its lactone. In using these substances as polymerase inhibitors we found that any change in the molecule diminished inhibition. A complete loss of inhibitory activity was displayed by the analogue which lacks one carboxylic group. Taken as a whole, the results are in agreement with Mildvan's conception of the indispensable three bonds to Mn^{2+}: where only two bonds can be formed, no binding and thus no inhibition occurs. The same applies to dephosphorylated thuringiensin which was found to be noninhibitory and nontoxic already at an early stage of our work (Ref. 8). In the case of analogues containing glucaric or ribaric acid the three bonds can be formed but the conformation of the inhibitors differs from that of ATP: the binding is less tight and the inhibition is lower than in the original compound. However, the lactone of thuringiensin does not fit into this series. It inhibits rather strongly in spite of the fact that its conformation - due to the rigid lactone ring - is dissimilar from thuringiensin or ATP. The resason for this behaviour is under study at present.

Summarizing our results on the structural requirements of the elongation site of DNA-dependent RNA polymerase the following two features have to be stressed: (1) The active site for elongation does not require the triphosphate group as a prerequisite of binding. It binds equally well also carboxylic functions provided their conformation is correct. (2) The spatial requirements of the active site are rather strict.

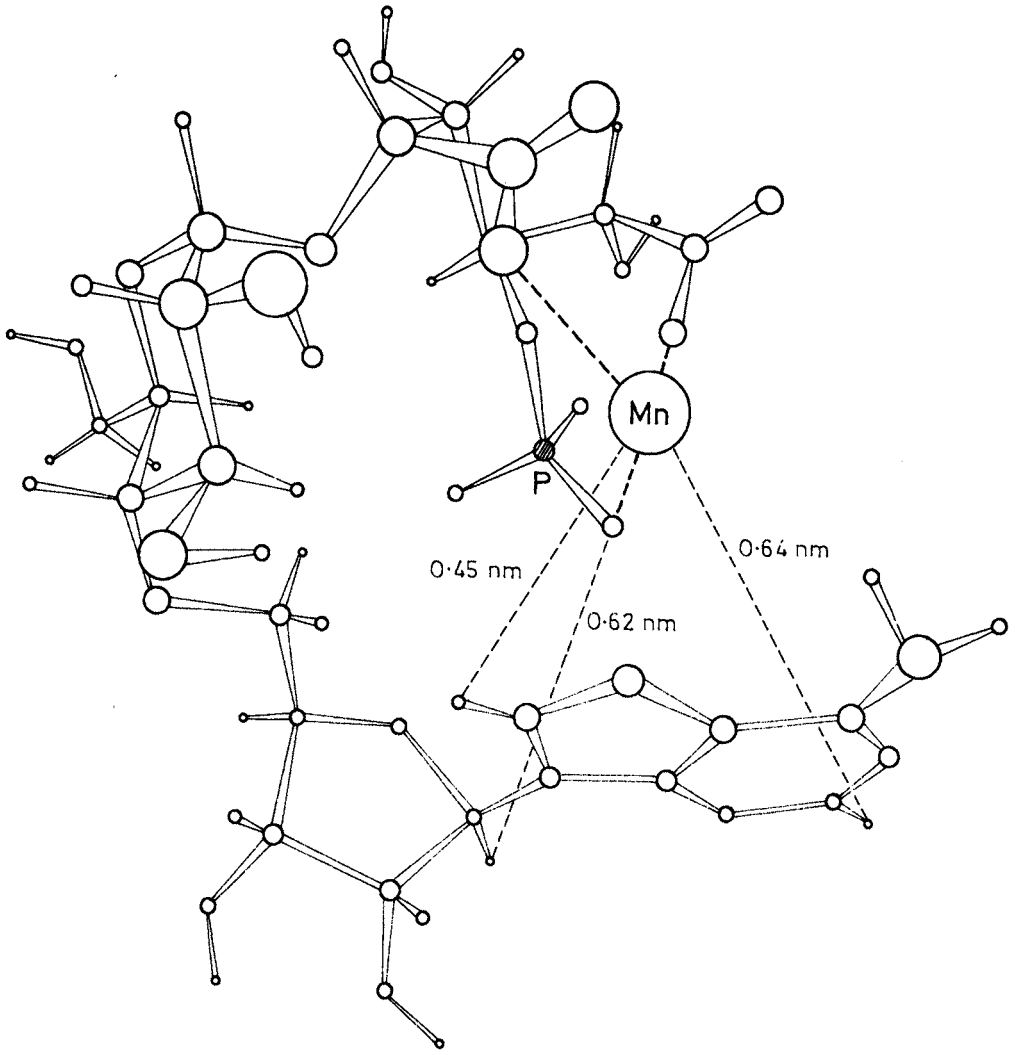


Fig. 5. Tentative conformation of thuringiensin in the active site for elongation of DNA-dependent RNA polymerase (Ref. 16).

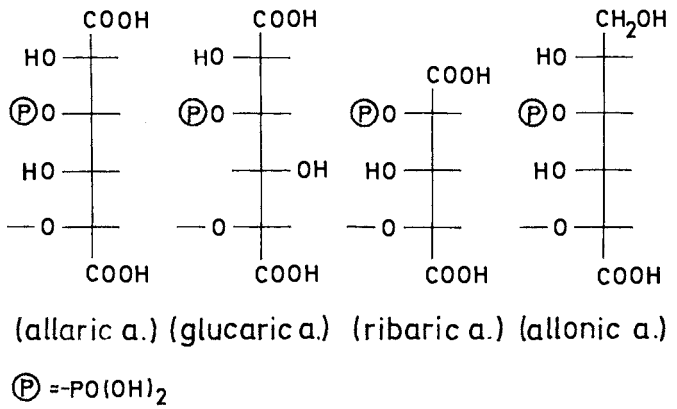


Fig. 6. Acidic moiety of thuringiensin and some of its analogues.

The experiments outlined above were performed with the DNA-dependent RNA polymerase of Escherichia coli. The strictness of the spatial requirements of the elongation site permitted us to compare the degree of similarity of active sites belonging to polymerases of different origin. Eukaryotic polymerases were tested for the inhibition by thuringiensin several times before but the results were somewhat controversial (Refs. 17, 18, 19). We used therefore highly purified preparations of DNA-dependent RNA polymerase A and B from calf thymus. It may be seen (Tab. 1) that both polymerases are by at least one order more sensitive towards the inhibitor than the bacterial enzyme. The nucleolar polymerase (A) which is responsible for the

TABLE 1. Inhibition of different DNA-dependent RNA polymerases by thuringiensin.

DNA-dependent RNA polymerase	K_i (μM)	Conc.of thuringiensin at 50 % inhibition (μM)
Calf thymus A	0.4	1.0
Calf thymus B	1.5	4.0
Escherichia coli	25.0	50.0

synthesis of ribosomal RNA is more sensitive than the nucleoplasmic one (B). Experiments with polymerase C are under way. These results reflect a similarity but not identity of the relevant active sites.

The inhibitory activity of thuringiensin is apparently limited to DNA-dependent RNA polymerases only. Other enzymes using nucleoside triphosphates for the formation of the 3',5'-phosphodiester bond are not affected by the analogue. Two cases where inhibition was reported (adenyl cyclase (Ref. 20) and an unidentified enzyme involved in processing of RNA (Ref. 21)) need further confirmation.

In the last part of my talk I should like to draw your attention to the practical use of thuringiensin in biochemistry and molecular biology. In several laboratories including our own, thuringiensin has recently been used as an inhibitor of DNA-dependent RNA polymerases in vitro and in vivo (Refs. 17, 19, 21, 22). The results of in vivo experiments are in good agreement with the proposed mechanism of action. After the application of the substance biosynthesis of the different species of RNA is decreased in direct relation to the sensitivity of the single polymerases towards the inhibitor. Consequently it is the synthesis of ribosomal RNA which is affected most considerably.Noteworthy is the long duration of the inhibition. The studies of the nuclei isolated from livers of animals which received a single dose of thuringiensin indicate that the activity of polymerase A and B is inhibited for 10 hours following the administration of the analogue. The considerable prolonged effect is interesting mainly because of the repidly decreasing concentration of the inhibitor in the body due to excretion and dephosphorylation to an inactive compound (Ref. 19).

It may be expected that thuringiensin will increasingly be used for the study of transcription and RNA processing especially on account of its specific mechanism of inhibition. Moreower, the relationship between the structure and biological activity of thuringiensin may furnish new incentive for the synthesis of a novel type of analogues.

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PROBLEMS OF STUDIES OF SPECIFIC CELL AUTOREGULATORS (ON THE EXAMPLE OF SUBSTANCES PRODUCED BY SOME ACTINOMYCETES)

A. S. Khokhlov

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow 117988, GSP-1, USSR

Abstract - Naturally occurring substances specifically controlling biological processes (so-called, bioregulators) form an important domain of bioorganic chemistry. These substances can be conveniently divided into three classes depending on their role in Nature: 1) ecological bioregulators, 2) physiological bioregulators, and 3) intracellular regulators. Intracellular regulators of actinomycetes are a promising field for study, owing to versality of biosynthetic processes in these organisms and a considerable number of morphological variations. Systematic study of blocked Streptomyces griseus mutants led to discovery of a bioregulator called "Autoregulating factor" (A-factor). Its structure was elucidated as 2S-isocapryloyl-3S-hydroxymethyl-γ-butyrolactone and confirmed by the synthesis of its racemate. A number of its analogues were also prepared. Their study showed the A-factor structure to be very specific. In experiments with mutant strains there is the risk of substances being produced which are not typical of the original organism. A special study was, therefore, performed on the occurrence of the A-factor in Nature. It was found to be synthesized by all streptomycin-forming Str. griseus strains but not by its inactive mutants. Most of the latter produce streptomycin when grown in a medium to which the A-factor has been added. Mutants in which synthesis of the A-factor is blocked markedly differ from original strain in many morphological and biochemical properties; for example, they do not form spores, the activities of a number of their enzymes are changed, etc. The differences disappear or markedly decrease in most blocked mutants, when they are grown in the presence of A-factor, making the mutant strain resemble the original one. A tentative hypothesis has been proposed regarding the mode of action of the A-factor. A similar approach has been used to show the existence of bioregulators in other actinomycetes, for example Streptomyces galbus.

One of the important chapters of bioorganic chemistry is the investigation of specific natural bioregulators, which control course of definite biological processes. From our point of view, it is reasonable to divide all the bioregulators into three main classes, according to their principal role in the nature: 1) ecological bioregulators; 2) physiological bioregulators, and 3) intracellular bioregulators.

It is possible to include into the first class such regulators which take part in a chemical control of relations between different organisms. Their characteristic feature is the formation by an organism and action on receptors in an other organism. To this now the best known class of bioregulators belong sex attractants, pheromones, natural repellents, antibiotics, toxins, signal substances and other natural products.

One can include into the second class such regulators which secure the chemical control of interactions between different organs, tissues and cells in the same organism. It is characteristic of them that they are produced by a certain type of cells and are acting on receptors, located in cells of other target organ in the same organism. Human hormones are

the best known example of physiological bioregulators.

There is no doubt in existence of the third regulator class, namely of intracellular bioregulators, which participate in the chemical control of processes occurring out in the cell itself. For them it is characteristic that they are produced by a cell and are interacted with receptors in the same cell. Some compounds of this type are the subject of this communication.

The intra-cellular bioregulators seem to be widely distributed in the nature, since without special system of chemical control existence of cell and its development are impossible. However at present time we know very little about bioregulators of this type in comparison with regulators of the first and second type. This shortness of our knowledge may be the result of some peculiarities of these bioregulators, which make their studies especially difficult. Such peculiarities include the more troublesome separation (in time and space) of processes of their biosynthesis and their consumption by the same cell, greater difficulties of their biological assay, the necessity to apply the unusual experimental technique, especially, finding out and use of specific mutant organisms, in which the processes of biosynthesis of such regulators and their utilization by cell were separated. At the same time the use of deficient mutants in which the synthesis of an essentially important product is blocked and therefore one or some biological functions are absent is rather troublesome. Such mutants are often very capricious, they undergo poor development, often they perish and so on.

If to evaluate different organisms as possible sources of intra-cellular regulators, we can conclude, that the unicellular organisms have many advantage for such studies, and especially actinomycetes. These unique microorganisms combine the primitiveness of prokaryotes and very rich diversity of morphological and biochemical processes. Therefore we chosed different actinomycetes producing antibiotic streptomycin, as the first subject of our studies. They included high-productive (selectioned) strains of Str. griseus (which were able to produce streptomycin as much as 3000 mcg/ml) and many mutants, derived from active producers under influence of different mutagens, as it is shown in Table 1.

TABLE 1. Mutants of Str. griseus derived under the action of various mutagens

Mutagen	Number of colonies investigated	Zero mutants		
		total	AF^+-mutants	AF^--mutants
1. N-nitroso-methyl-urea	5470	20	1	19
2. N-nitroso-methyl-biuret	10979	85	4	81
3. Ethylenimine	2270	1	0	1
4. Chloroethylenimine	4160	4	0	4
5. Diethyl sulphate	9376	9	0	9
Total	32255	119	5	114

In all, about 32 000 mutants were obtained and studied in our laboratory in more or less details. (Refs. 1-3). Thus some complementary pairs of practically inactive mutants were found. Among them the pair of mutants No. 1439 and No. 751 was investigated rather thoroughly.

As is shown in Table 2, the first mutant (No. 1439) produced no streptomycin at all, whereas the second mutant (No. 751) synthesized it in small amount. By combined fermentation they produced antibiotic in quantity approximately equal to amount formed by the parent high-active strain.

TABLE 2. Streptomycin biosynthesis in mixed fermentation of mutant strains No. 751 and No. 1439

Strain and mode of fermentation	Streptomycin, mcg/ml		
	72 h	96 h	120 h
773 (control)	800	2 500	3 000
1439 (alone)	0	0	0
751 (alone)	7	10	28
Mixed fermentation (1439 + 751)	800	2 000	3 000
Mycelium of 751 transferred to the broth of 1439	0	10	10
Mycelium of 1439 transferred to the broth of 751	800	1 840	3 200

utant No. 1439 gave great amounts of streptomycin also in the presence f small quantity of cultural broth of mutant No. 751 (Refs. 3,4). These ata showed that the latter produced a bioregulator acting on the mutant o. 751. We obtained this bioregulator (which we named "autoregulating actor" or shortly "A-factor") in pure state, elucidated its structure nd performed a total synthesis of its racemate. Recently when the pure ynthetic A-factor became available and the optimal methodics of its assay ere elaborated, it was stated, that 0.001 mcg of A-factor, added in ime of inoculation to mutant No. 1439, caused the biosynthesis of 1000 mcg f streptomycin, i.e. the induction coefficient was equal to 1 million. hus, the A-factor is a very potent bioregulator, which biological action ay be detected in concentration about 8.10^{-13} molar.

s was mentioned above, the use of mutants, in which some important functions were blocked, is rather difficult and connected with many dangers. ne of them is the probability that a mutant can produce instead of normal etabolites such substances, which are alien to the normal parent organism. nvestigation of such products of changed metabolism may lead to erroneous onclusions. Therefore it was necessary to know whether A-factor was roduced only by mutant No. 751 or also by other strains of Str. griseus. he studies of the great number of actinomycetes have shown, that the -factor was formed by all investigated industrial strains of Str. griseus, roducing streptomycin, by all wild (natural) strains, and by all low- ctive (in streptomycin) mutants. On the contrary, almost all the inactive utants, which lost the ability of streptomycin biosynthesis, as well as trains of other streptomycin-producing actinomycetes (Str. galbus Okami, tr. mashuensis) could not synthesize the A-factor. It is worth emphaizing that the overwhelming majority (114) of zero strains (119), seected from the 32.000 mutants restore their ability to produce streptoycin, if A-factor is added by inoculation. So, it may be taken for ranted that A-factor is necessary to regulate the antibiotic biosynthesis n all the streptomycin-producing strains of Str. griseus.

n addition to the streptomycin biosynthesis the A-factor influences trongly the formation of related compounds. So, zero mutants do not prouce streptidine and phosphostreptidine (in A-factor absence) but produce hem in its presence. The activity of transamidinase - an enzyme taking art in the streptidine biosynthesis - markedly increases in the presence f the A-factor. In general, the pathway of glucose utilization in zero trains changes strongly in the A-factor presence; this phenomenon will be ater discussed in more detail. Thus, the A-factor plays a regulatory ole in many biochemical processes in Streptomyces griseus.

owever, the A-factor is essentially important for producing actinomycetes ecause it influences not only the streptomycin biosynthesis, but also he various processes of differentiation of the parent microorganism, specially the formation of submerged and aerial spores. In the course of he development of usual strains of Str. griseus (which have the A-factor)

in submerged culture in 5-6 days the long filaments of the mycelium desintegrate and the numerous submerged spores resease. On the contrary, the zero strains (which have no A-factor) under similar conditions behave themselves quite differently: they preserve the long filaments of mycelium and the free submerged spores do not almost release. However, if the zero mutant is grown in A-factor presence it develops quite similarly to the normal strains. The influence of the A-factor on the differentiation processes in Str. griseus may be followed more clearly by the cultivation of normal strains and mutants on agar media. Without the A-factor the deficient mutants form rather large diffuse colonies fully devoid of spores. On the contrary, in its presence mutants deficient in this bioregulator formed well-shaped colonies with abundant sporulation. These colonies are smaller in size and more compact in shape. The colonies of deficient mutants of the same age grown in the absence and in the presence of the A-factor differed in size, shape, colour and sporulation so strongly, that they seemed to be the colonies of different species (Ref. 5).

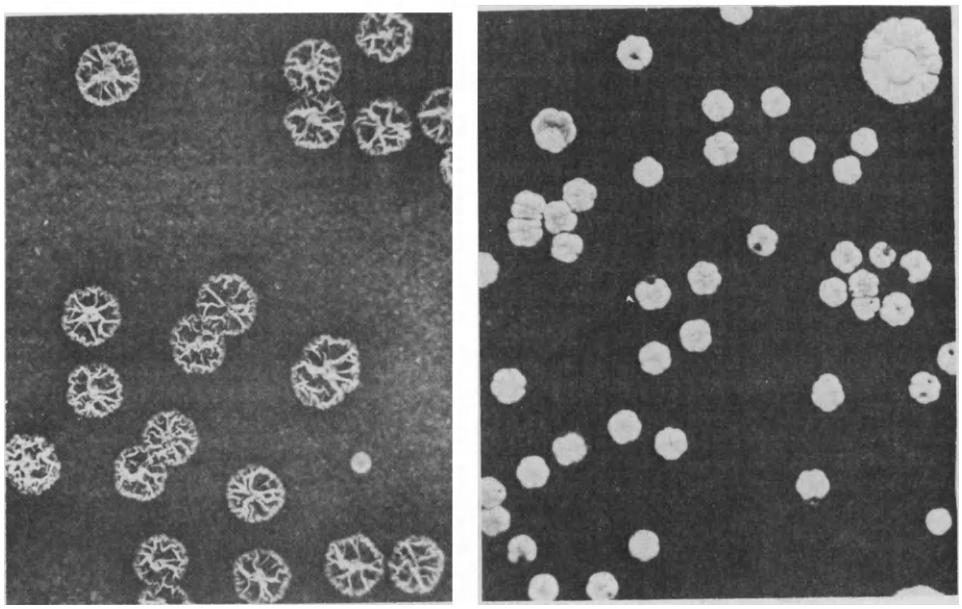


Fig. 1. Colonies of A-factor deficient mutant (mutant No. 143) of Streptomyces griseus grown on agar media for 12 days. Left: Medium without the A-factor; Right: Medium with the A-factor (10 mcg/ml)

Spores formed by asporogenous mutants in the A-factor presence and transferred on the usual agar medium without A-factor developed into asporogeneous colonies which produced no streptomycin. Therefore the A-factor effect is not connected directly with the alteration of the genetic material of actinomycete. These experiments also show that a unique model is found to study the processes of actinomycete sporulation. This model consists of three entities: 1) the well-sporulating normal parent strain; 2) an asporogenous mutant, derived from previous strain; 3) the same asporogenous mutant, which give spores in the presence of normal bioregulator of the parent strain - A-factor.

The worked out system for searching regulators of sporulation was applied to an other actinomycete species - to Streptomyces gelbus Okami. It was shown that this actinomycete had not the A-factor but its own regulator of sporulation. Its existence may be demonstrated as follows. From usual strain of Streptomyces galbus asporogenous mutants were prepared, which did not sporulate in the presence of the A-factor (and A-factor producing actinomycetes), but gave abundant sporulation under the influences of substances produced by parent strain of Streptomyces galbus; at the same time these compounds did not cause the sporulation in asporogenous mutants of Streptomyces griseus (in particular, in mutant No. 1439), which

sporulated very well in the presence of the A-factor.

The profound distinctions in the course of a number of differentiation processes in normal strains and in deficient mutants were revealed also by electron microscopy investigation. The majority of these distinctions were diminished, or even disappeared, when the mutants grew in the presence of the A-factor. The most spectacular distinction between the parent strain and the zero mutant was seen in the formation of intracellular structures (polysomes, membranes a.o.). In 3-4 days of culture development the normal strain had numerous analogous structures, whereas they were practically absent in the zero mutant. However, if the zero mutant grew in the presence of the A-factor, it behaved as the normal strain. A striking feature of zero mutants, when they were developing in the presence of the A-factor, was the appearance of specific tubular structures, which were always seen in normal strains in the period of streptomycin biosynthesis and were absent in zero strains growing without the A-factor. These structures seem to be connected with the antibiotic biosynthesis. Thus the A-factor is necessary for the producing actinomycete to control not only some biochemical reactions, but also cardinal processes of its development and differentiation.

Quite naturally, after the discovery of an autoregulator so important for microorganism producing it we decided to elucidate its structure. This task was rather hard because A-factor was produced in very small amounts; it is, probably, a common property of intra-cellular autoregulators. In 0.5 ton of cultural broth (obtained after fermentation in 1 cubic meter fermentator) there were only about 20 mg of the A-factor together with 25 kg of solid residue, which contained also its lower homologues almost devoid of specific biological activity. Therefore, we had to treat cultural broth from many fermentators to harvest sufficient amount of material for chemical investigations (Ref. 6). Rather active oily preparations of the A-factor were isolated by the combination of multiple extractions and different chromatographic procedures; they were able to stimulate the streptomycin biosynthesis very strongly, but were not homogeneous and showed by mass-spectroscopy the presence of some homologues. Therefore a number of A-factor derivatives were prepared and purified. Among them its diphenylcarbanilate obtained by interaction with phenylisocyanate was isolated after crystallization in homogeneous state. Its empirical formula $C_{27}H_{32}N_2O_6$ showed that the A-factor itself had the formula $C_{13}H_{22}O_4$.

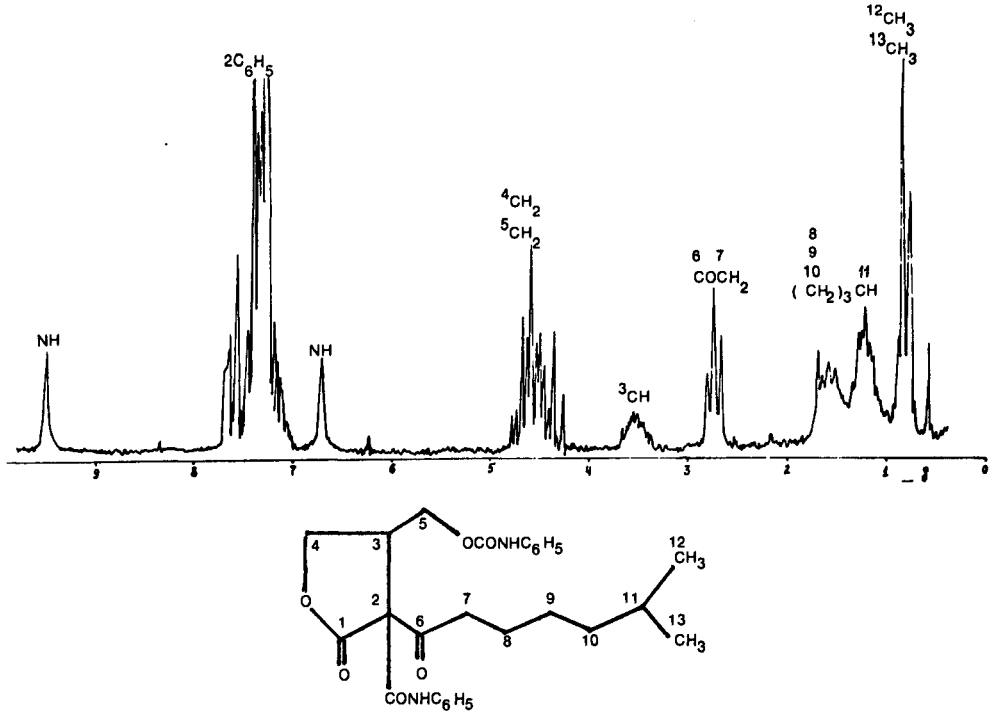


Fig. 2. NMR-spectrum of the A-factor diphenylcarbanilate.

The main information about dicarbanilate and parent A-factor structures was obtained by different physico-chemical methods. So, study of IR- and UV-spectra of diphenylcarbanilate and products of its mild hydrolysis demonstrated that it contained γ-lactone cycle; in β-position to its carbonyl the second carbonyl was located. By different methods the A-factor was shown to contain a hydroxymethyl group. Thus, the nature of all the four oxygen atoms in its molecule was determined. Investigation of dicarbanilate PMR-spectrum (Fig. 2) confirmed the presence of 32 protons including 7 protons of iso-propyl group, 10 protons of two phenyl residues and two protons of NH-groups, i.e. the presence of two phenylcarbanyl groups in it. Study of 4 proton signals (multiplet at 4.2-4.8 m.p.) showed, that they belonged to two methylene groups, both of them adjoining to O-acyl-groups. Both methylens interacted with the same proton, which had a multiplet signal in the region 3.4-3.6 p.m. All these evidences together allowed to deduce that the A-factor is 2-isocapryloyl-3-hydroxymethyl-4-hydroxybutanoic acid lactone (or according to the other terminological system 3-isocapryloyl-4-hydroxymethyl-γ-butyrolactone) (Ref. 7).

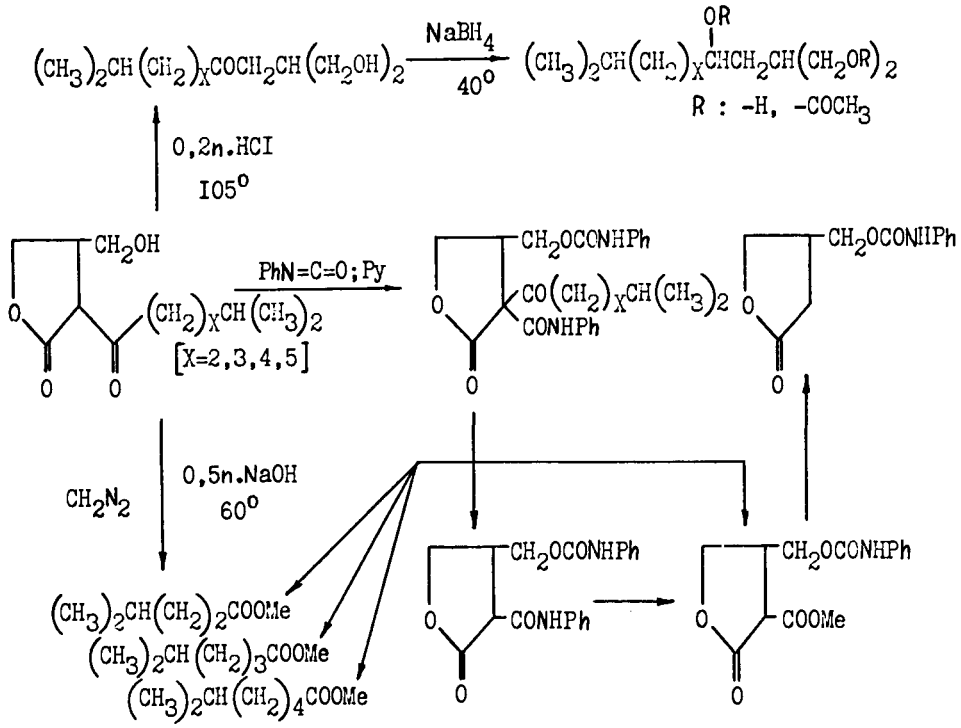


Fig. 3. Chemical transformations of A-factor.

The deduced formula of the A-factor is in good accordance with a number of its transformations, summarized on scheme 1 (Fig. 3). A mixture of homologous branched fatty acids was found out as a result of acid or alkaline hydrolysis of the crude A-factor and its non-crystallized diphenylcarbanilate. These data were a confirmation of the presence of its lower homologues in parent preparations.

A-Factor contains two asymmetric centers: C-2 and C-3. In its spectrum of circular dichroism two positive Cotton's effects (at 285 and 225 nm) are present; the second of them is due by lactone chromophore. According to the well-known sector lactone rule the sign of this effect is determined mainly by S-configuration of the substituents at C-3. As to the orientation of acyl substituent, the more stable trans-isomer (also with S-configuration) must be predominant in solution in consequence of easy epimerization of C-2. Naturally, it is necessary to have in mind the existence

of the enol form, which causes shoulder at 250 nm. The spatial structure of the A-factor can be expressed by formula on Fig. 4.

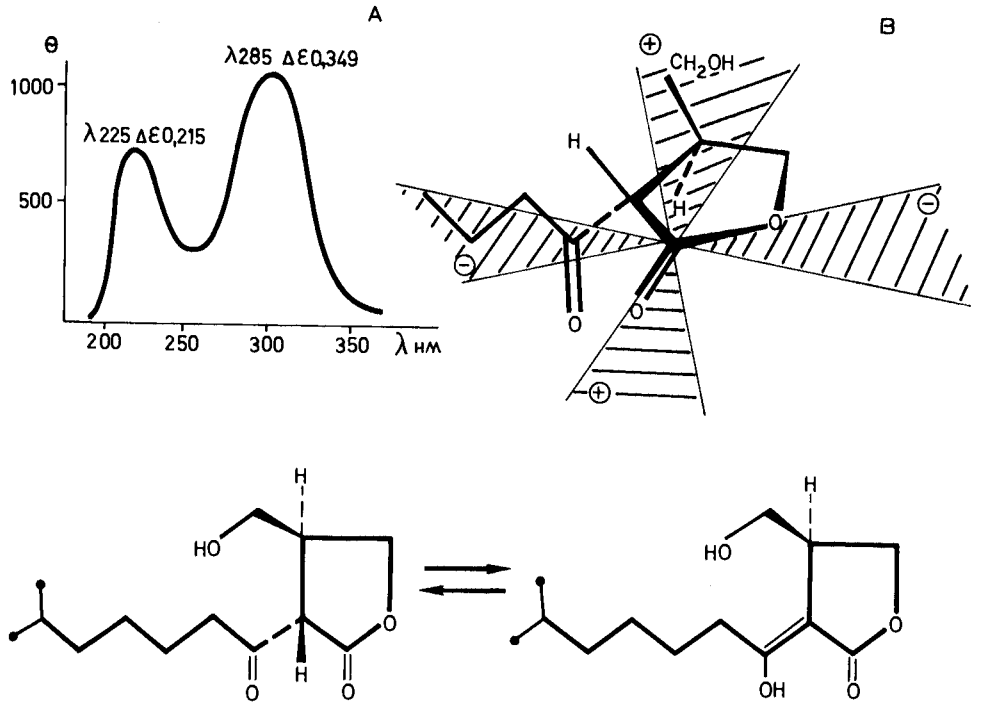


Fig. 4. Spatial structure of the A-factor.

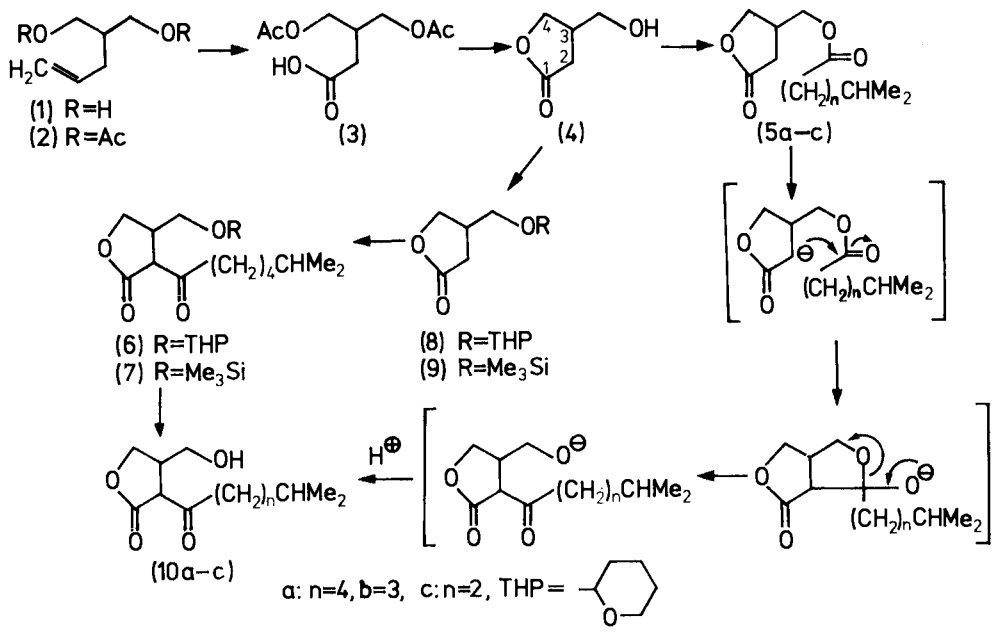
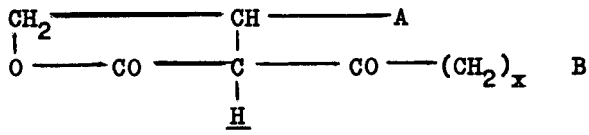


Fig. 5. Scheme of synthesis of the A-factor and its analogues.

The synthesis of A-factor racemate, shown on the scheme 3 (Fig. 5), was a decisive proof of the suggested formula. According to the first variant of the synthesis prepared 3-hydroxymethylbutyrolactone was transformed into the racemic A-factor with a preliminary protection of hydroxyl. The protected compounds were condensed with methyl isocaprylate in the presence of dymmylsodium. Obtained compounds were hydrolysed easily into the racemic A-factor. The better yields were, however, achieved without the preliminary protection of hydroxyl by intra-molecular C→O acyl migration (Ref. 8).

TABLE 3. Specificity of the A-factor (dependence of stimulating activity on structure)



A	X	B	Activity (%%)
CH_2OH	4	$CHMe_2$	100
CH_2OH	3	$CHMe_2$	0–10
CH_2OH	2	$CHMe_2$	0
CH_2OH	5	$CHMe_2$	0–15
CH_2OH	4	Me	0
CH_2OH	4	CH_2Me	0–20
CH_2OH	4	CH_2CH_2Me	0–10
CH_2OH	4	$(CH_2)_3Me$	0
CH_2OH	4	$(CH_2)_4Me$	0
H	4	$CHMe_2$	0
CH_3	4	$CHMe_2$	0
CH_2OAc	4	$CHMe_2$	100
$CH_2OCONHC_6H_5$	4	$CHMe_2$	0
H→Ac			
$CH_2O\overline{CH(CH_2)_4O}$	4	$CHMe_2$	0
H→Ac			

A number of homologues and analogues of the racemic A-factor was synthesized by this and similar methods. Their biological assay showed that the A-factor molecule was rather specific. As one may see on the table 3, any alteration of its structure leads to strong decrease or even disappearence of stimulating activity.

Now we are studying the localization of an A-factor receptor in actinomycete cell. The radioactive sample of the A-factor is used, which has been prepared by treatment of the synthetic A-factor with thermally excited tritium atoms. The level of A-factor binding by actinomycete cells was measured after a short-term treatment of 24 hr old mycelium of mutant No. 1439 with the radioactive A-factor. The same amount of the A-factor was found to be bound firmly with cells (approximately 0.18 mcg of bioregulator per 1 g of dry mycelium) after the treatment of the mycelium of mutant No. 1439 with rather different concentrations of the A-factor and subsequent careful washing. According to the preliminary data the A-factor was bound mainly with a protein component of cytoplasm.

We are also studying the biochemical role of the A-factor and the molecular mode of its action. The results of comparative investigations of enzymes of carbohydrate metabolism in normal strains and deficient (zero) mutants were essential for understanding of this problem. Among a great number of

above-mentioned enzymes, the strongest distinction between normal and mutant strains was found for D-glucose-6-phosphate: NADP-oxidoreductase (1.1.1.49), usually named as glucose-6-phosphate-dehydrogenase (G6PDH). The activity of this enzyme was shown to be very low in active streptomycin producers, so it was practically impossible to measure it by standard methods. On the contrary, activity of G6PDH was high in zero mutants. However, soon after the A-factor was added to the growing mycelium of such zero mutants, the sharp fall of the G6PDH-activity occured rather quickly and after approximately 12-16 hours it was impossible to measure it. At the same time, the pure A-factor added to crystalline G6PDH did not decrease its activity (Ref. 9). It was shown that a specific NADP-ase was synthesized or activated in the cells of zero mutant under the influence of the added A-factor. This NADP-ase destructed NADP (nicotinamide-adenine-dinucleotidphosphate) with liberation of free nicotinamide and adenosine-diphosphoribose phosphate. The last compound seemed to inhibit selectively glucose-6-phosphate-dehydrogenase.

It is well known, that adenosine-diphosphoribose and its phosphate influence greatly the development of different eukaryotic organisms, including, for example, the sporulation of fungus Neurospore crassa. By analogy it is possible to suppose that these substances may influence on the sporulation processes in Streptomyces griseus. In this case the A-factor acts as a trigger, which switches on the system of enzymes necessary both for sporulation and for streptomycin biosynthesis (Ref. 10).

Without any doubt these suggestions are very preliminary, but they may be evaluated as the first working hypothesis. On the basis of this hypothesis it is possible to understand reasons of many distinctions between parent normal strain (No. 773) and deficient mutant No. 1439 and reasons for decrease (or even disappearance) of these distinctions under the influence of the added A-factor, as is shown in Table 4. Thus, the A-factor influences directly or indirectly a number of biological processes, important for Streptomyces griseus which produces it. Therefore the A-factor is a bright example of intra-cellular autoregulators, i.e. it is a bioregulator of the third type according to the classification mentioned in the beginning of this communication.

TABLE 4. Comparison of properties of Streptomycin high producing strain No. 773 and zero mutant No. 1439 grown without the A-factor (AF) and in its presence

Properties	mutant 1439		strain 773
	without AF	in presence of AF	
Formation of			
Streptomycin (mcg/ml)	0	3000	3000
Streptidine	-	+	+
O-Phosphorylstreptidine	-	+	+
NADP-ase	-	+	+
Dark pigment	-	+	+
Aerial spores	-	+	+
Intracellular structures:			
Membranes	low	high	high
Polysomes	low	high	high
Tubular organelles	-	+	+
Transamidinase activity (units per 1 mg of protein)	0.12	2.0	3.9
G6PDH activity	high	low	low
Mycelium basophility	high	low	low
Release of spores from hyphae	-	+	+

The search in Nature, studies of biological properties and elucidation of chemical structures of specific intra-cellular autoregulators seem to be of great importance, since they give the possibility of better understanding the mechanism and the very essence of processes, taking part in the cell itself and may allow to control voluntarily these processes.

In conclusion I wish to fulfill my peasant duty and give the names of my co-workers, whose personal interest in this research, experimental skill and patience made these labour-consuming investigations possible. From the very beginning of these sutides Dr. I.I.Tovarova has not only been at the head of all the biochemical aspects of work, but, together with me, she is the initiator and pitiless critic of our work. Also from the very beginning Dr. L.N.Anisova has been leading the genetic and selection aspects of work and takes active part in working out of general conceptions. Very active have been working many members of our laboratory: O.L.Voronina, L.A.Shevchenko, E.Ya.Kornitskaya, O.I.Krassil'nikova, S.A. Ostroumov, O.V.Efremenkova, T.S.Popova, I.N.Blinova and others. In the elucidation of A-factor structure took part V.V.Onoprienko and his coworkers E.M.Kleiner, S.A.Pliner and V.S.Soifer. The preparation of crude A-factor was made in the pilot-plant of our institute under the supervision of G.M.Smirnova. The electron-microscopic investigation was performed together with a group of electron microscopy (head - Dr. P.L.Zaslavskaya) in National Institute of Antibiotics. All the mentioned persons are the co-authors of corresponding parts of this communication.

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BIOLOGICAL IMPLICATIONS OF RECENT DISCOVERIES IN THE MARINE STEROL FIELD

R. M. K. Carlson, C. Tarchini and C. Djerassi

Department of Chemistry, Stanford University, Stanford, California 94305, USA

Abstract - Marine sterols exhibit widely varied and often complex patterns of alkylation, dealkylation and side chain cyclopropane construction. These marine sterols occur at concentrations which reflect a structural role in the membrane. Only a single sterol, cholesterol, has been used in the majority of membrane biological and biophysical studies. In this paper the possible functional significance of the unusual structural features unique to the various marine sterols is explored. Also, the extent of the existing survey of sterol compositions of marine organisms is evaluated, important unexamined groups of marine organisms are pointed out, and the importance of marine sterol distributions to various biological fields is discussed.

INTRODUCTION

Sterols are essential compounds for the maintenance of all forms of eukaryotic life (Ref. 1). Indeed, two basic schools of thought exist concerning the evolutionary origins of the eukaryotic cell. One theory (Ref. 2) holds that eukaryots arose by a process of endosymbiosis of numerous prokaryotic organisms, while the other theory (Ref. 3) holds that eukaryots arose out of selective pressures to compartmentalize in a single ancestral blue-green alga which lost its cell wall and developed the ability of phagocytosis. The development of a fluid plasma membrane by ancestral pre-eukaryots is a central requirement in both theories. One of the recent and fundamental achievements of membrane biophysicists and biochemists has been the demonstration of the basic role of sterols in maintaining plasma membrane integrity (Ref. 4-7). Prokaryots do not contain sterols (Ref. 8); therefore, the development of sterol biosynthesis by a pre-eukaryot may have been a key step in the evolutionary process that resulted in the sudden rise of the eukaryots about 1.5 billion years ago, and the subsequent development of all higher forms of life.

That sterols were excellent fundamental "raw materials" for the development of biologically active compounds by evolving eukaryots is apparent from the existence today of the extraordinary number of steroid classes which have diverse yet basic biological functions and which are all derived from a common sterol precursor. Steroids (Ref. 9,10) are involved in calcium metabolism (vitamin D), digestion (bile acids), arthropod molting mechanisms (ecdysones), poisons (saponins and insect poisons), reproduction in fungi (antheridiol and ooginol)(Ref. 11), and ionic regulation and reproduction in mammals; still other classes of steroids exist in nature which have as yet unknown biological functions (e.g., polyhydroxy marine sterols (Ref. 12)). It is interesting and significant that whereas many steroid classes have regulatory roles in the interaction of organ systems in higher organisms, sterols appear to have a basic structural role in the membrane at the purely cellular level. The primary site of cell-cell interactions and the final interface of a cell with the outside world is the plasma membrane; therefore, a clear and comprehensive understanding of the functional role, chemical environment, and dynamic interactions of sterols and other membrane constituents would be an important and fundamental achievement. However, the general concepts of membrane stabilization and permeability modifications by sterols have been proven only recently, and the structure-function relationships behind these properties is an area of intense current research.

The majority of studies concerning the role of sterols in membranes have employed only a single sterol, cholesterol (25) (Ref. 4-7). A few membrane studies have considered the properties of certain of the terrestrial phytosterols (Ref. 7); however, to the present time the only naturally occurring sterols to be considered in membrane biochemical or biophysical studies have been from terrestrial sources. If we draw a generalized terrestrial sterol (Fig. 1a) and for the present neglect the various patterns of unsaturation (some of which represent biosynthetic intermediates) and side chain stereochemistry, we find that the only basic differences in the substitution pattern of terrestrial sterols is found at

C-24 (where R_0 represents a hydrogen for sterols produced by terrestrial animals, and a one or two carbon substitution for terrestrial plants). The picture is very much different for a generalized marine sterol (Fig. 1b). No less than five different sites of possible alkylation (C-22, C-23, C-24, C-26 and C-27), three different sites of possible dealkylation (C-19, C-26, C-27), and four different modes of possible side chain cyclopropane construction (involving C-20, C-22 and C-23; C-22, C-23 and a nonsqualene carbon; and C-23, C-24, and a nonsqualene carbon; and C-25, C-26 and C-27) have been demonstrated in sterols from marine sources.

The rapid developments that have occurred over the past couple of years in the marine sterol field have been summarized in numerous reviews (Ref. 13-21); however, the rate of developments has been so swift that even the most recent works are outdated (Ref. 22). In Tables 1 & 2 we list the complete set of marine sterols which had been reported at the writing of this paper (Aug., 1978). The Tables are used as a reference for discussions in the remainder of this text. It is not our purpose to again review the general subject of marine sterols. We refer the interested reader to the long list of reviews cited above. Instead, we intend to explore several aspects of marine sterol chemistry which we believe to be of potential importance to other areas of scientific investigation, particularly membrane physiology, marine biology and geochemistry. The discussion presented below arose out of a comparison of the generalized structures presented in Figure 1a & b.

The profound complexity of the generalized marine sterol as compared to the generalized terrestrial sterol, and the tremendous number of different marine sterol structures (Tables 1 & 2) suggest the two basic questions which we will consider: (1) What is the biological significance of the unusual structural features of the marine sterols? and (2) Why are marine organisms such superb sources of unusual sterols as compared to terrestrial organisms?

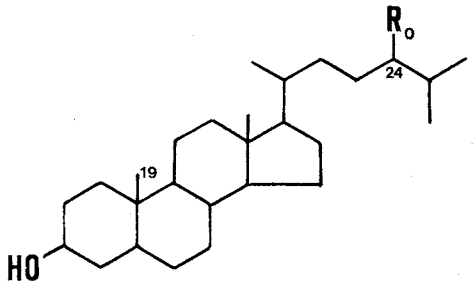


Fig. 1a Generalized Terrestrial Sterol

R_0 = H, Me or Et

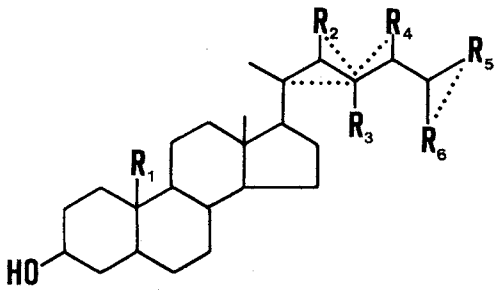


Fig. 1b Generalized Marine Sterol

R_1, R_2, R_3 = H, Me or is a cyclopropyl carbon as noted

R_4 = H, Me, Et, n-propyl, isopropyl or is a cyclopropyl carbon as noted

R_5, R_6 = H, Me, Et or is a cyclopropyl carbon as noted

IMPLICATIONS TO MEMBRANE BIOLOGY

A discussion of the biological significance of secondary metabolites, such as the sterols, can develop along several lines. The possible phylogenetic/taxonomic significance of the organismic distribution patterns of specific sterol structural features will be touched upon later. However, the principal questions relate to the functional biological significance, or physiology, behind specific biochemical modifications among the naturally occurring sterols. It could be argued that many times the structural diversity found in a specific class of secondary metabolites does not reflect various physiological requirements, but are rather the result of some nonspecific biosynthetic process (the amazing diversity among the indole alkaloids may be an example of such a case (Ref. 23)). However, as the series of biosynthetic modifications directed toward a sole product which belongs to a class of compounds having a specific and important biological function becomes more numerous and complex, and as the steady state concentration of that product in the tissues of an organism increases, the probability that the product reflects some accidental biochemical diversity with no real functional significance surely drops off sharply. Furthermore, by postulating physiological reasons for various biochemical modifications, functional hypotheses can be developed which are open to experimental testing. Bloch (Ref. 24) has taken

TABLE 1

(see Ref. 61)

MARINE STEROL NUCLEI

MARINE STEROL SIDE CHAINS	NUCLEI								
SHORT SIDE CHAINS		274 **1**							
		300 **2**							
		300 **3**							
		302 **4**							
		314 **5**							
		316 COMERIBOL **6**		318 **7**					
		330 **8**							
C_{29}		370 Δ^{22}-24-NOR-CHOLESTEROL **9**	370 ASTEROSTEROL **10**	372 **11**		358 **12**			
		24-NOR-CHOLESTEROL **13**							
		382 **14**							
C_8		384 OCCELASTEROL **15**	384 AMURESTEROL **16**	386 PATINOSTEROL **17**					
		384 Δ^{22}-CHOLESTEROL **18**	384 **19**	386 22,23-DEHYDRO-CHOLESTANOL **20**	382 **21**	372 **22**			
		384 DESMOSTEROL **23**	384 **24**						
		386 CHOLESTEROL **25**	386 LATHOSTEROL **26**	388 CHOLESTANOL **27**	386 7-DEHYDRO-CHOLESTEROL **28**	374 19-NOR-CHOLESTANOL **29**	372 19-NOR-CHOLESTEROL **30**	386 ZYMOSTEROL **31**	388 COPROSTANOL **32**
			396 **33**						
		398 PINCSTEROL/ CRINOSTEROL **34**	398 STELLASTEROL **35**	400 22,23-DEHYDRO-CAMPESTANOL **36**	396 **37**				
C_9		398 BRASSICASTEROL **38**	398 5,6-DIHYDRO-ERGOSTEROL **39**	400 NEOSPONGOSTEROL **40**	396 ERGOSTEROL **41**	386 **42**			
		398 CODISTEROL **43**							
		398 CHALINASTEROL/ OSTREASTEROL **44**	398 EPISTEROL **45**	400 24-METHYLENE-CHOLESTANOL **46**		386 **47**			
		400 CAMPESTEROL **48**	398 **49**	402 CAMPESTANOL **50**	398 7-DEHYDRO-CAMPESTEROL **51**		386 **57**	400 **58**	
		400 22,23-DIHYDRO-BRASSICASTEROL **52**	400 FUNGISTEROL **53**	402 ERGOSTANOL **54**	398 22,23-DIHYDRO-ERGOSTEROL **55**	388 **56**			
		410 CALYSTEROL **59**							
		412 STIGMASTEROL **60**	412 (α)-SPINASTEROL/ NITOPESTEROL **61**	414 **62**	410 CORBISTEROL **63**	400 **64**	398 **65**		
		412 PORIFERASTEROL **66**	412 CHONDRILLASTEROL **67**						
		412 FUCOSTEROL **68**	412 Δ^7-FUCOSTEROL **69**	414 FUCOSTANOL **70**					
		412 AVENASTEROL/ 28-ISOFUCOSTEROL **71**	412 Δ^7-AVENASTEROL **72**	414 28-ISOFUCOSTANOL **73**					
C_{10}		412 CLEROSTEROL **74**							
		412 Δ^5-DEMETHYL-DINOSTEROL **75**				400 **76**			
		412 24,28-DIDEHYDRO-APLYSTEROL **77**							
		412 23-DEMETHYL-CORGOSTEROL **78**		414 23-DEMETHYL-CORGOSTANOL **79**					
		414 (δ)-SITOSTEROL **80**	414 SCHOTTENOL **81**	416 STIGMASTANOL (δ)-SITOSTANOL **82**	412 7-DEHYDRO-SITOSTEROL **83**	402 **84**		414 **85**	416 **86**
		414 CLIONASTEROL/ γ-SITOSTEROL **87**	414 DINYDROCHON-DRILLASTEROL **88**		412 7-DEHYDRO-CLIONASTEROL **89**				
		414 APLYSTEROL **90**							
C_{11}		426 CORGOSTEROL **91**	426 ACANTHASTEROL **92**	428 CORGOSTANOL **93**					
		426 29-METHYL-28-ISOFUCOSTEROL **94**							
		426 29-METHYL-FUCOSTEROL **95**							
		428 $\Delta^{7,24}$-24-ISOPROPYL CHOLESTEROL **96**							
		428 24-ISOPROPYL-CHOLESTEROL **97**							

this approach in suggesting that demethylation at C-4 and C-14 of the primary squalene cyclization products lanosterol and cycloartenol evolved as a means of improving the membrane stabilizing capabilities of sterols, and that further demethylation of C-19 and C-18 did not evolve due to a hypothetical destabilizing effect of such sterol β-face demethylations. In subsequent experiments using a series of 4, 14 tri-, di- and mono-methyl sterols and a C-19 demethyl sterol, he has shown (Ref. 25,26) that the stabilizing and destabilizing effects which he predicted were in fact exhibited in model membrane systems.

Model membrane and biological studies by numerous researchers have produced a list of generalizations (Ref. 4-7) regarding the structural requirements for sterol function in biological membranes. To the present time membrane physiologists and biophysicists have not considered the possible significance of the various marine sterols, and indeed, the existence of a number of marine sterols contradicts several of the sterol membrane function requirement rules which have been developed. We will discuss these apparent contradictions and consider the possible functional significance of some of the unusual marine sterol structural features. It is our hope that this discussion will stimulate experimental studies which explore the role of sterols in the membrane systems of marine organisms. We believe that such studies will help clarify various aspects of the general role of sterols in biological membranes.

To function in membrane stabilization a sterol must (i) reduce the effective molecular area of membrane phospholipid molecules (known as the sterol condensing effect); (ii) mobilize phospholipid fatty acid chains for phospholipids in the ordered gel state, but reduce mobility of fatty acid chains for phospholipids in the fluid liquid crystalline state; (iii) reduce nonionic permeability for phospholipids in the liquid crystalline state, but increase nonionic permeability for phospholipids in the ordered gel state. The net effect of these sterol activities is to increase membrane fluidity and stability and to modify membrane permeability in a way essential to eukaryotic life. The general biological requirement is that a "sterol" must support life in "sterol" deficient mutants. It is important to note that even nonsteroids might function as membrane stabilizers in certain organisms (Ref. 4,8,9). It is generally thought that the stabilization of membranes by sterols is the result of interaction between the sterols and other membrane constituents (Ref. 27). Furthermore, it has been found that to be effective in membrane stabilization a sterol must possess certain very specific chemical and stereochemical features, and these required features are summarized in Figure 2 along with examples of marine sterols which contradict these structural requirements.

TABLE 2 RECENTLY REPORTED MARINE STEROLS

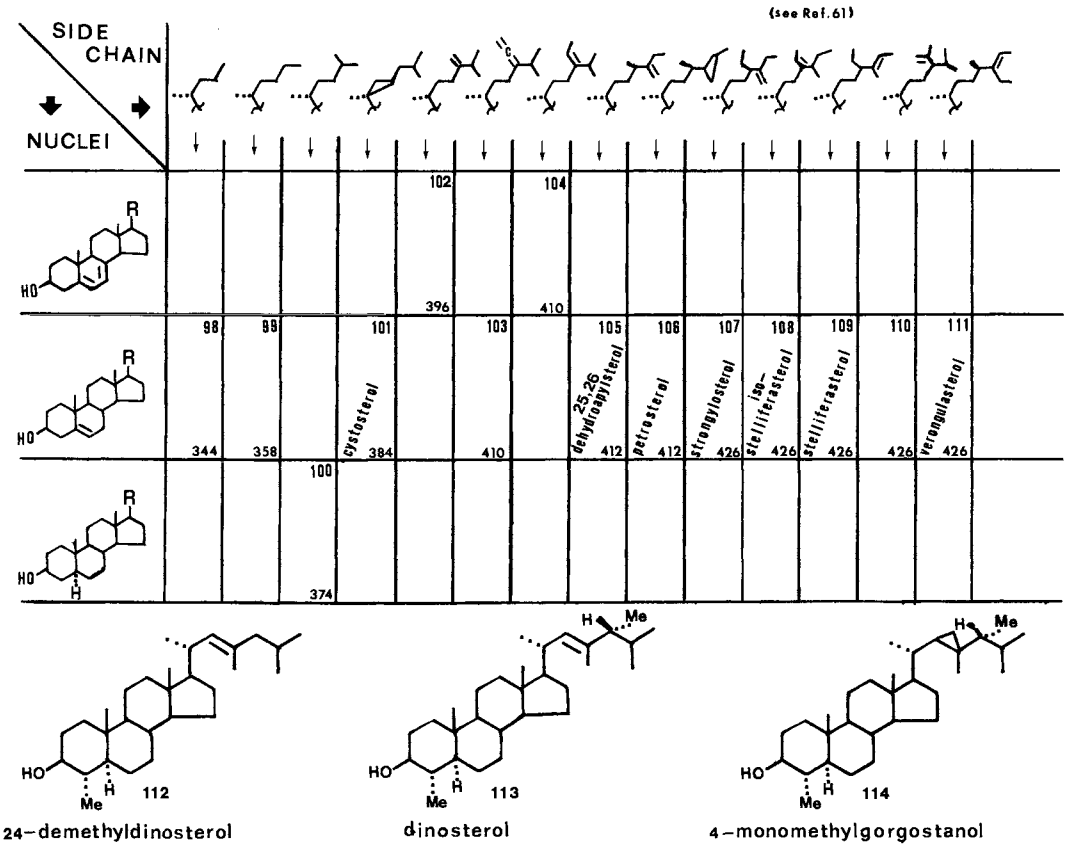
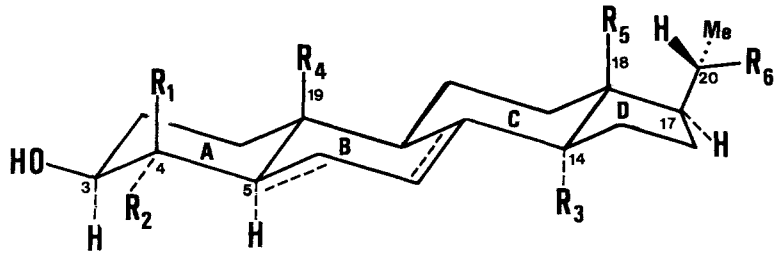
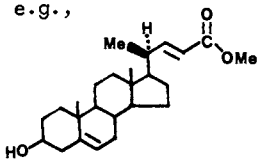
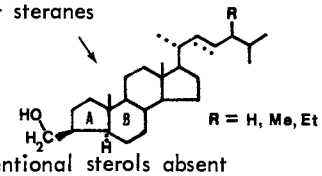


FIG. 2 GENERALIZED STEROL STRUCTURE-FUNCTION RELATIONSHIPS AND SEVERAL MARINE EXCEPTIONS



#	REQUIREMENT	MARINE EXCEPTIONS	REFERENCES requirement/exception
1.	Planar Steroid Nucleus -trans ring junctions A/B, B/C, C/D	5β-stanols, 32, 86 -minor constituents -known bacterial metabolites Probably no membrane role	(4-7)/(51)
2.	20R and 17β configurations	20-iso steroids, sargasterol (C-20 epimer of 68) now thought to be an incorrect assignment -ptilosarcus steroids e.g.,  probably no membrane role	(9)/(19, 29)
3.	3β-hydroxy group (3α-hydroxy, 3-keto and C-3-fatty acid esters are not functional)	A-nor steranes  R = H, Me, Et -conventional sterols absent -steranes occur at "sterol" concentrations Probable membrane role	(4-7)/(14, 30)
4.	4,14 demethyl nucleus i.e., $R_1 = R_2 = R_3 = H$	dinosterol 113 major sterol of a marine dinoflagellate Probable membrane role	(24-26)/(31)
5.	Nucleus must possess C-18 and C-19 angular methyls, i.e., $R_4 = R_5 = Me$	19-nor stanols, 12, 22, 29, 42, 47, 56, 87, 84 -conventional sterols absent -occur at "high" sterol concentrations Probable membrane role also 30, 57, 65 (minor sterols)	(24-26, 33)/(32, 51)
6.	Side chain must contain greater than 5 carbons i.e., $R_6 \geq C_3$	Marine sterols 1-8 -minor components Probably no membrane role	(4-7, 34, 35)/(37)
7.	Side chain must contain less than 10 carbons, i.e., $R_6 \leq C_{10}$	C_{30} marine sterols 91-97, 103-111 -major, sometimes only sterol, -high concentrations Probable membrane role	(4-7, 35, 36)/(13-22)

The first rule specifies that a sterol must possess a planar nuclear structure to function in membrane stabilization. Marine sterols (e.g., 86, Table 1) are known which possess the 5β-stanol nuclear structure of coprostanol (32, Table 1), but these sterols occur at minor or trace levels, are known bacterial metabolites and are, therefore, probably waste products which do not function in the membrane systems. Closely related to the requirement of a planar steroid nucleus is the second requirement that a functional sterol must possess the R configuration at C-20 (Ref. 9,28). The 20S configuration forces the sterol side chain out of the plane of the nucleus. Sterols with this "unnatural" configuration do not function as membrane stabilizers (Ref. 9). Marine sterols with the 20-iso configuration were once thought to exist (sargasterol, the 20-iso epimer of fucosterol 68), however that assignment is now thought to be incorrect (Ref. 19). The existence of marine steroids which have the 20-iso configuration [e.g., (20S)-chola-5,22-dien-24-oic acid methyl ester, from Ptilosarcus gurneyi (Ref. 29)] has been established, although the highly functionalized side chains of these compounds strongly indicate that these steroids are not membrane constituents. The third sterol structural requirement for function in membrane stabilization is the 3β-hydroxy group. Steranes, 3α-alcohols and 3-keto containing sterols are not functional (Ref. 4-7). The marine sponge Axinella verrucosa contains A-nor-steranes instead of sterols (Ref. 30). The occurrence of these unusual compounds at concentrations comparable to those of sterols in other animals, and the lack of conventional sterols in this sponge, strongly suggest a membrane role for the compounds.

The interactions of the sterol polar substituent with other membrane constituents are not clearly understood (Ref. 5). Studies of the naturally occurring A-nor-steranes and possibly various synthetic analogs could provide some insight into those interactions. The fourth requirement for sterol membrane function was mentioned earlier, that is, that sterols possessing 4 or 14 methyl groups remaining from the primary squalene cyclization products, lanosterol or cycloartenol, do not function well in membrane stabilization. Furthermore it was predicted (Ref. 24) that methyl sterols would not be found functioning as sterols in organisms. Minor levels of methyl sterols are commonly found in organisms since methyl sterols are the biosynthetic precursors of the sterols. However, the major sterol of the marine dinoflagellate, Gonyaulax tamarensis, has been found to be a 4α-monomethyl sterol, dinosterol (113) (Ref. 31). The high, "structural" concentration of dinosterol in G. tamarensis suggests a role for dinosterol in the cellular membranes of that dinoflagellate.

The phospholipids of G. tamarensis may differ from those used in current model membrane studies since methyl sterols do not function well in those systems. If a structural complimentarity does exist between dinosterol and other membrane lipids in G. tamarensis, a careful study of that complimentarity might result in a better understanding of the interactions between the polar "head group" regions of the various membrane lipids.

The fifth sterol structure/function requirement is that the nuclear demethylation process (of requirement 4) should not extend to the C-18 and C-19 angular methyls of the sterol nuclear "β-face" (Ref. 24-26). However, 19-nor-stanols have been discovered in the marine sponge Axinella polypoides (Ref. 32). Again, the absence of conventional sterols and the high structural concentrations of these 19-nor-stanols suggests a structural role of these sterols in the cellular membranes of this sponge. It has been suggested by Huang (Ref. 27,33) that the β-face of sterols interacts preferentially with the unsaturated fatty acid chains of membrane phospholipids whereas the sterol α-face interacts with saturated fatty acid chains of membrane phospholipids. The adjacency of the C-19 sterol angular methyl and the Δ^9 fatty acid-phospholipid double bond was suggested by Huang to be important in the β-face interactions. The presence of 19-nor-stanols in A. polypoides suggests that an examination of the phospholipid-fatty acids of that sponge (if the sponge does in fact contain phospholipids) might reveal a complimentary structural perturbation at C-9 on the fatty acid chain.

The final structural requirement for sterol membrane function concerns the side chain and is perhaps the most vague of all the generalized requirements (Fig. 2). Experimental studies have shown that sterol side chains must contain more than five carbon atoms (Ref. 34), preferably eight carbon atoms (Ref. 35), and not more than nine or ten (Ref. 36). In Table 1 many examples of sterols with side chains apparently too short to be functional (1-8, 98, 99) or too long to be functional (C_{10} and C_{11} side chains as specified in the table) are shown. Furthermore, marine sterol side chains exhibit a great variation in structure (summarized in Fig. 1).

We recently considered in detail the possible origins and functions of the short side chain marine sterols (Ref. 37). These sterols with biosynthetically unusually short side chains (fewer than the eight carbon atoms expected for primary squalene cyclization products) occur in a wide range of invertebrates but at very minor concentrations (<5% of the sterol mixtures). Careful evaluation (Ref. 37) suggests that these minor sterols may arise by an in vivo autoxidative process and are therefore probably not membrane constituents. In contrast the marine long side chain sterols almost undoubtedly play a structural role in the cellular membranes of marine organisms. This is because many times the C_{29} and C_{30} marine sterols are major sterol constituents, occurring at the high concentrations necessary for a structural

membrane component. Many of the unusual C_{29} and C_{30} marine sterols have been isolated from marine sponges (e.g., aplysterols 77 and 90, stelliferasterol 109, verongulasterol 111, isostelliferasterol 108 and strongylosterol 107 - Table 1) (Ref. 22). Sterols with synthetically extended side chains do not function well in mammalian-type model membrane systems composed of phospholipids typically found associated with cholesterol (Ref. 36). This suggests that the natural marine sterols with extended side chains may occur in membrane systems composed of phospholipids (or another class of polar lipids) which differ from the ones found in mammalian membranes. Furthermore, these sterols almost invariably possess a "cholesterol" nucleus which suggests that the structural perturbations in the membrane phospholipids might be expected to occur in the terminal fatty acid chains of the hypothetical sponge phospholipid rather than in the polar "hand-group" area. The discovery (Ref. 38) of unusually long fatty acids (C_{24} - C_{30}) in many marine sponges may be an example of the kind of complimentary structural variations suggested above.

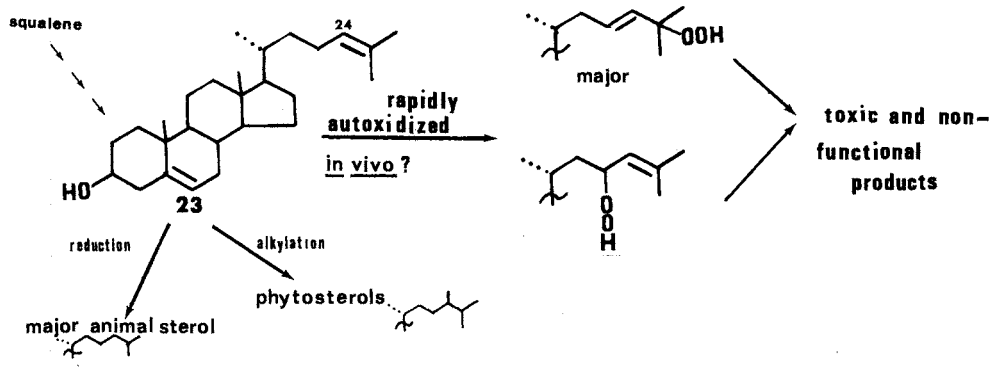
Conversely C_{29} and C_{30} side chain alkylated sterols do not function well in certain other marine animals which apparently require C_{27} sterols. These marine invertebrates have evolved enzymic mechanisms of sterol side chain dealkylation (Ref. 15), a mechanism common in insects. It is interesting that certain minor marine sterols (103) are inhibitors of C-24 dealkylation (Ref. 39), while others are possibly dehydrogenase inhibitors (59) (Ref. 40). These C-24 sterol dealkylations may be a reflection of the sixth sterol structure-function requirement in Fig. 2. However, it seems clear that a modified rule will be developed for organisms possessing sterols with C_9 - C_{11} side chains.

It is significant that certain marine organisms possess C_{27} sterols (15-17) which are apparent C-25 rather than C-24 dealkylation products of C_{28} sterols. There is nothing evolutionarily sacred about the C_8 side chain of cholesterol and removal of alkyl (methyl) groups attached to C-25 could simply be another means of shortening a sterol side chain. Alternately, if we take the view that sterol side chain modification represents a "fine tuning" of the structure for a specific but presently not understood structural compatability with other membrane constituents then in light of the large number of elaborately alkylated marine sterol side chains, it is not surprising that dealkylation at C-25 has evolved as a means of modifying a sterol structure.

One way of testing the functional specificity of sterol side chain structures is to take the approach that Bloch used (Ref. 24-26) with the 4,14 methyl sterol biosynthetic precursors, that is, test the relative membrane functionality of the various sterol side chain biosynthetic precursors. As discussed at the beginning of this section, the apparent complexity of the biosynthetic sequences (Ref. 22) leading to certain marine sterol side chains may be functionally significant.

These apparently complex and specific biosynthetic processes may reflect a very real requirement for the specific structure generated. Such an hypothesis is clearly open to experimental testing with biosynthetic precursor sterols or synthetic analogs, but it should now be clear that such studies are only valid in the organism or in model membrane systems derived from the lipids of the organism in which the sterol is found.

A final feature of sterol side chain biosynthesis which has interested us since we began to consider (Ref. 37) in vivo autoxidation as a possible source of the marine short side chain sterols is the apparently ubiquitous metabolism (either reduction or alkylation) of the Δ^{24} double bond present in primary squalene cyclization products, lanosterol and cycloartenol. We have found that desmosterol (23) and other sterols containing a Δ^{24} double bond (even Δ^{24}-5β-cholestene) are autoxidized much more rapidly than other side chain unsaturated sterols (e.g., 60 and 68). The initial autoxidation products are Δ^{23}-C-25 and Δ^{24}-C-23 hydroperoxides which give rise to polar products which are known to be either toxic, biosynthetic inhibitors, or non-functional in membranes. Desmosterol seems to function well in membrane systems that require C_{27} sterols. Perhaps the universal metabolism of desmosterol to either side chain saturated or alkylated analogues is a reflection of the susceptibility of the Δ^{24} bond to natural oxidative attack.



FURTHER BIOLOGICAL IMPLICATIONS

We will turn now to the second question which was posed earlier: Why are marine organisms such superb sources of unusual sterols as compared to terrestrial organisms? The answer is really quite simple, but yet has profound implications in all other areas of natural products chemistry. Chemical taxonomists hold that the phylogenetic diversity of organisms is generally reflected by a corresponding basic chemical diversity. This should be particularly true in the "lower more primitive" groups where the phylogenetic differences are the greatest. The ocean is a much richer source of these diverse and primitive groups compared to the land. A careful consideration of the sterol composition (Table 3) of the kingdom Metazoa (the true multicellular animals comprising about 30 phyla) reveals that whereas the sterol compositions are many times very complex, their complexity almost certainly arises from sterols obtained from the Protist (Fig. 3) (eukaryotic microorganisms) groups either through the food chains (Ref. 41) or through symbiotic associations (Ref. 42), and that the Metazoans either do not possess de novo sterol biosynthetic machinery, or biosynthesize only cholesterol. It is significant that the Metazoans are thought to have arisen from a single ancestral Metazoan. In contrast, the extraordinarily diverse biochemistry (Ref. 43) of species within the kingdom Parazoa (sponges or Porifera) which are thought to have extremely diverse polyphyletic origins (Ref. 44), seems to reflect their apparent polyphyletic diversity. In the sterol field the sponges have been an exceptionally good source of novel sterols (with sterol 12,22,29,42,47,56,58,59,64,76,77,84,85,90,96,97 and 103-111 in Table 1&2 coming exclusively from this group). The structure (Ref. 44) and mode of feeding (Ref. 45) of sponges is such that their membrane surfaces are truely immense compared to other organisms, and since sterols are membrane constituents, the multitude of sterol structures could also reflect the over-riding importance of membranes in the life of the sponges.

An extension of the question concerning the abundance of unusual sterols in marine sources is: How many new marine sterols will be found in the future? Is the field nearly exhausted or is it likely that many new sterols will be found? These correlative questions can be approached from two angles (i) biosynthetically - how many sterols are possible? or (ii) biologically - how many organisms are left to examine, that is, how good is the present survey? We attacked the first question several years (Ref. 46,47) ago by using a computer program to generate all the possible sterol side chains and then to "prune" the number to those which are "biosynthetically reasonable". By this pruning process less than 1800 3β-hydroxy sterols with side chains ranging from 7-11 carbons were generated, which is certainly formidable but by no means unmanageable. It is important to note that we were concerned primarily with side chain generation and used the set of sterol nuclei which were known at that time; obviously, the discovery of any new nuclear structures, which is certainly possible (Ref. 48), would add to this number. Since our predictions of the possible new structures, over a dozen new sterols have been discovered. The fact that all these structures were contained in the list of predicted structures lends some support to the actual existence in nature of biosynthetic constraints used in our generation (Ref. 46,47) of the list. However, a great many novel structures predicted by the computer evaluations have not yet been discovered, and if only a small percentage of these predicted structures are found, it would certainly include some very important new sterols.

If we turn to the question of how good the existing survey of the sterol compositions of marine species is, we find a similar situation, that is, many marine organisms which have a good potential of containing novel sterols have never been examined. This is in spite of the fact that the marine sterol field is perhaps the oldest and best studied branch of marine natural products (Ref. 48). The most thoroughly studied kingdom of marine organisms, the Metazoa, contains less than a thousand analyzed species (Table 3). When it is realized that there are approximately 200,000 species of named marine invertebrates and a vast number of species which have not been described (Ref. 49), the number of analyzed Metazoan species is seen to be quite small. However, what is more important is that the reported analyses of the marine Metazoa do not even constitute a good survey of that group. Out of 31 described phyla of marine invertebrates, not a single species of 19 entire phyla have analyses reported in the literature (Ref. 50). Another important consideration is that many of these analyses were performed prior to 1960 (Ref. 48), and therefore without the aid of gas-liquid chromatography (glc), mass spectrometry (ms), nuclear magnetic resonance (nmr), or high pressure liquid chromatography (hplc). Since marine sterol extracts are often complex mixtures which require the above techniques (Ref. 51) for a reliable analysis, many of the old results must be considered unreliable (an important example of this problem is aplysterol (90) mistaken for β-sitosterol (80) in the older literature (Ref. 52)).

Although the bulk of sterol analyses of marine organisms have concerned a limited number of metazoan phyla, the Protists, for reasons discussed above, are probably the true sources of any unusual sterols discovered in the Metazoa. Therefore, it is most pertinent to consider the extent of the marine sterol survey among the Protist divisions. The kingdoms of living marine organisms are depicted in Figure 3 along with plausible phylogenetic interrelationships (Ref. 53) between the Protist divisions. Figure 3 together with Table 3 illustrate the extent of the marine sterol survey among the Protist divisions, and delineate the most important marine divisions. The only marine protist divisions for which reasonable surveys

TABLE 3

SUMMARY OF MARINE METAZOAN STEROL ANALYSES (see Ref. 62)

Phylum	Class	Approx. Number of Living Species	Marine	Marine Species Analyzed
Porifera[g]		5000	*	1.8%
	Calcarea	400	100%	0.5%
	Hexactinellida		100%	W
	Demospongiae		>90%	2%
	Sclerospongiae		100%	0
Cnidaria		9000	*	0.7%
	Hydrozoa	3000	>90%	0.1%
	Scyphora	200	100%	3.5%
	Anthozoa	6000	100%	0.9%
Mesozoa		67	100%	0
Platyhelminthes		15,000	*	0
Gnathostomulida		125		0
Nemertea		700	>90%	0.2%
Acanthocephala		800	+	0
Rotifera		2000	**	0
Gastrotricha		500	**	0
Nematoda		10,000	**	0
Nematomorpha		50	6%	0
Kinorhyncha		100	*	0
Entoprocta		125	>90%	0
Annelida		8600	65%	0.1%
Echiuroidea		70-100	100%	0
Sipunculida		275	100%	0
Mollusca		106,000	54%	
	Gastropoda	90,000	48%	0.15%
	Bivalvia	15,000	87%	0.3%
	Cephalopoda	600	100%	1.0%
	Scaphopoda	350	100%	0
	Aplacophora	250	100%	2.5%
	Monoplacophora	10	100%	0
Tardigrada		350	**	0
Arthropoda		830,000	†	
	Chelicerata	52,000	**	††
	Pycnogonida	500	100%	0
	Crustacea	27,000	*	0.2%
	Labiata	760,000	0.5%	0
Priapulida		8	100%	0
Brachipoda		260	100%	0.38%
Phoronida		20	100%	0
Bryozoa		4000	100%	0.03%
Chaetognatha		50	100%	0
Echinodermata		6200	100%	1%
	Crinoidea	650		0.5%
	Asteroidea	2000		1.5%
	Ophiuroidea	1800		0.2%
	Echinoidea	850		1.2%
	Holothuroidea	900		0.8%
Pognophora		80	100%	0
Hemichordata		80	100%	0
Chordata		44,000		
	Tunicata	200	100%	0.6%
	Cephalochordata	13	100%	0
	Vertebrata	42,000	†	

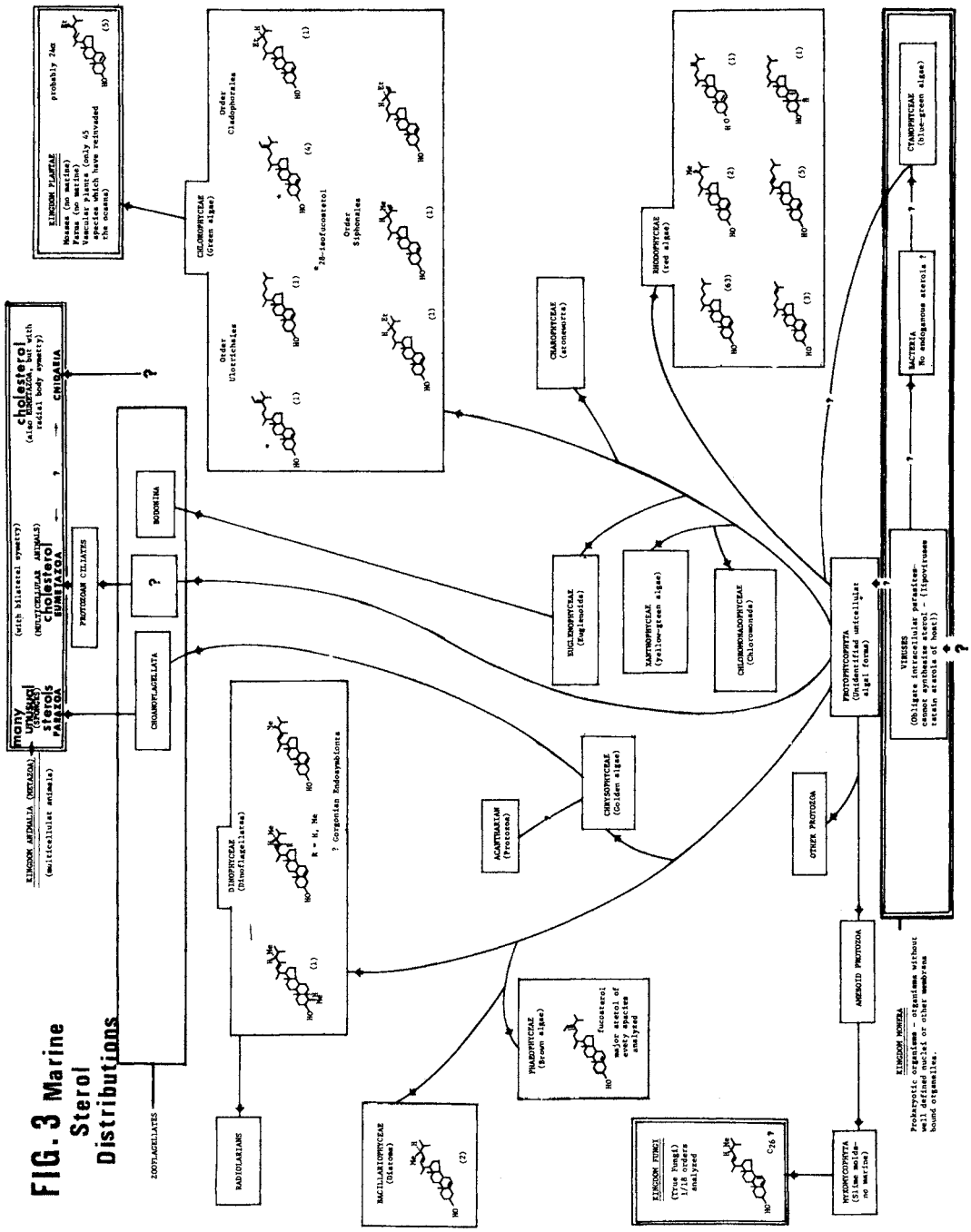


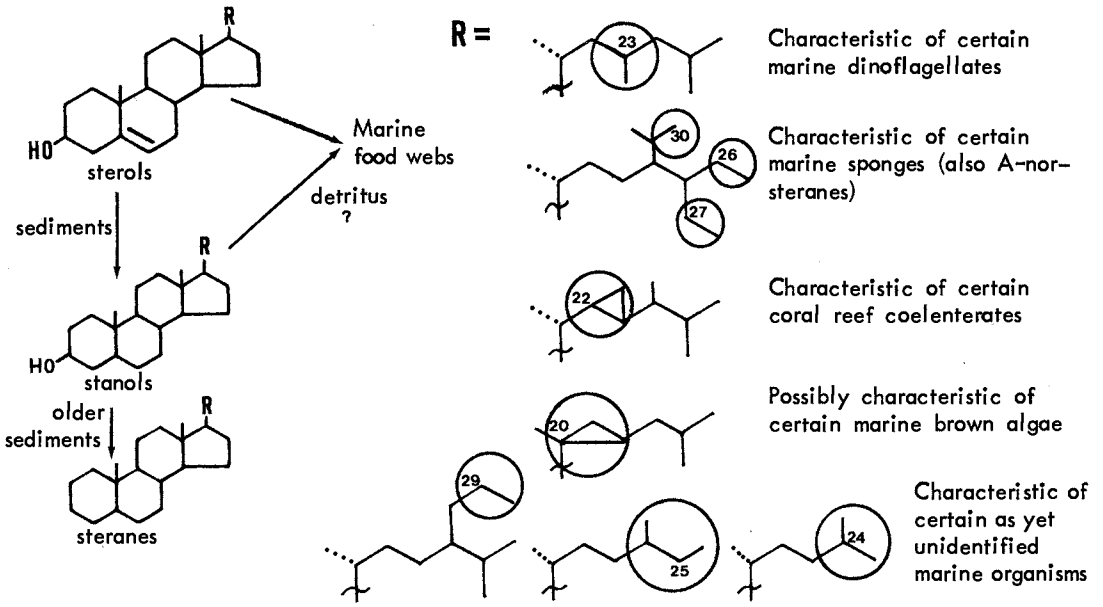
FIG. 3 Marine Sterol Distributions

TABLE 4

SUMMARY OF MARINE PROTISTA STEROL ANALYSIS (see Ref. 62)

DIVISION	CLASS	APPROX. # OF SPECIES	% MARINE	% MARINE SPECIES ANALYZED
Protozoa (Phylum) (one celled animals)		28,000	†	0
Pyrrophyta				
	Dinophyceae (dinoflagellates)	>1100	93%	0.2%
	Cryptophyceae (cryptomonads)	100	†	0
Chrysophyta				
	Chrysophyceae			
	Golden Algae	650	20%	0
	Coccolithophorids	200	96%	0
	Bacillariophyceae	6000-10,000	30-50%	0.1%
Euglenophyta				
	Euglenophyceae (euglenids)	400	3%	0
Xanthophyta (yellow-green algae and chloromonads)		600	15%	0
Chlorophyta				
	Chlorophyceae (green algae)	7000	13%	1%
	Charophyceae (stonewarts)	76	13%	0
Phaeophyta				
	Phaeophyceae (brown algae)	1500	99.7%	3%
Rhodophyta				
	Rhodophyceae (red algae)	4000	98%	2%
Myxomycota		450	0	-

Fig. 4 Several Unique Marine Sterol Alkylation Patterns of Phylogenetic Ecological and Geochemical Importance



exist are the Phaeophyceace (brown algae) and Rhodophyceace (red algae). Every species of the hundred brown algae examined (Table 4) contains the single sterol fucosterol (68 in Table 1) as the major sterol. A similar statement can be made for the red algae: the majority of red algae contain cholesterol (25 in Table 1) as the major sterol, while several species contain other well known, widely distributed sterols (Fig. 3). Since every order of both the red and brown algae has been surveyed, the discovery of new sterols as major components in species in these divisions seems less likely than for the numerous unstudied divisions. However, it is important to note that the analysis of the minor and trace sterol components of marine sterol mixtures is a new area with the potential of making possible the detection of new sterols in nearly any marine extract and we have concentrated on this aspect of marine chemistry in our laboratory (Ref. 54). Indeed, recently a new sterol, presumably 101 in Table 2, was discovered as a minor component of the sterol mixture of a brown alga (Ref. 55).

Many protist divisions have so far received little or no attention from marine natural products chemists. The prime reason for the neglect of these important groups is that they are unicellular and require culturing. However, the few cases where cultured species have been analyzed have resulted in significant new discoveries. A prime example is dinosterol 113 (4α,23,24R-trimethyl-5α-cholest-22-en-3β-ol) isolated as the major component of a marine dinoflagellate (Ref. 31). The occurrence of this sterol at concentrations indicative of a structural role in cellular membranes raises important questions (noted in the previous section) concerning the possible but unexpected (Ref. 24,25) role of 4-monomethyl sterols in biological membranes and is also an example of the very unusual C-23 alkylated sterols.

A possible means of selecting protist divisions for sterol analysis would be to use the plausible phylogenetic interrelationships suggested by biologists as an indication of which divisions (based on possible affinities with groups known to possess unusual sterols) are most likely to yield novel sterols. An example would be to trace back the supposed ancestry of a group, such as the Parazoa (sponges), which is presently known to contain interesting sterols. Such a strategy (Fig. 3) would suggest that certain protozoa (the choanoflagellates) and even coccolithophorids (marine Chrysophyceae) may have a high potential of possessing interesting sterols. Likewise, protozoans (such as the Radiolaria) which may have arisen from the Dinophyceae would also deserve careful investigation.

Another means of selecting protist groups for analysis would be to consider more carefully the more phylogenetically plastic groups. The Chlorophyceae or green algae is such a group compared to the red or brown algae considered above (Ref. 56). The Chlorophyceae contains a number of orders for which no analyses are reported. The several analyses that have been reported have yielded a number of interesting results, for example the discovery of codisterol (43 in Table 1) in Codium fragile (Ref. 57). The variety of sterols discovered so far among the Chlorophyceae perhaps demonstrates the divergence within this division compared to the red and brown algae where a single sterol predominates (Ref. 56), and suggests that further analyses of Chlorophyceae species may result in the discovery of important new sterols.

A third criterion which could be used to select marine protist groups for sterol studies would be to consider those groups most important to the oceanic biomass. The diatoms (Bacillariophyceae), dinoflagellates and coccolithophorids all contribute greatly to the biomass and economy of the seas. This fact alone, particularly when viewed in the light of important contributions through the marine food chains justifies the study of these groups. Finally, careful analysis of the unstudied groups depicted in Fig 3 would have a good potential for discovering new sterols, and may help in clarifying the phylogenetic interrelationships within the various divisions. Such considerations have been of use in evaluating some phylogenetic interrationships within the Parazoa (Ref. 58). Furthermore, if specific sterol structural features can be shown to be restricted to a certin group of marine organisms then the occurrence and abundance of sterols with that specific structural feature may well be a good means of assessing the contribution of a particular protist to a specific food web. If C-23 alkylation is shown to be restricted to certain dinoflagellates, then C-23 alkylated sterols will be a natural tracer for the presence of dinoflagellates in a food web. The reason sterols may be used as these kinds of tracers is that sterols are very stable molecules, and there is evidence that sterols are carried intact through marine food webs (Ref. 59). An extension of this concept is that if certain sterol alkylation patterns found in various marine organisms (e.g., C-23 alkylation in dinoflagellates, C-26 and C-27 alkylation in certain marine sponges, or gorgosterol-like stanols (78, 91) in coral reef coelenterates possessing zooxanthellae) are shown to be restricted to specific groups (which now seems likely), then those structural features which surely evolved many hundreds of millions of years ago may have given rise to "chemical fossils" (stanols and steranes (Ref. 60)) with the same alkylation patterns as the corresponding unique sterols. The presence of such stanols and steranes in geochemical deposits would then be an excellent indication of the origin of these deposits (Fig. 4).

CONCLUSION

The marine sterol field appears to be far from exhausted, and many new discoveries are not only possible but very likely. It also seems probable that many of the unusual marine sterol structural features will be proven to be characteristic of specific groups of organisms and may therefore be useful in evolutionary phylogenetic considerations. The unique nature and intrinsic stability of certain of the marine sterol structural features may also make them useful as natural tracers which will help clarify complex marine food webs, and as chemical fossils to help determine the origins of geochemical deposits. Finally, the existence of many of the unusual marine sterol structural features recently encountered and many even more unusual ones that we believe will be uncovered as a result of ongoing investigations will probably initially create something of a dilemma for membrane physiologists and biophysicists. However, in the long run, careful investigations of the membrane properties of these unusual sterols may well offer the key to the resolution of the general role of sterols in biological membranes.

ACKNOWLEDGMENTS

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61. In Tables 1 and 2, bold face numbers are numbers used (and underlined) in the text to refer to the indicated structures. Small numbers are the molecular weights of the represented sterols. The common trivial names are also given for structures which possess them. See References 13-22, and 50 for information and original references for any specific structure.
62. The symbols used in Tables 3 and 4 have the following meanings: * mostly marine; + all parasitic, but parasitic marine forms known; ** mostly non-marine; † large numbers of marine and non-marine; W one marine species analyzed; †† two marine species analyzed; ▮ considered by many to be a separate kingdom, the Parazoa.

PROBLEMS OF REGULATION, SYNTHESIS AND METABOLISM OF NATURAL COMPOUNDS

A. S. Sadikov

Institute of Bioorganic Chemistry of the Academy of Sciences, UzSSR, Tashkent, GSP, USSR

Abstract - Regulatory systems in the living cell depend on protein-nucleic acid interactions the state of which, in turn, depends on relationships between low-molecular compounds, proteins, nucleic acids, various membrane components and other substances. The study of chromatin functions by the technique of reconstruction from its components has revealed the presence of certain nonhistone groups of proteins active on the specific template of a given nucleohistone. Nucleohistone containing a partially denatured histone preparation has an abnormally high template activity. Changes in the structures histones caused by denaturation in the process of their isolation in different experiments were registered by CD, DOR, H and ^{13}C-NMR-spectroscopy. It was shown that compounds of plant origin influence the state of protein-nucleic acid systems in some cases directly, in others indirectly. This capacity is manifested in antiviral, antitumor and immuno-depressive activity of gossypol and its derivatives. The present paper lists our findings characteristic of the specific effect of extracellular receptors on nucleoprotein systems. Specific relationships between alkaloids and eukaryotic cell proteins lead to various biological effects in membranes, ribosomes, polysomes and chromatin, whereas a multitude of conformational forms and interconversions of alkaloids ensures a high rate of specific regulation at the compartmentalysis level.

Clarification of the nature of regulation in the functioning of various intracellular molecular systems and co-ordination of the stages in their performance constitutes one of the key problems which have arisen with the development of molecular biology and bioorganic chemistry. Expedient and efficient utilization of cell resourses saves energy and substrates which otherwise would have been used up for the synthesis of compounds not used in the process at the given moment. Untimely formation of these compounds may have a toxic effect, but nevertheless the presence of competing metabolic processes accounts for the effect of physiologically active substances. As illustrated by numerous experimental data the regulative reactions have a common chemical basis - the specific interactions between biopolymers: nucleic acids and proteins, proteins of various types (basic, phosphoproteins, glycoproteins, lipoproteins), polysaccharides, lipids, which are realized in various cellular organellas. Specific modification of the state of these systems can be achieved through enzymatic or non-enzymatic interaction with a fixed set of effectors. At the Institute of Bioorganic Chemistry of the Uzbek Academy of Sciences comprehensive studies are being conducted on the high and low molecular compounds which participate at various levels in the regulation of genetic information realization processes. These studies include chemical and structural investigation as well as works on clarification of functional role of substances. One of the subjects of research is chromatin isolated from various types of tissues. Researchers at present know some of the peculiarities in the structure of most of the chromatin components. However the biological function of the latter can be judged only on the basis of their structural peculiarities in the course of interaction with other components (Fig. 1).

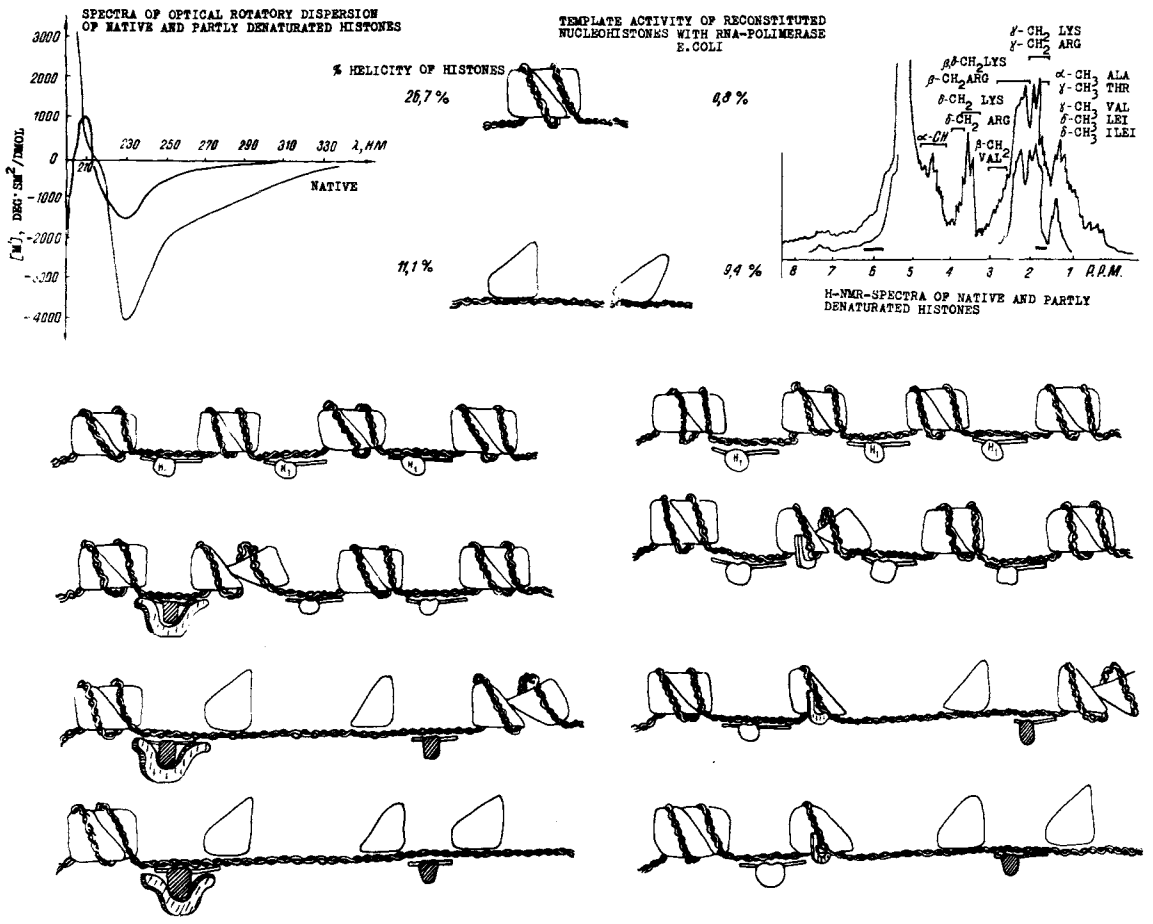

Fig. 1. The model of cooperative activation of genom.

Assuming that in the course of transcription the histones do not dissociate from the DNA complex, it is natural to take the various conformation states of the nucleohistone complex as factors inhibiting genetic information read-out (Refs. 1, 2). The conformation of various sites of the nucleohistones may vary due to the enzymatic modification of the histones, their formation into complex with regulation molecules, including non-histone proteins, as well as due to influences facilitating alteration of the secondary structure or the octamer-forming histones and the tertiary structure of the nucleosome. Experiments with nucleohistone reconstruction from their components have shown that reconstructed nucleohistones incorporating partially denaturated histones possess greater matrix activity as compared with free DNA. Conseauently, if it is not the free DNA, that is the matrix in the transcription process, but the nucleoprotein complex (the basic component of chromation), then the activly transcribed part of the genom differs in its conformation state. There may be suggested a model of cooperative activation of a nucleosome group, which forms part of the genom locus, with the help of an effector - a non-histone protein possessing specific affinity with the nucleohistone. The labile and strongly bounded non-histone proteins differ in degrees of affinity with various chromatin components (Fig. 2). The labile bound non-histone proteins, which are the most active elements in matrix synthesis, contain fractions possessing affinity with all the chromatin components (Refs. 3,4), whereas the less active bound chromosomic proteins are enriched with fractions which are bound only with nucleohistone. There apparently exist regulative nuclear proteins which do not interact with either the DNA or the histones, but which interact selectively with the nucleohistone complex of the chromatin. This has been illustrated by the method of affinity chromatography of various non-histone chromosomic protein fractions by affinity with immobilised DNA, histones and nucleohistone complex.

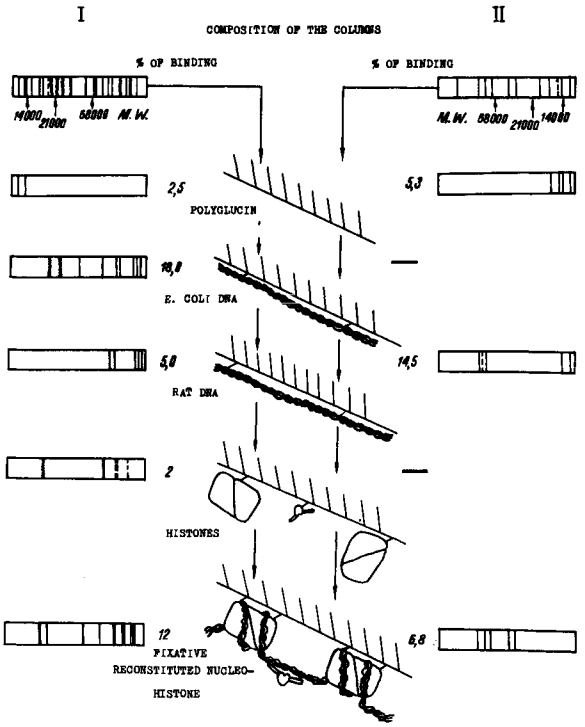

Fig. 2. Affinity of non-histone proteins to the different immobilised components of chromatin.
I. SDS-PAAG-electrophoretic patterns of the whole and immobilized-sorbent-binding 0,35m-NaCI Soluble nonhistone chromatin proteins.
II. SDS-PAAG-electrophoretic patterns of the whole and immobilized-sorbent-binding 1-3 m NaCI-soluble nonhistone chromatin proteins.

Conformation data on various proteins which determine the functional state of cells depending on the phase and features of their interaction with nuclear components and other heterogeneous components of the cell, make it possible to undertake a functional classification of proteins: replicative, transcriptional, epigenetic, structural, information, processing proteins, modifying enzymes, receptor system proteins. The active role played by membranes in regulating the functions of various subcellular organellas and molecular systems has prompted researchers to study such problems as details of their molecular structure, the role of membranes in providing and regulating the delivery of substances into and from the cell, the nature of excitation and the mechanism of enzymatic regulation of bioenergy processes within the membrane. These and other problems have been studied on the basis of such protein compounds as post- and pre-synaptic active toxins, various cyto- and cardio-toxins, numerous phospholipases isolated from various venomous animals and also alkaloids and their derivatives. The venom of arthropoda, as that of elapidae, is marked for the fact that along with high-molecular weight toxins it contains low-molecular weight components which play an important role in intoxication and present unique models of the "intimate" mechanism of action within the living organism. Some of the enzymes contained in the venom of arthropoda bring about various pathophysiological changes in the organism. For instance, the venom of snakes (Ref. 5) and arthropoda contains a group of vasoactive polypeptides - kinins which are produced enzymatically in the organism (Fig. 3). We have discovered and studied the highly active components of the kinin system in the venom of a number of arthropoda, particulary the centipede, which split off the residues of arginine and phenylalanine with arginine from the C-terminal of bradikinin. The specific activity of this kininase is 1,000 times higher than the activity of kininase contained in the venom of other animals. We studied the structural features of the active surface of a number of post-synaptic membranes, in particular the enzymes (acetyl and butyril-cholinesterase and choline receptors) by the method of sub-

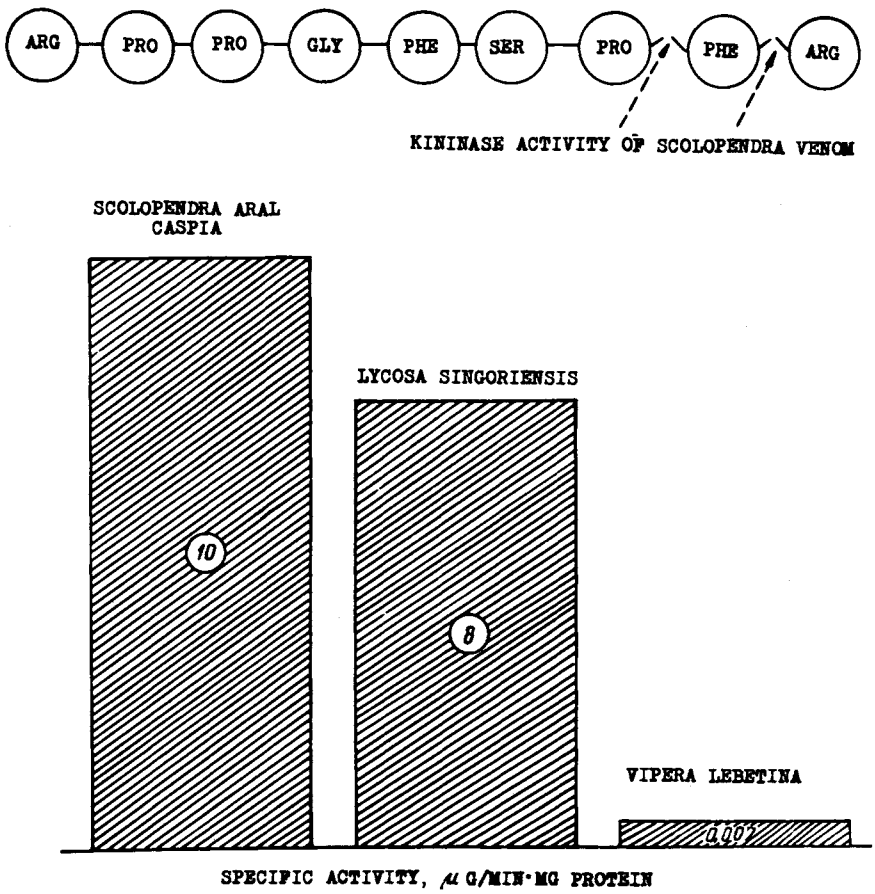

Fig. 3. Relative activity of kininase from different animals.

strate-inhibiting analysis with the application of a wide range of alkaloid derivatives of the piperidine, dipiperidile, quinolisidine series (Refs. 6, 7). In comparing data on the rate of cholinesterase hydrolysis and the nicotinomymetic activity of the quarternary derivatives of substituted piperidilecholines it has been established that the hydrophobic surrounding of the esterase section in the nicotine choline receptor resembles the hydrophobic region at the esterase centre of butyrilcholinesterase. At the same time the hydrophobic framing of the receptor anion centre bears similarity with the non-polar sections at the acetylcholinesterase anion centre (Refs. 8, 9). No comprehensive studies have hitherto been conducted on the biological functions of many compounds isolated from plants (e.g. alkaloids, terpenoids, polyphenols, flavonoids, lactones, coumarines, catechins, antocyanes, saponines, etc.). It seems it is easier to clarify the biological function of some new protein with a very complicated structure, than to explain the expediency of one or another type of low-molecular weight natural compounds, which at first glance are not essential for the normal functioning of the plant organism. This is connected with their chemical polyfunctionalism and the difficulties entailed in the controlled development of an extra-cellular model system where their functions could be fully manifested. It may be assumed that some of the above-mentioned compounds serve as regulators of biochemical processes in membranes, cytoplasma, mitochondria, chloroplasts, nuclei, etc. It is important to note that their appearance is closely connected both with the metabolic transformations of the basic metabolites and the state of the cell high-molecular weight components. Natural polyphenols of Malvaceae plants which we have isolated and studied, constitute a big group of chemically polyfunctional compounds which have been shown tautomeric (Refs. 10-12), the formation of various quinoide and condensed forms (Ref.13) (Fig. 4). Although the physiological role of phenol compounds in the life of plants has not been studied well enough, we believe that their most probable function is that of selective dissociation on oxydation phosphorylation. The existence of such a variety of dissociation agent forms in plants is conditioned by the speci-

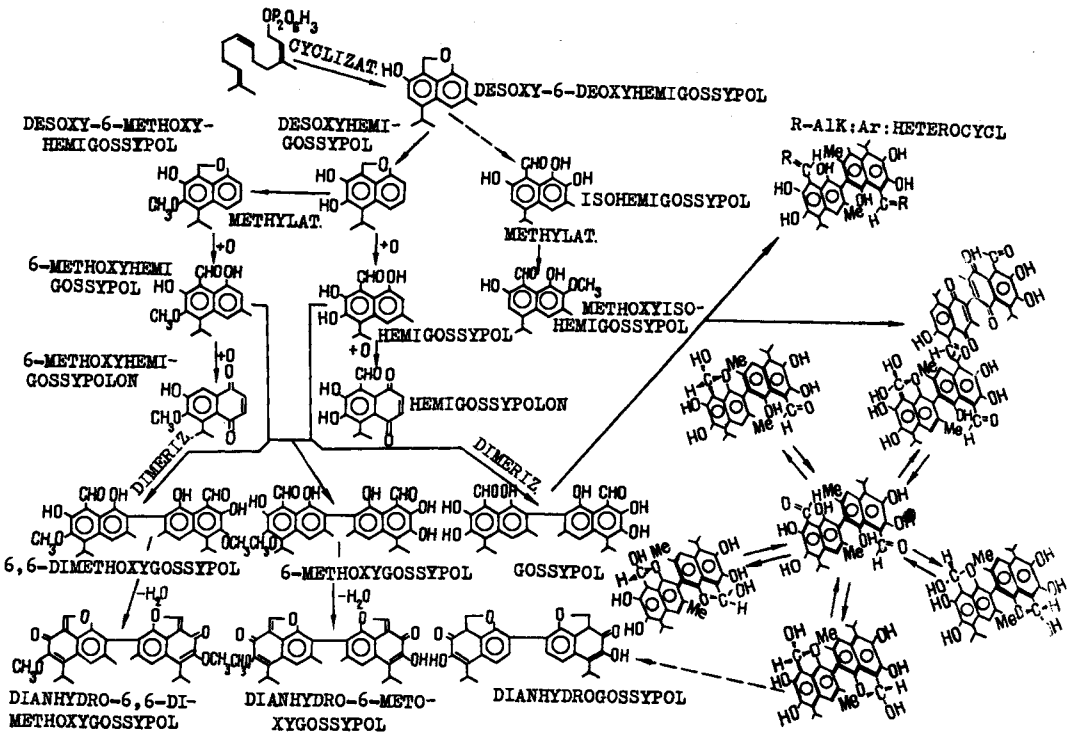

Fig. 4. Structures and biochemical relationships of phenolic compounds of cotton (gossypium)

fic features in the process of photophosphorylation, which depends on the type of tissue, the age, nutrition, growing conditions, etc. (Ref.14). All this requires a sufficiently large set of specific regulators. This may be corroborated by the clearcut dependence of the dissociation action of substances on the structure of polyphenols which has been observed during in vitro experiments. Apparently the specific features of interaction of polyphenols and their various modifications with proteins, their capacity for fine regulation of phosphorylation process explain attempts to find a dependence between the resistance of plants to various pathogenes and the accumulation of phenol compounds in their tissue. From cotton plants infected by V.dahliae we have isolated several phenol structure compounds which are not present in healthy plants and are formed only in affected ones. The degree of their phytotoxic action with regards to the fungus V.dahliae depends, to a great extent, on the structure (Refs.15-18). Active dissociation agents of oxydation phosphorylation display the greatest toxic action (Ref.19). The polyphenol compounds of natural origin in the heterological systems are specific physiologically active substances which affect the state of protein-nucleic systems directly in some cases and indirectly in others. This peculiarity is manifested in the antiviral,antitumoral and immuno-depressive activities of gossypol and a number of its derivatives. It has been established that gossypol, batriden, megosin, gossypolon and several other compounds have a wide range of activity on DNA and RNA-containing viruses (Ref.20). Gossypol, megosin and gossypolon display a relatively high inhibiting rate on the growth of tumours of various aetiology and in tissue culture of a stable cellular strain of cancer of the human pancreas sustained in a monolayer. In both cases the greatest effect is observed with gossypol (Ref.21). Further research in the physiological activity of gossypol and its derivatives has shown that some of them are active immunodepressor which inhibit humoral and cellular immunity of animals in the transplantation of tissue, kidneys and lung. It has been established that these compounds inhibit antibody formation much more effectively than conventional immunodepressors (Ref.22). This wide range of biological activity of natu-

ral polyphenols and their derivatives coupled with their relatively low cytotoxic action leads us to the assumption that all these forms of activity share a common feature - influence on the regulation of a certain type of replicative intracellular processes. The latter depend on the derepression of a group of genes which determine the phasespecific synthesis of proteins essential for the segmentation of cells, i.e. they interact directly with DNA and RNA. The physiological action of the polyphenols is also phase specific. Hence it has been assumed that being introduced into the organisms these substances produce a synthesis of proteins which slow down the transition of animal cells into the segmentation stage and inhibit the replication of viruses while retaining the ability of the cells to normal

TABLE 1. Interferon inductive activity of gossypol and GSN in chick embryo fibroblast cultures

Gossypol concentration mg/ml	Contact time of preparation with cells, hr	Interferon titers PIU 50/ml	GSN concentration, mg/ml	Contact time of preparation, with cells, hr	Interferon titers PIU 50/ml
250	24	10-15	250	24	640
250	4	20	250	4	15-20
250+	24	18-100	250+	24	2500
125+	24	120-140	125+	24	320
Poly (I) Poly (C)	6	64-128			
Sindbis virus	5-7 PFU/cell	512			
VSV	5-7 PFU/cell	320			

+ - inassistance of DEAE - dextran;
VSV - vesicular stomatitis virus.

functioning at the stage of rest G_0. Such discriminatory proteins, as is known, are interferons. Within the culture of chicken embryo fibroblasts, human embryo fibroblasts as well as in mice and monkey cells gossypol and megosin produce a high interferon titer (up to 640 UE%/ml) (Ref. 23 Table 1, 2). Synthesis of interferon is a process which includes many stages. It is put into operation by various inductors and is connected with the suppresion of interferon genes. After the transcription and translation of informational RNA for the interferon the latter comes out from the cells into cultural fluid (Refs. 24, 25I. It should be recognized that genes responsible for the interferon formation (localized in human cells in 2 and 5 chromosomes) and RNA, possessing genetic information for its synthesis, are the major parts of chain reaction resulting in production of interferon (Ref. 26). Isolation of these informational RNA for interferon with matrix activity is of prime importance in realization of molecular nature of biosynthesis of this inducible protein. The study of transcription and translation mechanisms in homological and heterological cell system as well as in a protein synthesizing system without cells would apparently give the answer for the question concerning the role of carbon component in the molecules of interferon to show its activity and specific difference (Refs, 27, 28). After the GSN treatment of cell culture we succeeded in isolating functionally active parts of informational RNA of interferon which formed specifically different interferon after translation in homological and heterological cell systems as well as in protein synthesizing systems without cells out of wheat germs. A lot of research work was conducted on the state of protein biosynthesis and nucleic acids in cells after treatment by gossypol and its derivatives. Gossypol displayed a high inhibitory rate on incorporating of marked predecessors of RNA and DNA biosynthesis (H^3 - uridine and H^3-timidine) depending on concentration. GSN in similar concentrations inhibited incorporation of marked predecessors 3,5 - 4,5 times higher than

TABLE 2. INTERFERON PRODUCTION IN MAMMALIA CELLS INDUCED BY GSN

Cell type	GSN concentration mg/ml	Interferon titers, PIU 50/ml
HEF	125	200-300
	250	640
Human	250	320
tonsils	125	80
	62	60
Human	250	80
spleen	125	20
	62	10
Human	250	40-60
thymus	125	20
	62	10-20
FS-4	250	500-640
	125	100-250
L-1210	250	280-320
BSC-1	250	320-480

HEF - human embryo fibroblast

gossypol. Induction of interferon synthesis by gossypol derivatives makes it possible to determine the range of their biological activity. Indeed, blocking by interferon of replicative protein synthesis at the segmentation stage slows down the transition of the cells to the segmentation phase, i.e. it leads to suppression of proliferation. This is observed when interferon affects normal cells and other tumour cells (Ref. 29). The suppression of tumour cell segmentation by interferon depends on the following factors: whether the tumour cells possess functioning interferon receptors and whether these modified receptors produce a synthesis of inhibitors of transcription and translation of replication nuclear proteins (Refs. 30, 31). The action of polyphenols and their derivatives as immunodepressors can also be explained by the induction of interferon and its effect on immunological processes. Suppression of the first stages of immunocyte proliferation by interferon prevents their differentiation. Eventually this effect is registered by the sharp decrease of antibody synthesis and the number of effector cells (Ref. 32). On the contrary, interferon induction at stages, when antibody synthesis cells and effector T-cells have already appeared, prevents their transition to the segmentation stage. This in turn is conducive to retaining the number of functioning immunocytes and augmenting their functions at the stage of rest, i.e. increases the number of tissue compatibility antigens on the surface of lymphatic cells. This deduction is confirmed by the changing nature of immune response when gossypol and megosin are introduced before and after immunization of the test animal. Polyphenol induced interferons stabilize the state of rest of animal cells that prevents synthesis of early virus proteins directly interacting with DNA and RNA which are identical functionally with the cell proteins at the segmentation stage (Refs. 33, 34). There are several facts to corroborate this assumption. The most interesting one (effective induction of anti-viral activity with 95-98% suppression) is observed only in those cases when the interferonogenis added to cells that had already formed a monolayer, i.e. had entered the stage of rest. When the interferon or interferonogen is added to the cells immediately after trypsinization of the culture, reproduction of viruses is suppressed by only 20-40%. Apparently this is due to the fact that part of the cells have already entered the stage of segmentation (Ref. 35). Thus the above-mentioned data characterize the specific action of the extracellular effectors on the nucleoprotein systems whose changing state leads

to the realization of part of the information related to the genom locus which is responsible for cell differentiation. Apparently these effectors have no direct relation to the protein whose synthesis they induce and hence by analogy they can be classified with molecules of plant and animal hormone effectors, cyclic nucleosides, prostaglandins etc. The directional expression of differentiation genes results in the biosynthesis of a big set of various chemical compounds. At present we know the structure and properties of over 150,000 natural compounds which are formed by differentiated cell systems of various organisms. The biological function of toxins, flavonoids, pigments, polyphenols, hormones and alkaloids which are concentrated in various cellular formations is usually displayed when these natural compounds emerge into the cytoplasm or the extra-cellular space due to changing structure or their modification. In studying a number of Central Asian plants we have isolated about 200 alkaloids, investigated their metabolism, biological action, spatial structure and dynamics of conformational changes (Ref. 36). A comparison of base structures in related plant species show that their spatial forms and modification vary over a wide range as a result of biochemical oxydation, dehydration, condensation, hydrolysis, etc. The alkaloids we have studied may be divided into two classes which differ in the nature of activation of secondary metabolism: alkaloids which originate with the introduction of aromatic amino-acids (phenylalanine, thyrosine, triptophane) (Ref. 37) and alkaloids which originate with the introduction of aliphatic aminoacids (lysine, ornithine) (Fig. 5, 6).

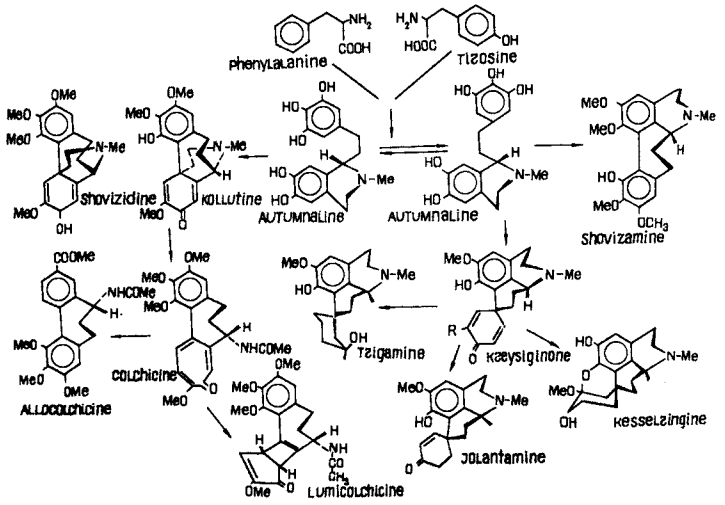

Fig. 5. Biogenetic pathways of alkaloids of the plants wurmbacoideae.

The secondary metabolism of aminoacids produces big groups of alkaloids of the dipiperidile, isoquinoline, quinolisidine series. One of the characteristic structural features of these bases is their participation in complex inversion-conversion transformations in solutions which we have studied in detail by the ^{13}C-NMR-spectroscopy methods (Ref. 38). For instance, stable trans-quinolisidine systems in concentrated hydrochloric acid solutions display two forms of lupinine (Fig. 7), more than three forms of pachycarpine (Fig. 8), three forms of sophoridine (Fig. 9). Determination of the spatial structures of these conformers has made it possible to approximate the mechanism of their transformations which includes inversion processes of nitrogen atom and cycle conversion (Refs. 38-39) (Fig. 10). Alkaloids maintain a specific interaction with various proteins and nucleoprotein system of eucaryotic cells which results in interesting biological processes. Colchicine, colchamine, vinblastin, vinchristin and other phenylalanine-thyrosine metabolism bases, as well as quinolisidine alkaloids of the matrine group form compounds with a number of nucleic proteins and inhibit mitosis. The antimitotic activity of the above-mentioned bases is very high. Inhibition of 50% in tissue culture is achived at picomol concentrations of alkaloids. On the other hand, natural bases which are produced from the metabolism of nicotinic acid, lysine, ornithine and thyrosine have specific interaction with the cholinergic (nicotine), acetyl-choline dependent (pilo-

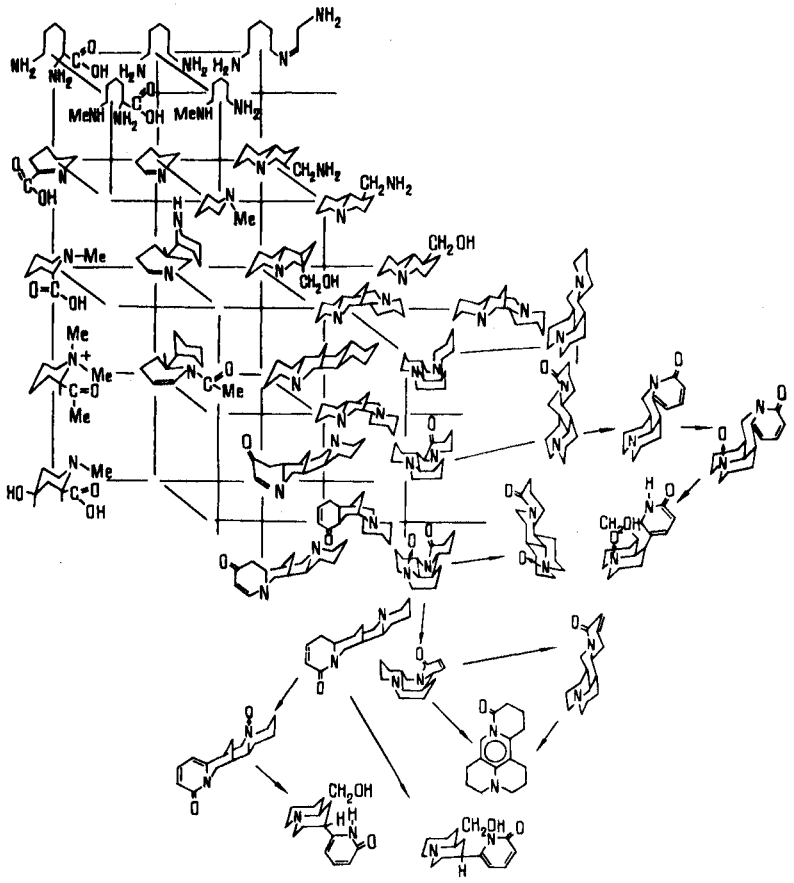

Fig. 6. Biogenetic pathways of lysine into quinolizidine alkaloids and its interconversion.

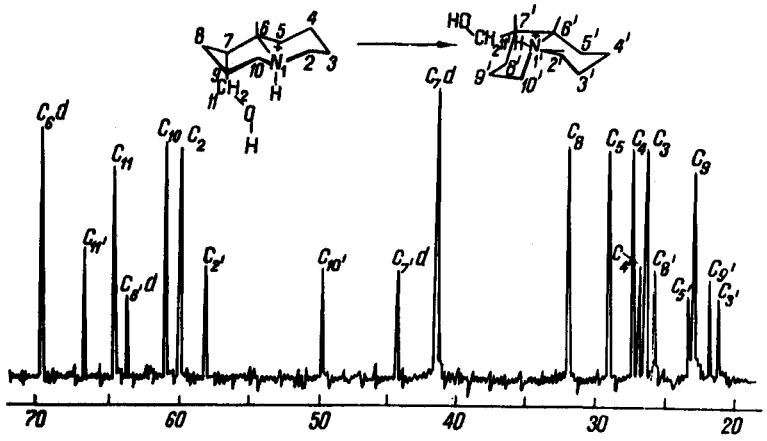

Fig. 7. Conformational equilibrium shift of lupinine (trans quinolizidine system) in conc. HCl solutions.

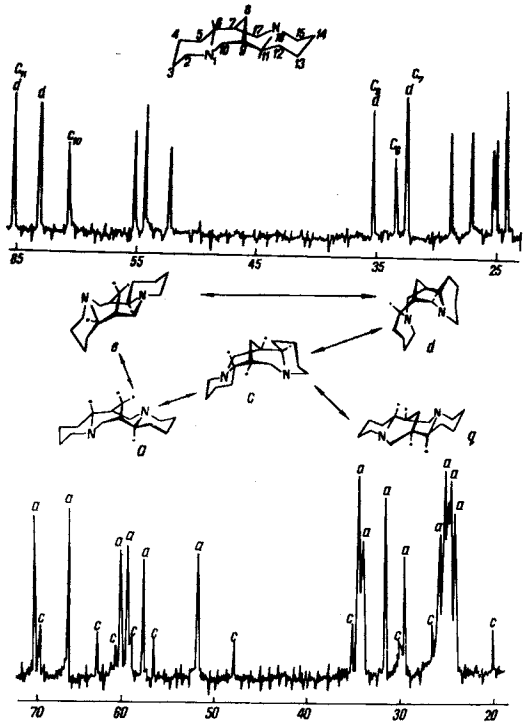

Fig. 8. Conformational transitions of sophoridine.

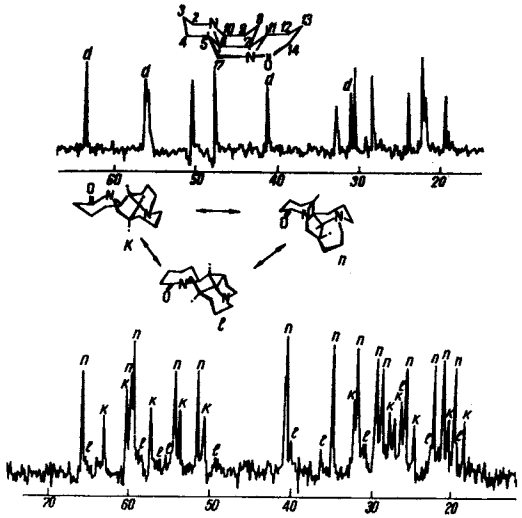

Fig. 9. Transformation of 1,3 nonane system of pachycarpine due to protonizations.

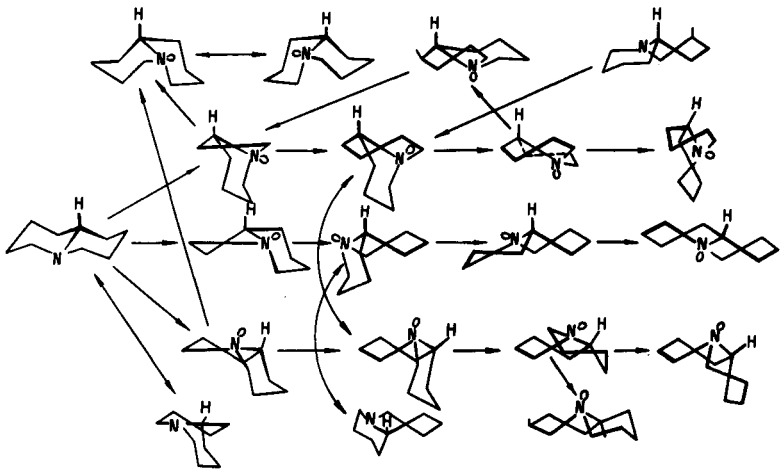

Fig. 10. Inversion - conversion transformations of quinolizidine systems.

carpine, etc.) glycine (arsenic), and prostaglandin (morphine) receptors. More often than not the alkaloids are bound to these receptor 5-10 times stronger than their corresponding effectors. Apparently the most interesting feature of this series of alkaloids which contain bi-cyclic quinolisidine, decahydrochinoline and pyrrolisidine systems is their ability to selective interaction with ribosomes of animal cells. This results in the effective inhibition of various stages of elongation in the process of protein synthesis at the polysomes. Multiplicity of conformation forms of quinolisidine bases may be of great importance for regulating this type of interaction inasmuch as change in the state of the medium often results in a sharp displacement of conformation equilibrium. Apparently this property of quinolisidine bases may be important for directional compartmentalysis and controlled emergence of quinolisidine alkaloids into the intracellular and intercellular space. Accumulation of alkaliods is symbatic to processes of protein biosynthesis retardation, but is not correlated with the increase in the free aminoacid pool. We believe that the most probable biological function of most of the alkaloids is their regulative role in the process of biosynthesis of certain proteins at the translation level: the alkaloid form bonds with incomplete polypeptide chains of proteins of their operon on polysomes and thus can either inhibit or activate the process. The existence of complicated structural spectra of alkaloids in plants, the diversity of spatial forms and dynamic conformation transformation ensure the high rate and specificity of regulation. In this connection we found it interesting to study the system of enzymes which govern the biosynthesis and interconversion of these complex compounds. Radioactive alkaloids peculiar to the given plant were introduced at certain stage of its development (Refs. 40-42). A rapid redistribution of tagged atoms was observed throughout the entire alkaloid spectrum. The introduction of alkaloid not peculiar to the given plant species showed that these bases took almost no part in the general system of metabolism. These data testify to the high specificity of interaction between alkaloids of the quinolisidine series and enzymes which realize their metabolism. Thus the most interesting and most important intracellular processes are realized with the participation of heterogeneous cellular formations. It remains as the main task to establish the structural aspects (including the conversion dynamics of these systems) which determine the process of recognition or conformity between the components. The solution of this task calls for investigations that would draw a connection between the biological function of the given natural substance and its physiological action on the organism of other species. In our research we proceeded from the assumption that there must exist a certain correlation between these aspects in the functioning of biomolecules. Comprehensive studies in this field will enable researchers to fully understand the regulative mechanisms which determine the state and functioning of living organisms.

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SOME NEW RESULTS IN THE TOTAL SYNTHESIS OF ESTROGENS

A. R. Daniewski and M. Kocór

Institute of Organic Chemistry, Polish Academy of Sciences, 00-961 Warsaw, Poland

Abstract - A review of the methods of the total synthesis of estrogens carried out in our laboratory is given. The first chapter is presenting a modification of Torgov - Ananchenko synthesis leading to 11-keto-estranes. The next chapter describes the application of the coupling reaction between diazoketones and trisubstituted boranes for the total synthesis of estranes, and the last chapter presents a new short and efficient synthesis of these compounds, which has not been published as yet.

INTRODUCTION

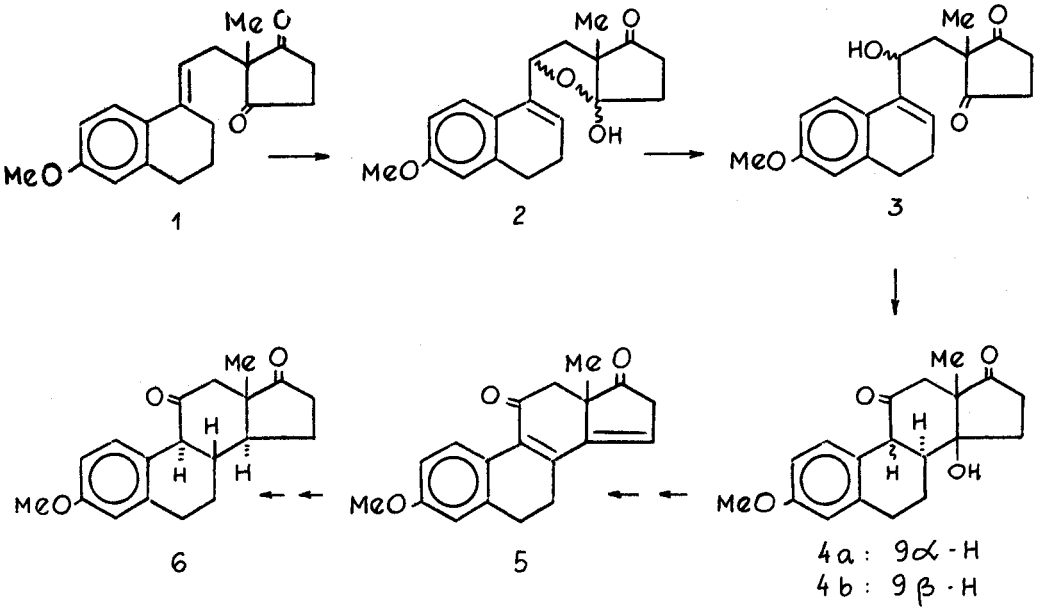
Since the first synthesis of an estrogen i.e. of equilenin in 1939 by Bachmann [1] many new approaches towards the total synthesis of estrogens have been elaborated in many laboratories. Because the estrogens and the androgens are interconvertible, the number of different total syntheses of these compounds exceeds now several dozens. Some of them strike by their genius, the other ones by their elegance or simplicity resp., but only very few of them have found practical i.e. industrial application. Among the latter ones the total synthesis of estrone published by Torgov and Ananchenko first in 1959 [2] is by far the most versatile, and with Gibian modification [3] leading to optically active natural estrogens it is used on industrial scale for manufacturing the oral contraceptives, the anabolic drugs and other biologically active steroids.

TOTAL SYNTHESIS OF 11-KETOESTRANES

In the introductory part of the well known monograph on total synthesis of steroids by R. T. Blickenstaff et al. published in 1974 [4] one can find the remark, that "Torgov and Ananchenko and their colleagues as well as many others have explored this reaction in depth and have found it particularly efficacious for synthesis of aromatic A-ring steroids". Just a couple of months after the appearance of this monograph and later we demonstrated the truth of this remark in a series of our papers published in J. Org. Chem. [5]. An exemplification of this i.e. an efficient synthesis of 11-oxidized steroids is presented on the Scheme 1.

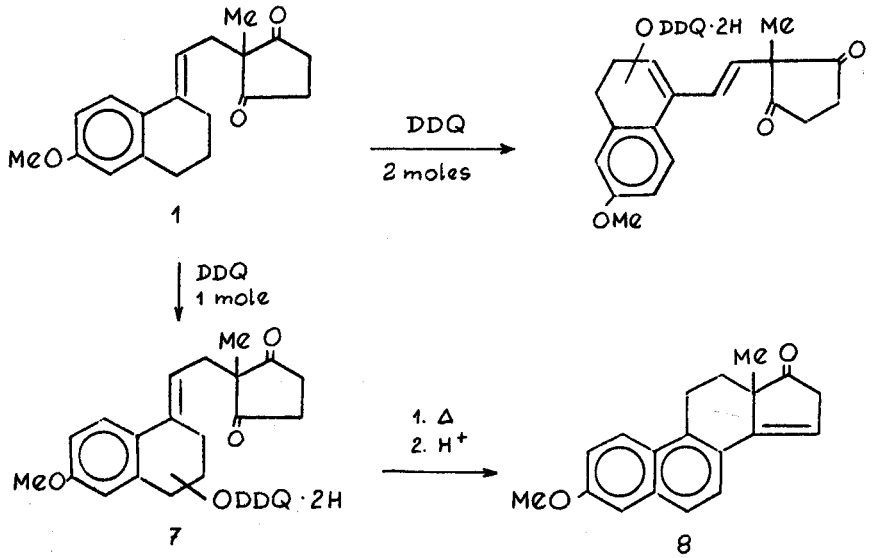
The main feature of this synthesis is the introduction of an oxygen function prior to cyclization of the Torgov dione 1, and the use of Meeerwein reagents i.e. trialkyloxy-borotetrafluorates R_3OBF_4 as cyclization reagent. Although the synthesis gave as the intermediate products compounds 4 with unnatural geometry at C-8, their further transformations could lead to the formation of rac. 11-ketoestrone methyl ether 6, thus offering the possibility to an easy access to 11-oxygenated steroids.

Scheme 1



The same Torgow dione 1 was used for a very efficient and simple synthesis of rac. 14-dehydroequilenin methyl ether 8 as indicated on the next Scheme [6].

Scheme 2

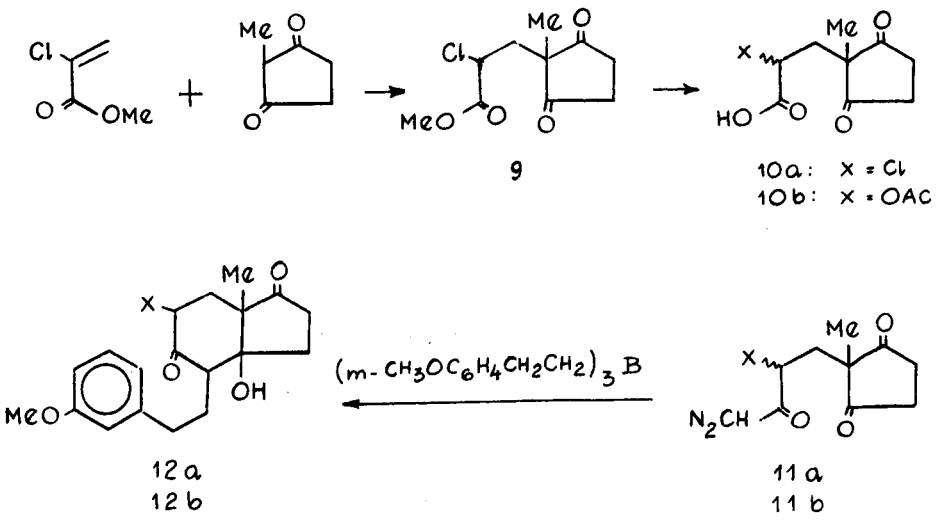


We have tried to carry out these two syntheses with optically active "secolone", obtained by microbial reduction of one keto group in Torgov dione 1; the results were similar but the yields significantly lower [5d].

THE APPLICATION OF COUPLING REACTION OF TRISUBSTITUTED BORANES WITH DIAZOKETONES

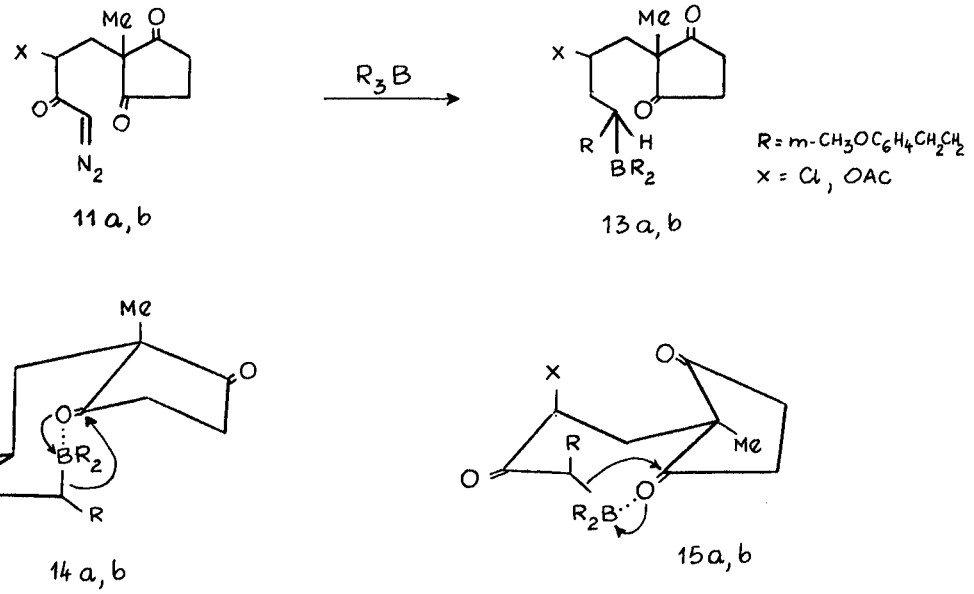
Since on the other hand our intention was to work out a purely chemical synthesis of optically active estrogens not involving any microbial step as in the case of Torgov - - Gibian synthesis, we elaborated an entirely different total synthesis whose goal, however, were initially also 11-oxidized estrogens. As it can be seen very easily this new approach /Scheme 3/ gives on the very beginning of the synthesis a possibility of resolution of the Michael adduct 9 after its transformations into the free acids 10a,b into both enantiomers by standard methods i.e. by salt formation with organic bases. This chance has been exploited by us [7] and we used for further transformations either the acids 10a or 10b resp., which formed insoluble salts with /-/ - ephedrine or the racemates. The other enantiomers 10 were not wasted - by suitable substitution reactions at the future carbon atom C-11 they could be reconverted into the right enantiomers needed for the synthesis described below /Scheme 3/. The acids 10a,b have been first transformed via their acid chlorides with diazomethane into the diazoketones 11a or 11b resp. The crucial step in the further synthesis was now the coupling of the diazoketones 11 with tris-[2-(3-methoxyphenyl)-1-ethyl] borane; a reaction which was applied earlier for the synthesis of ketones from acid chlorides [8].

Scheme 3



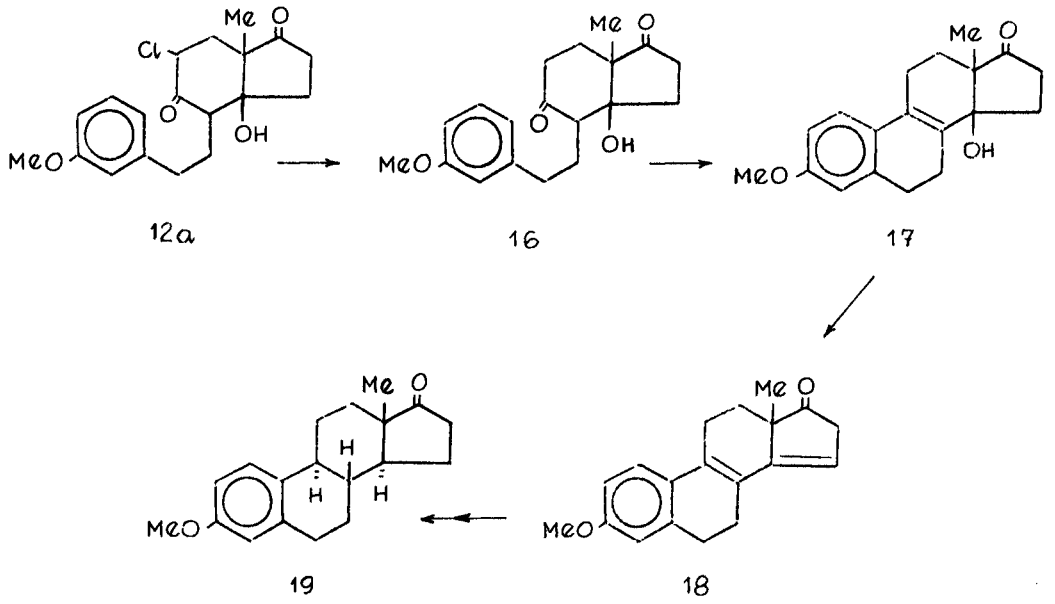
In our hands this reaction did not give the expected chain ketone 30 but the tricyclic compounds 12a,b with the correct geometry at the future carbon atoms C-8 and C-13 and with 14β-hydroxy group. The stereospecifity of this coupling reaction was practically 100%. The mechanism of this two steps reaction involves most probably a preliminary formation of β-ketoboranes 13. These compounds can exist in a number of conformations, from which the most rigid and most stable ones would be those with the coordination of boron with one of the carbonyl groups i.e. 14 and 15 as suggested by N. Cohen [9]. However, conformation 15 is less favourable, because of the diaxial interaction of 11 S substituent with phenylethylene residue in the supposed intermediate.
Thus the role of 11 S configuration in the asymmetric induction is evident, and as the consequence of its influence 14β-OH is formed and the correct geometry at the pro-C--8 and pro-C-13 is created in compounds 12a,b.

Scheme 4



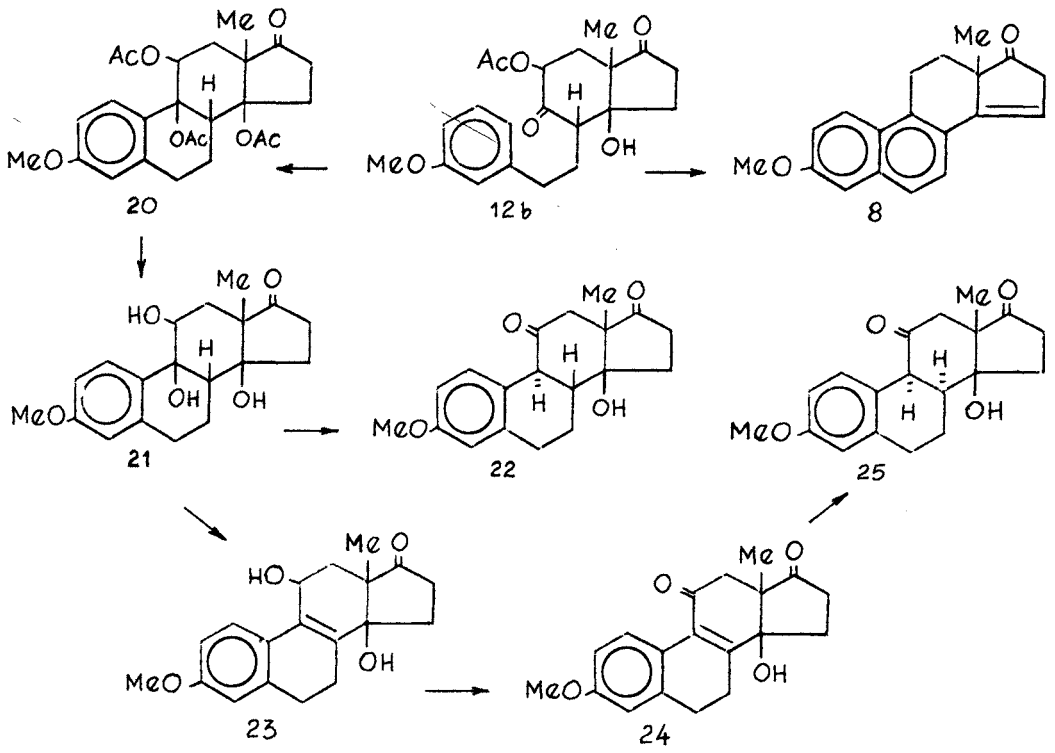
For further synthesis of estrone[10] /Scheme 5/, the chlorine substituent at C-11 has been removed with zinc and acetic acid and the intermediate 16 cyclized in the presence of strong acids, preferentially with 100% perchloric acid in acetic acid to the known Torgov pentaenone 18. In some instances using milder reaction conditions also the isolation of the intermediate 17 in poor yield was possible. The overall yield of this reaction sequence with respect to the resolved or racemic acids 10a was 40% i.e. approximately the same as in Torgov - Gibian synthesis, but our procedure omits the technologically unpleasant step of microbial reduction.

Scheme 5



The strong tendency towards aromatization was visible, when the 11-acetoxyketone 12b was treated with mineral acids -14-dehydroequilenin was formed easily. Treatment of 12b with p-toluenesulfonic acid and acetic anhydride /acetyl p-toluenesulfonate/, however, resulted in a smooth formation of the tetracyclic triacetoxyketone 20 without any noticeable elimination or rearrangement.
The striking difference in the cyclization of 16 and 12b can be explained in the following way:
In compound 16 the attack of phenyl ring on the positive centre at C-9 occurs from the less hindered upper side producing a nonisolable intermediate with 9α-hydroxy group with axial configuration. This intermediate fullfils the requirements for trans-diaxial elimination of water, which really takes place with such a rate that the intermediate 9-OH compound cannot be isolated.

Scheme 6



In compound 12b the 11β-acetoxy group seems to create a great steric hindrance for an attack of phenyl ring from the upper side; consequently it has to occur from the rear, producing a 9β-hydroxy group with equatorial configuration. Therefore instead of water elimination only acetylation takes place and compound 20 is formed.
The latter compound could be converted into desired 11-ketoestrogens in two alternative ways with comparable yields /Scheme 6/.

a/ Alkaline hydrolysis of the acetoxy groups gave a ketotriol 21 which smoothly underwent dehydration with p-toluenesulfonic acid to yield the unsaturated ketodiol 23, whose chromic acid oxidation gave the diketone 24. The catalytic hydrogenation of the latter produced the ketone 25, identical in all respects with the ketone 4a, obtained in our modification of Torgov synthesis. The dehydration of the diketone 24 gave as before the bisunsaturated diketone 5, whose transformation into 11-ketoestrone methyl ether was described before.

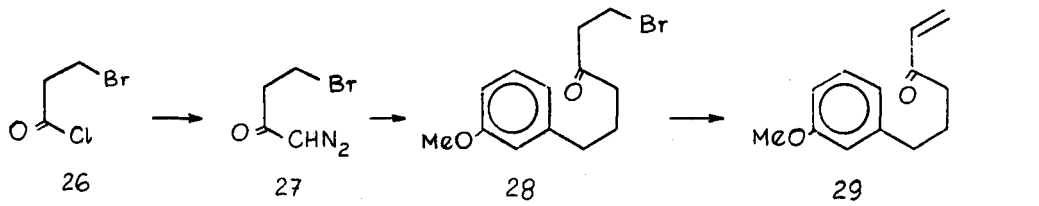
b/ The same ketotriol 21 on treatment with boron trifluoride etherate underwent easily a rearrangement to the new diketone 22 with natural geometry at C-8.

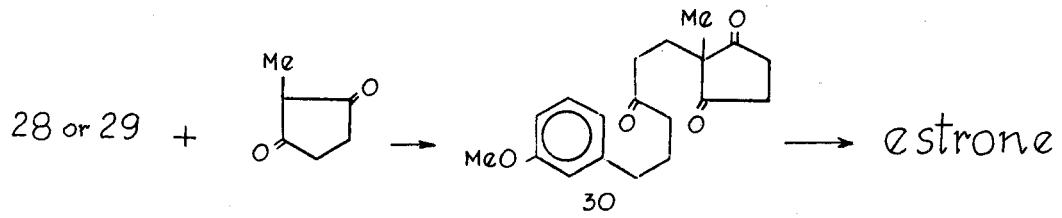
This rearrangement is very characteristic for the vicinal cis-diols, thus additionally proving the configuration of the hydroxyl at C-9 to be beta.

This new method of total synthesis of estrogens and their 11-ketoderivatives has a negligible disadvantage from laboratory scale point of view, which, however, causes some inconveniences on the industrial scale of production. This is the consumption of three moles of m-methoxystyrene per one mole of diazoketone. We found that a great part of m-methoxystyrene can be recovered, when after the coupling reaction the mixture is treated with tert. butylhydroperoxide, because along with 12a,b also 2-(m-methoxyphenyl)- ethyl alcohol can be isolated and reconverted into m-methoxystyrene, but this procedure requires some further studies. The same regeneration procedure was also used in our next nonpublished as yet approach presented on Scheme 7.

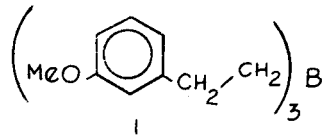
A NEW SHORT AND EFFICIENT SYNTHESIS OF ESTRANES

Again the crucial step in this reaction sequence is the coupling of the easily accessible diazoketone 27 with the same borane as before yielding the monocyclic ketone 28. Although this reaction gives excellent yields, we found it to be less suitable for industrial purpose for the same reason as before i.e. because of the high consumption of m-methoxystyrene and the necessity of its regeneration.
We expected a drastic reduction of the molar amount of the latter reagent by replacing two m-methoxyphenylethyl groups in the borane with catechol. Unfortunately the yield of the coupling reaction with this modified borane was 40% only i.e. half of the yields obtained before.

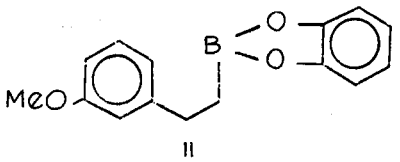




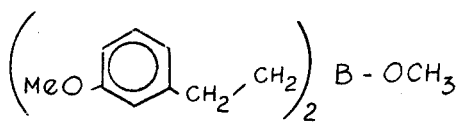
Coupling reagents



I



II



III

Due to an experimental mistake we prepared di-(m-methoxyphenylethyl)-methoxyborane III, and this compound proved to be a very good coupling reagent /70%/, only slightly inferior to I /80% yield/. So, although we were not able to reduce the amount of m-methoxystyrene to 1/3, a 33% reduction is also significant, and the presented method can be applied on industrial scale without the need of recovering the m-methoxystyrene.

Thus the very important intermediate 28 was synthesized. Its further conversion into the steroidal molecule is simple and it is effected by its reaction with 2-methylcyclopenta-1,5-dione under alkaline conditions to give 30. The reaction mechanism can be explained either as the alkylation of methylcyclopentadione with 28 or as Michael reaction of 29 which is easily formed under alkaline conditions.

The intermediate 30 is a symmetric prochiral compound. Its asymmetric aldol condensation in the presence of optically active organic bases has been already described [9] by other authors, so formally the last Scheme presents a new total synthesis of estrone.

We would like to emphasize that this is the most efficient synthesis of estrone, and therefore it can find the practical application not only for the synthesis of estrogens but also for 19-norsteroids, used commonly as oral contraceptives and anabolic drugs.

The Schemes 1 and 5 present the synthesis of 11-ketoderivatives of estrane system with natural and unnatural chirality - it is possible that among these compounds some new hormonal drugs can be found.

Acknowledgement - We would like to thank our colleagues Drs M. Guzewska and B. Aweryn, Mr. D. Jinaraj /NCL, Poona, India/ and our technician Mr. A. Kinowski for their contribution in performing the experiments.

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SYNTHETIC APPROACHES TO 14-HYDROXYSTEROIDS

I. V. Torgov

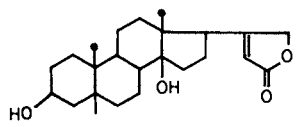
Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow 117988, GSP-1, USSR

Abstract - The synthesis of 14-hydroxysteroids of estrane series by cyclisation of the 2-substituted cyclopentane-1,3-diones or cyclohexane-1,3-diones has been described. Application of this method to 2-aminomethylcyclopentane-1,3-dione allows to obtain steroids with 14,18-azepine ring. Reduction of some hydroxysteroids leads to the compounds with 8β, 9α, 14β-configuration characteristic of cardenolides. A scheme for preparation of steroids with 17β-butenolide chain on the model of dehydroepiandrosterone has also been presented.

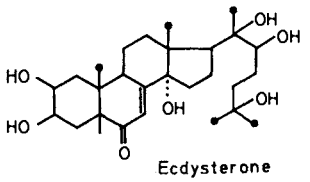
At present practically all the principal types of steroids can be prepared by total synthesis, although only very few procedures are capable of competing with partial synthesis from naturally occuring substances. Such are the total syntheses of compounds of the estrone series and of 19-nor-steroids; and the future is bright for the total synthesis of androstane derivatives. There is less certainty about other classes of steroids.

I should like to draw your attention to the synthesis of 14-hydroxysteroids among which there are such interesting and important compounds as cardenolides, ecdysons, bufadienolids and some alkaloids.

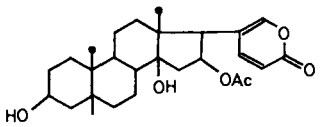
Scheme 1



Digitoxigenin



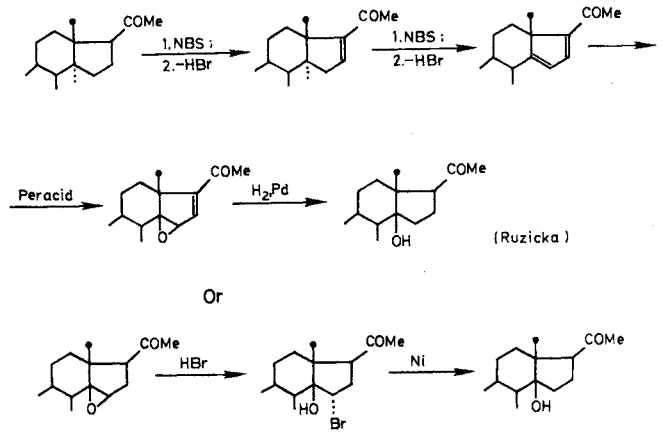
Ecdysterone



Bufotalin

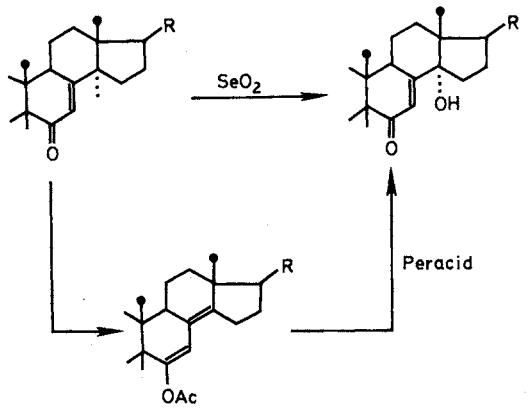
There are three general ways for introducing a hydroxy group into position 14. The first one prepared by Ruzicka starts from $\Delta^{14,16}$-dienes which (after selective hydrogenation or directly) are subjected to epoxidation (Ref.1); the resultant 14,15β-epoxide is reduced or transformed to the 14β-hydroxy-15α-bromide (by treatment with HBr) and then debrominated (Ref. 2) (see Scheme 2). This method always led to 14β-hydroxyderivatives and consequently was used in the total or partial synthesis of cardenolides.

Scheme 2



The second method involves direct incorporation of the hydroxy group by allylic oxidation of Δ^7-steroids with selenium oxide or with peracides (Ref. 3). It has found application in the partial synthesis of ecdysone and its analogues.

Scheme 3



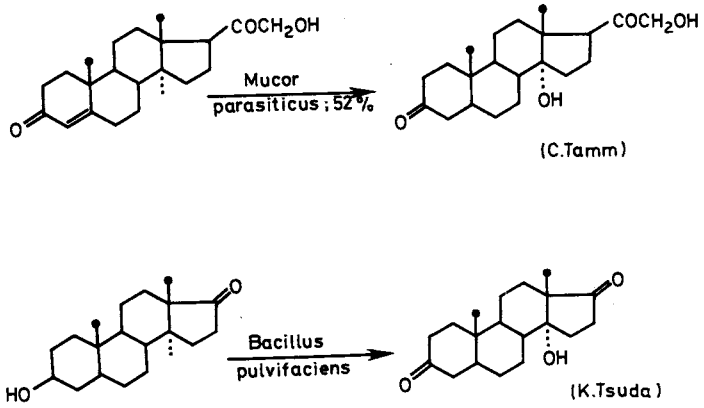
Both methods proved to be useful for transforming pregnane or cholestane steroids.

The third - microbiological method was quite successful in the case of desoxycorticosterone (Ref. 4) and less so in the case of testosterone and dehydroepiandrosterone (Ref. 5) (see Scheme 4).

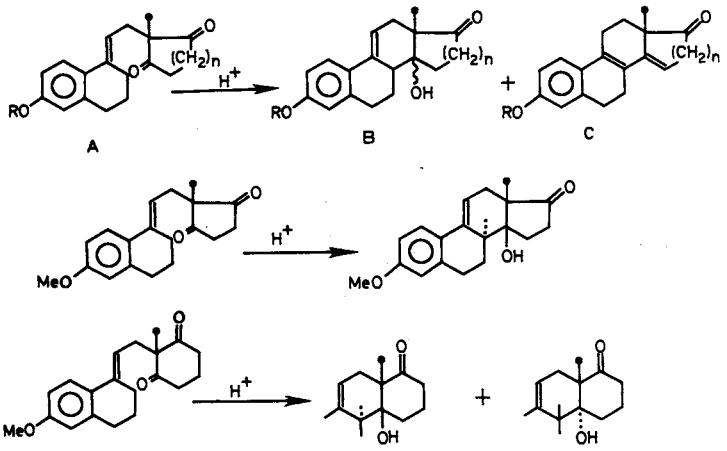
Some time ago we proposed (Ref. 6) a rather simple way of preparing 14-hydroxysteroids of the estrane and D-homoestrane series, namely, cyclization of secodiketones of type (A). Besides 14-hydroxycompounds (B), bisdehydrocompounds of type (C) are formed (see Scheme 5).

The simplicity of the reaction conditions and the possibility of varying the starting secoketones (A) prompted us to study this reaction in detail, in particular its scope, limitations and stereochemistry, First of all it was found that configuration of the hydroxy group depends on the size of ring D. If n=1, i.e. the ring D is five-membered, the 14β-hydroxyisomer

Scheme 4



Scheme 5

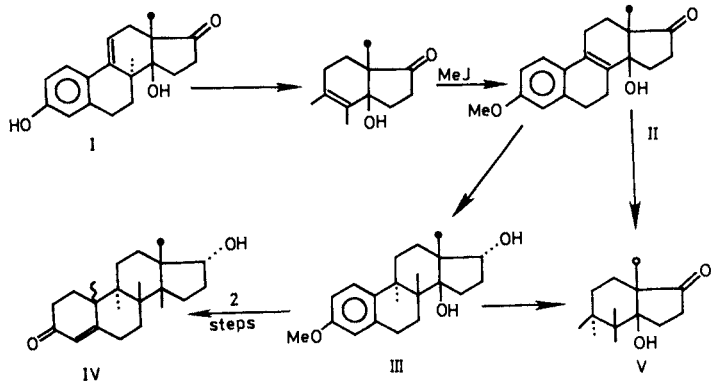


is practically the only one formed (the 14α-isomer was not detected in the reaction mixture) whatever the substituents at positions 3 and 18. If n=2, i.e ring D is six-membered, both 14α- and 14β-isomers are formed, their ratio depending on the nature of the substituents.

When a methoxy group was at position 3 the yields in both examples did not exceed 25% (the rest consisted of bisdehydroderivatives); the presence of a 3-hydroxy group increased the yield to 60% which makes the reaction a preparative method. In other more complicated cases the yields depend also on the nature of the 18-substituent, but, as a rule, exceed 50%.

The hydrogen atom in position 8 is always trans to the 14-hydroxygroup and, therefore, cyclization leads to 14β-hydroxysteroids with "unnatural" configuration. We certainly tried our best to pass from "the unnatural" to the "natural" cardenolide configuration, i.e. to 8β, 14β-compounds, and this indeed proved possible thanks to the mobility of the double bond (Ref. 7).

Scheme 6

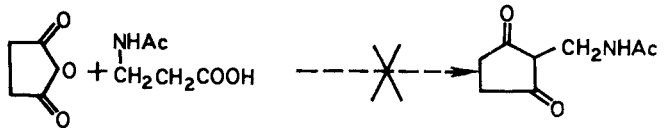


When the $\Delta^{9(11)}$-double bond of the 3-methoxy compounds is sufficiently stable (Ref. 8) it easily migrates to position 8(9) in 3-hydroxycompounds like (I). After methylation the ketol (II) was reduced with sodium in liquid ammonia to the diol (III) with 9α, 8β, 14β-configuration. Its subsequent Birch reduction afforded the Δ^{4}-3-ketosteroid (IV). It is interesting to note that hydrogenation of the ketol (II) on a Pd-catalyst also gave rise to the compound with the natural configuration, namely ketol (V). In this case nothing remains but to suggest the formation of an equilibrium mixture of the $\Delta^{9(11)}$-8β-and $\Delta^{8(9)}$-compounds (under the influence of the catalyst), the former compound already being easily hydrogenated to the ketol (V).

In a further extention of this method we obtained 18-functionalized 14-hydroxysteroids the synthesis of which by other means is very difficult or simply impossible. To achieve this goal it is necessary first of all to have the corresponding cyclic diketones of the types (VI) and (VII).

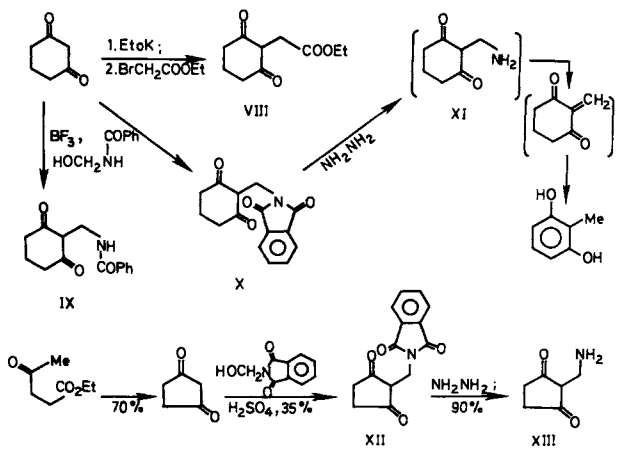
Scheme 7

R = COOR' ; CN ; NR''_2



We investigated several different ways of preparing these intermediates. Attempts to utilize cyclization of "complicated" aliphatic compounds unfortunately led to no positive results (one of such unsuccessful route is given as illustration in the Scheme 7). "Alkylation" of cyclopentane-1,3-dione and cyclohexane-1,3-dione proved to be much more fruitful. Incorporation of a carboxymethyl group was a rather facile problem, and, for instance, condensation of the potassium derivative of cyclohexane-1,3-dione with ethyl bromoacetate gave rise to the ketoester (VIII).

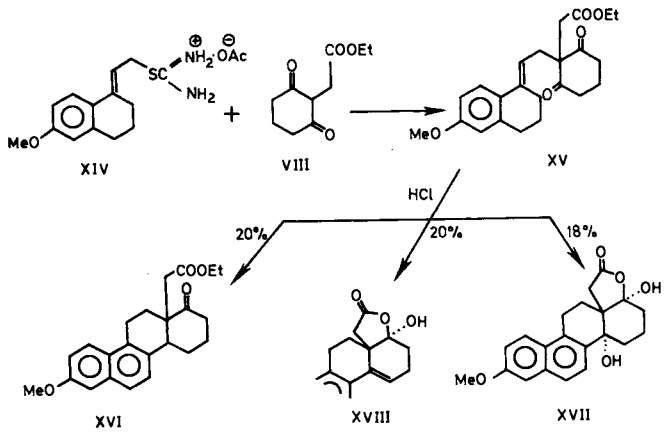
Scheme 8



It was more difficult to insert an amino-methyl group. However, condensation of the diketones with hydroxymethyl benzamide or, better still, with hydroxymethylphthalimide in the presence of strong acids proceeds with formation of the desirable products (IX-XII). Elimination of the protective group is achieved with hydrazine. It was just in this way that the aminodiketone (XIII) - a very interesting compound for syntheses - was obtained. Some attempts to prepare its cyclohexane analogue (XI) failed due to β-elimination of a molecule of ammonia and the subsequent spontaneous isomerisation of the resultant 2-methylencyclohexane-1,3-dione to 2-methylresorcinol.

Condensation of the diketoester (VIII) with the thiouronium salt (XIV) proceeds to a well known scheme giving the desired diketone (XV) which underwent cyclization upon acid treatment. In the presence of hydrogen chloride in methanol D-homoequilenine derivatives, namely, the ketoester (XVI), the hydroxylactol (XVII) and its dehydration product (XVIII), are formed.

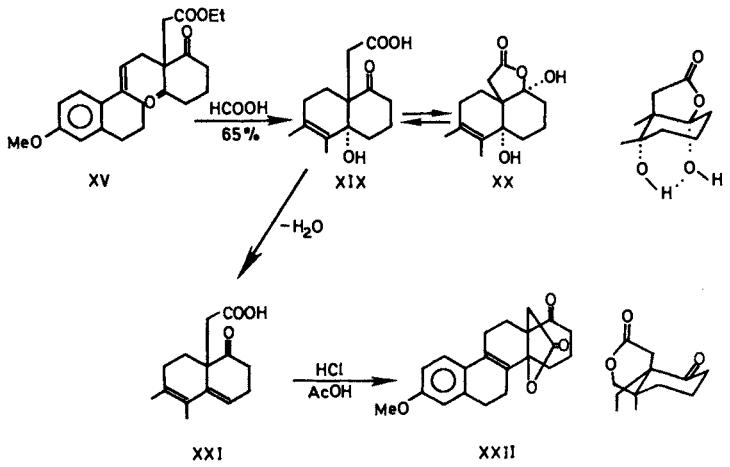
Scheme 9



Cyclization and dehydration also occur under the influence of formic acid but dehydrogenation is not observed. The hydroxyacid (XIX) in crystalline form exists as a lactol, but both forms give rise to the same methyl ester. The hydroxy group was ascribed the 14 α -configuration because it formed a strong hydrogen bond in the lactol (XX), as revealed in the IR spectrum of this compound. The corresponding 14 β -hydroxy-compound in

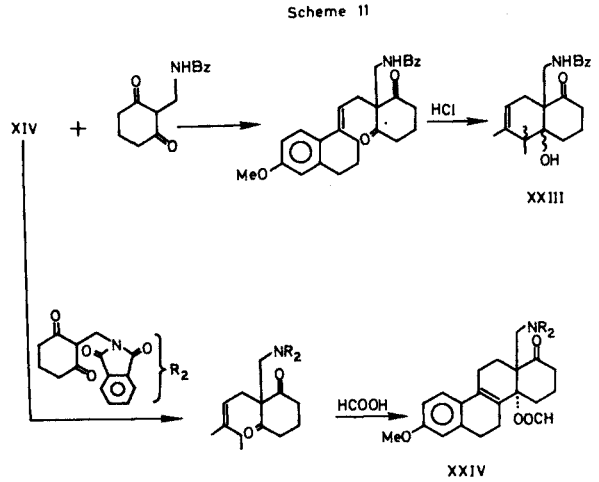
the lactone form (XXII) was obtained from the dehydration product of the hydroxy acid (XIX) by treating the latter with hydrogen chloride in acetic acid.

Scheme 10



In an analogous way the hydroxyketoamids (XXIII) and (XXIV) were prepared starting from amides and the thiouronium salt (XIV).

Scheme 11



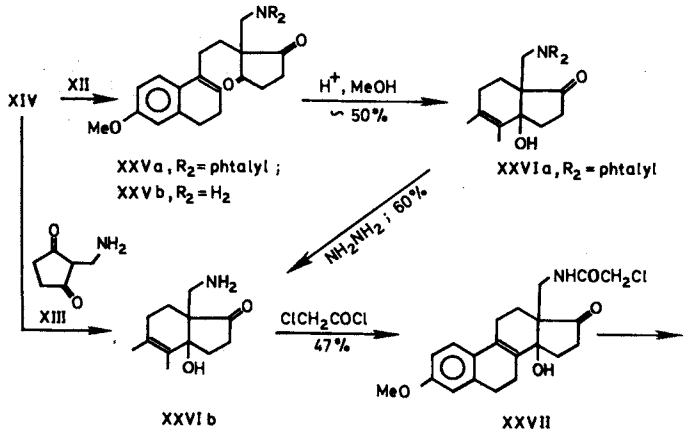
Since no hydrogen bonding was detected in the IR-spectrum of compound (XXIV) we ascribed to it the 14α -hydroxy structure.

Condensation of the thiouronium salt (XIV) with the cyclopentandione aminoderivatives (XII) and (XIII) proceeds smoothly on boiling in 50% isopropanol to give the aminodiketones (XXVa) and (XXVb) in 60 and 72% yields, correspondingly. On being treated with acid in methanol they cyclize to the 14-hydroxy derivatives (XXVIa) and (XXVIb) (see Scheme 12).

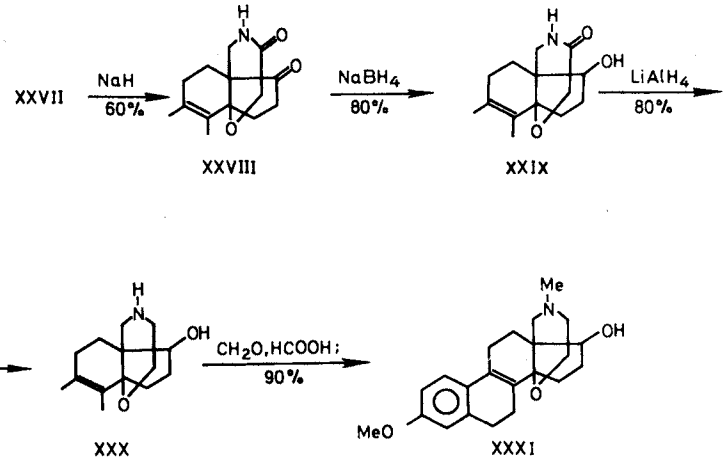
Theβ -configuration of the 14-hydroxy group was indicated by the facile formation of a 14,18-azepine ring. N-acylation of the hydroxyaminoketone (XXVIb) with chloroacetyl chloride and subsequent cyclization using sodium hydride led to the cyclic amide (XXVIII) which had no hydroxy group. Reduction of the amide and Wallach methylation afforded the interesting compound (XXXI) (see Scheme 13).

The availability of 14-hydroxyketones prompted us to carry out their further transformations so as to arrive at cardenolide systems. Total syntheses of

Scheme 12



Scheme 13



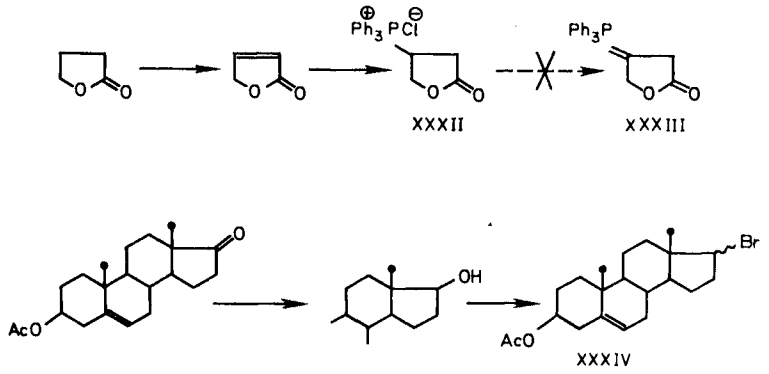
digitoxygenin (starting from pregnane or 21-norpregnane compounds) have already been described in the chemical literature. Here I should like to mention the schemes of Sondheimer-Danielli-Mazur (Ref. 9) and of Fritsch and coworkers (Ref. 10). No attempts, however, to carry out the synthesis from androstane or estrane compounds have been reported until 1973.

At first issuing from dehydroepiandrosterone as a model starting compound we intended to use the Witting reaction with the corresponding ylide (XXXIII) for insertion of the side chain. The phosphonic salt (XXXII) thus obtained could not, however, be converted to the desired ylide. Attempts to obtain a Wittig reagent from 17-bromo- Δ^5-androstene-3β-ol acetate were also unsuccessful (see Scheme 14).

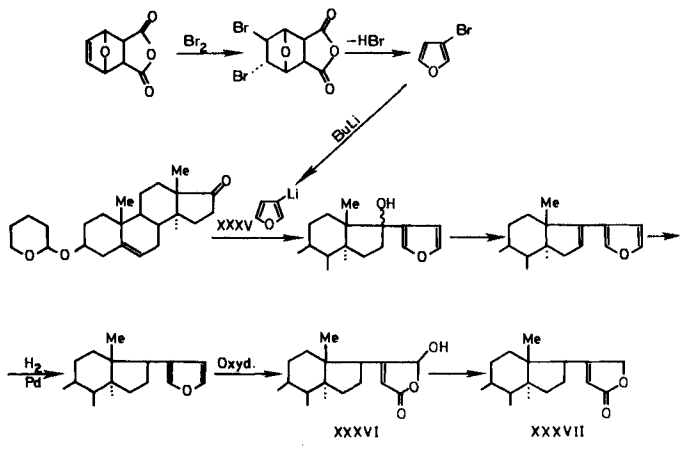
Perhaps the most convenient route to make the butenolide chain is insertion of a furan ring into the position 17, as suggested by Canadian chemists (Ref. 11). We carried out this scheme on the model of dehydroepiandrosterone (Ref. 12) (see Scheme 15).

The key steps in this scheme are: Grignard reaction of 17-ketocompound with β-furanlithium (XXXV) and (after dehydration and hydrogenation) oxidation of the furan ring to the hydroxylactone grouping (XXXVI). Its reduction with $NaBH_4$ leads to the butenolide (XXXVII).

Scheme 14



Scheme 15



In this way it becomes possible to convert androstane and estrane derivatives into the corresponding cardenolide analogues, which (particularly their glycosides) can be of medicinal interest.

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NEW PHYTOECDYSONES

N. K. Abubakirov

Institute of the Chemistry of Plant Substances, Academy of Sciences of the Uzbek SSR, Tashkent, USSR

Abstract - Several new phytoecdysones have been found in the following plants: Ajuga turkestanika (Rgl.) Briq. (Labiatae), Serratula sogdiana Bge. (Compositae), Rhaponticum integrifolium C.Winkl. (Compositae), Silene premixte M.Pop. (Caryophyllaceae). The structures of the isolated substances have been established.

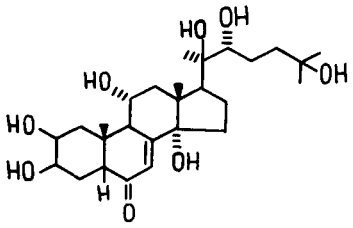
This short communication is devoted to ecdysones, which play a role of moult hormones in insects but were also found in plants. The ecdysones possess a large spectrum of physiological action. Together with juvenile hormones the ecdysones are of interest as potential insecticides devoid of the negative influence on a man and his environment. Nowdays they are applied in the silkworn breeding as the cocoons waving speeding up agents. Due to some anabolic effect of several ecdysons they can be used in growing of young animals in the cattle breeding. This property allows us to hope that some drugs similar to the anabolic hormones may be prepared on the base of the ecdysones. Ecdysones are of ten considered as exotic substances, which are typical only for pteridophyta. The investigation carried out in our laboratory permits us to make a conclusion that plants producing these substances are more numerous than it was supposed before. Now the moult hormones have been found in taxonomically different plants, some of them containing the phytoecdysones up to 2 per cent. We worked mainly with the flowering plants. Alongside with ecdisterone which is the predominant substance some new phytoecdysones of original structure have been found. There is no need in the short communication to discuss in detail the structure elucidation of new substances. It is enough to say that the analysis of the PMR and mass-spectra of the isolated substances, their acetates and acetonides have been of paramount importance.

We have carefully investigated the substances from Ajuga turkestanica (Rgl.) Briq. growing in the Middle Asia. Besides the known ecdysterone, ajugalacton, cyasterone and ajugasterone two new phytoecdysones: turkesterone (I) and 22-acetylcyasterone (II) (Refs. 1,2) have been isolated.

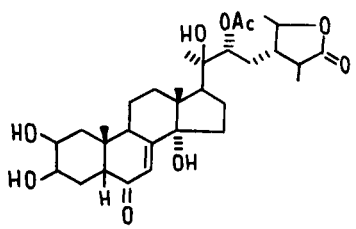
PHYTOECDYSONES OF AJUGA TURKESTANICA (Rgl.) Briq.

KNOWN : ecdysterone, cyasterone, ajugalactone, ajugasterone B.

NEW : turkesterone (I), 22-acetylcyasterone (II).



I. TURKESTERONE
$C_{27}H_{44}O_8$



II. 22-ACETYLCYASTERONE
$C_{31}H_{46}O_9$

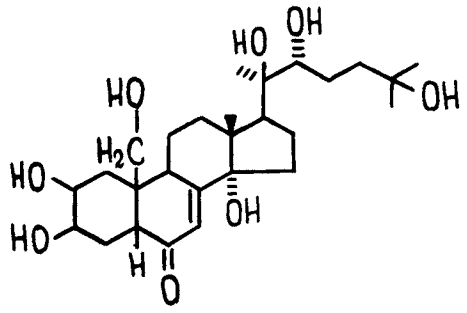
Turkesterone isolated from roots of the plant contains 4 hydroxyl groups, one of them being at 11α -position. The structure of the turkesterone side-chain is the same as that of ecdysterone. Therefore turkesterone can be called as 11α -hydroxyecdysterone.

The ecdysones from Serratula and Rhaponticum belonging to the Compositae family were also isolated and investigated. In the floscules of the Serratula sogdiana Bge. the known substances - ecdysterone and viticosterone E and a new ecdysone - sogdisterone (III) have been detected.

PHYTOECDYSONES OF SERRATULA SOGDIANA Bge.

KNOWN : ecdysterone, viticosterone E.

NEW : sogdisterone (III).



III. SOGDISTERONE

$C_{27}H_{44}O_8$

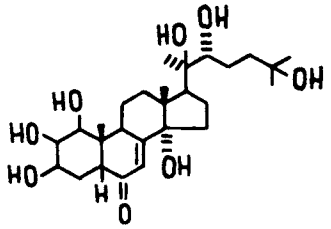
The characteristic feature of sogdisterone is the presence of hydroxyl group at C-19 as in some cardial aglycones like ouabagenin and strophantidol. There are no C-19 hydroxyl containing compounds known so far among ecdysones. The side-chain of sogdisterone is the same as that of ecdysterone. Thus, sogdisterone has a structure of 19, 20R-hydroxyecdysone (Ref. 3).

Several interesting compounds have been found in Rhaponticum integrifolium C.Winkl. This material as well as other flowering plants have been shown to contain the considerable amounts of ecdysterone. Besides the latter we succeeded in isolating of three new phytoecdysones: integristerone A (IV), integristerone B (V) and 24(28)-dehydromakisterone A (VI) (Refs.4-6).

PHYTOECDYSONES OF RHAPONTICUM INTEGRIFOLIUM C.Winkl.

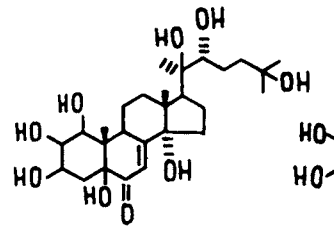
KNOWN : ecdysterone

NEW : integristerone A (IV), integristerone B (V), 24(28)-dehydromakisterone A (VI).



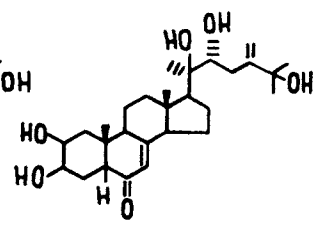
IV. INTEGRISTERONE A

$C_{27}H_{44}O_8$



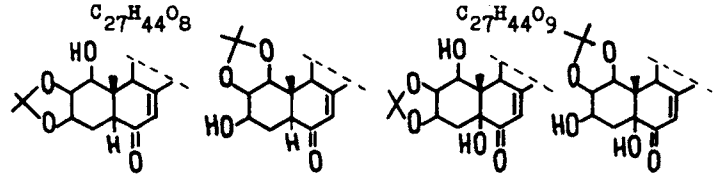
V. INTEGRISTERONE B

$C_{27}H_{44}O_9$



VI. 24(28)-DEHYDROMAKISTERONE A

$C_{28}H_{44}O_7$



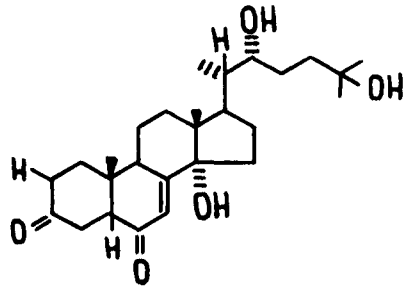
First two compounds are the most interesting ones. They contain a large number of hydroxyl groups in the cyclic part of the molecule. Integristerone A contains 6, and integristerone B - 7 hydroxyl groups. The possibility to obtain the isomeric diacetonides at 1,2 and 2,3 hydroxyl groups was determinant in the structure elucidation of both compounds, which are considerably hydrophylic. All above mentioned phytoecdysones possess the moulting activity.

In the plant Silene premixte M.Pop. (Caryophyllacese) 2-deoxy-α-ecdysone, 2-deoxyecdysterone and two compounds of little polarity have been detected. We have called them as silenosterone (VII) and premixisterone (VIII).

PHYTOECDYSONES OF SILENE PREMIXTE M.Pop.

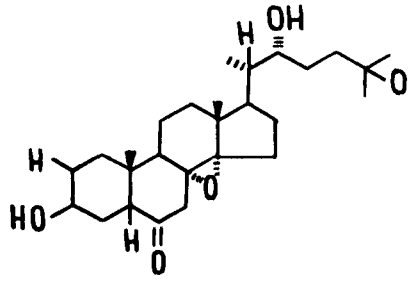
KNOWN : 2-deoxy-α-ecdysone, 2-deoxyecdysterone.

NEW : silenosterone (VII), premixisterone (VIII).



VII. SILENOSTERONE

$C_{27}H_{42}O_5$



VIII. PREMIXISTERONE

$C_{27}H_{44}O_5$

The first, in contrast to other natural ecdysones, contains the keto-group at the position C-3 instead of hydroxyl. The second compound does not contain a hydroxyl at C-14, but has an epoxy-group.

Thus, the total sum of ecdysones with the determined structures have reached 60, including 8 new compounds isolated by us. We believe that other ecdysone-producing plants and other ecdysones of different structures will be surely found.

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THE PROBLEM OF DIVISION OF BIOLOGICAL FUNCTIONS OF ENDOGENOUS SUBSTANCES

A. V. Kamernitzky

N. D. Zelinsky Institute of Organic Chemistry, Academy of Sciences of the USSR, Moscow, USSR

Abstract - The consideration of endogenous steroid hormones as morphofunctional system makes it possible to offer some general principles of their modification leading to the division of their biological functions. The testing of these assumptions has led to the division of the mineralocorticoid and spleenotropic activity of some steroids in one case and to the obtaining of a new class of steroid gestagens in the other one.

Problem confronting to steroid chemistry is the synthesis of compounds with division biological functions. It is well known that all attempts of native endogenous substances using as drugs fail because of the so called sideactions which sooner or later reveal themselves. The most striking example of such a case is, probably, the failure of cortisonotherapeutics which was very popular in the late 50ties and early 60ties. That's why practically all the works with native endogenous substances aiming at practical medical usage are carried on in the direction of their modification to eliminate or at least to lessen their side-action. Unfortunately, this search has to be made blindly as long as the dependence of the biological functions of the substance on its chemical structure is unknown. Certain references to the effect of the inclusion to or exclusion from a molecule methyl, hydroxy or some other group are not reliable and often give contradictory results. Evidently, it is necessary to obtain some general approach to the solution of the problem of purposeful modification of endogenous substances.

As an example of such an approach one may put forward the suggestion that native endogenous substances, being the products of the evolution of morphological structures, do represent the simpliest biological systems which should to some extent fall under the action of biological laws, and the regularities of evolutional morphology in particular.

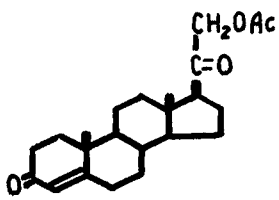
The inherent property of biological systems is their multifunctionality, that is their ability to perform in the organism not only one but a number of biological functions, one of them being the main and the other the minor ones (Ref.1). In the simpliest case the system may perform only two functions - the direct one and the feedback, the latter being directed at the regulation of the system itself.

As to native endogenous substances, the multifunctionality will mean the capacity of the substance to interact in parallel with the number of enzymes-receptors, each of these receptors being responsible for a certain branch of metabolism. Such a phenomenon is known for peptide hormones, where for the definition of the side of recognition of the same hormone by different receptors the notion of signature has been introduced (Ref.2,3).

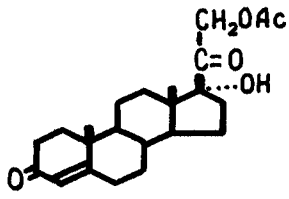
The modification of endogenous substances may lead to the preservation of the ability of the transformed compounds to react with some of the receptors and to the exclusion of such a reaction with the others, that is to the division of their biological functions. However, it is necessary to obtain a general criterion of the limits of transformation. As such one may take the principle (known from evolutional morphology) of the predomination of the accordance of structure of the system and its main function (Ref.1). For native endogenous substances, it means that endogenous compound, being the substrate, is complementary to its receptor just in the main function, where the greatest recognition degree is achieved. It is quite possible for the recognition degree of the substrate by the receptors of minor functions to be considerably lower.

In this case relatively small chemical changes of the endogenous substance molecule may prevent it from the efficient interaction with the receptor of the main biological function, the other capacities of performing minor bio-

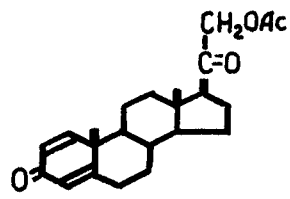
logical functions being intact. The subordination of biological functions can be drawn from levels of biological organization, that is organismal, tissual and cellular level.
The steroid hormones, being lowmolecular bioregulators with the wide spectrum of biological activity and giving the possibility of their systematic chemical transformations do represent good material for the verification of the suggestions made above.
In our laboratory two general ways of modification of steroid molecule were investigated concerning their influence on biological properties of the substances. These ways are as follows: firstly, flattening of the surface of the molecule by introducing extra double bonds and, secondly, steric shielding of the polar center of a molecule by fusing extra carbocycle.
It is known that mineralocorticoids such as 21-acetate of pregn-4-ene-21-ol-3, 20-dione (1), "DOCA" are able not only to sodium and water retention and to potassium excretion but that they also show the stimulating effect on the lymphatic system by promoting its growth and effectiveness (Ref.4).
From compound 1 and from 21-acetate of pregn-4-ene-17α, 21-diol-3,20-dione (2) Δ^{16}- and $\Delta^{1,16}$-analogues of 1 (5 and 6) and Δ^{1}-analogues of 1 and 2 (3 and 4) were synthesized.



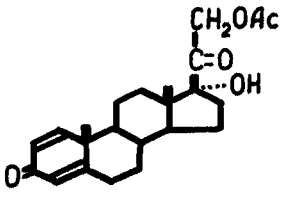
(1)



(2)



(3)



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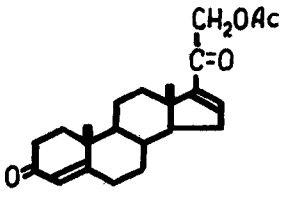

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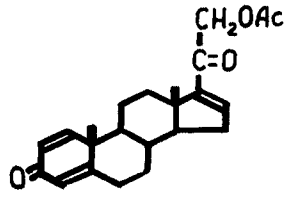


(6)

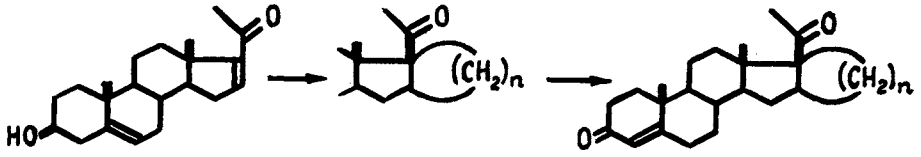
Compound 1 was dehydrated during the refluxion of its 3, 17,21-triacetate with dry AcOK in DMFA (Ref.5), whereas Δ^{1}-compounds (3,4 and 6) were prepared through the dehydration of DDQ (Ref.6).
Biological tests have shown that introduction of extra double bonds is accompanied by the reduction of sodium retention effect in compound 2, its disappearance in 5 or its inversion in 4. At the same time the transformation of 1 into 2 leads to the loss of potassiumurethic effect and further to the appearance of potassiumretention capacity in 5 and 6.
Thus, alteration in geometric structure of the molecule in this case is accompanied by sharp changes in its influence on the exchange of electrolytes, when the mineralocorticoid activity of it suppressed or inverted.
Meanwhile, all the compounds obtained retain the ability to stimulate the growth of the organs of lymphatic system, which proves a tropic effect general for all of compounds of such a type (Table 1).

Table 1
The tropic effect of 21-hydroxypregnenes acetates (1-6) on the spleen (dose 1.6-3.2 mg/kg for 7 days)

Compound	1	2	3	4	5	6
Weight of spleen (% to control)	130-140	125	120	130-150	-	115-130

Evidently, in this case the preservation of tissue unique response to the introduced modified steroid could indicate that the receptors which are responsible for the growth of a certain sort of tissues are less demanding

to the substrate structure than the ones responsible for the exchange of electrolytes in the whole organism.
The synthesis of a new class of biologically active steroids, 16,17α-cycloalkanoprogesterons or D'-pentarans (16,17α-cycloalkanopregn-4-ene-3,20-diones) which was worked out in our laboratory, could provide another example of purposeful modification of steroids. These compounds with extra 3-membered carbocycle D' fused with the steroidal skeleton in 16,17 -positions were synthesyzed from the pregna-5,16-diene-3ß-ol-20-one's (7) derivatives with the aid of cycloaddition (2+2), (2+3) and (2+4) reactions and further expansion or contraction of extra cycle (Ref.7).



(7) n=1 - 4 (8-11)

These compounds do not contain any additional polar groups, but their 20-carbonyl group is more or less hindered from the back side of the molecule. Biological tests have shown that compounds of such a type have strong gestagenous and contraceptive activity (Table 2), but it is not accompanied by reduction of adrenal glands function but by its stimulation, the contraceptive effect being explained by the delay of the ovulation.

Table 2

The gestagenous activity of 16,17α-cycloalkanoprogesterons (8-11) in Klauberg's test (dose 0,4 mg/kg)

Compound	Progesteron	8	9	10	11
n	0	1	2	3	4
Proliferation index	3	3,5	3	3	4

From these data one can probably conclude that in this case the action through the hypothalamus-hypophysis system takes place, that is there occurs the effect on the feedbac which regulates the biosynthesis of hormones rather than the progesteron replacing interaction with the receptors of endometrium.
The above experimental data and other ones demonstrate, to our mind, the possibility of division of biological functions of steroids and obtaining thereby information which concerns the relationship between chemical structure of endogenous substance and its biological properties.

Acknowledgment. The biological tests were carried out in the Scientific Research Institute for Biological Tests under A.I.Terekhina, Institute of Midwifery and Gynaecology of Academy of Medical Sciences of the USSR, under K.K.Korkhov and the Adaptation Department of the Far Eastern Scientific Center of Academy of Sciences of the USSR, under O.I.Kirillov.

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EXTANT FLORA WITH RESPECT TO ITS CHEMISTRY

V. Herout

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Flemingovo 2, 166 10 Praha 6, CSSR

Abstract - An attempt is made to follow the conditions of plant species existence with respect to the content of their secondary products. According to present data, an evaluation of the phytochemical contribution to the phylogeny of some developmental lines of the present-day flora is presented (e. g., of the Marchantiopsida, etc.). This synthesis is concentrated mainly to plant-plant, plant-microorganisms, and plant--animal interactions.

In deliberations on the maintenance of a plant species in the complex ecosystems, very different conditions are to be taken into account. For the present flora the environmental climatic, edaphic etc. conditions are usually competed also by a further factor, i.e. the presence of the secondary products of plant metabolism and their possible functions for survival and further development in the steadily changing conditions. For a similar study, I wish to choose a very antique group of plants represented in extant flora as liverworts (Marchantiopsida).

According to fossil records their presumed existence starts beyond reasonable doubts in carboniferous times (Apotreubia Hatt. et al., Treubiites Schust.), and may be extended with probability till the Devonian era (Pallaviciniites sp. Schust.). In a very detailed article, Schuster (Ref. 1) tried to explain the present distribution of some taxa of leafy liverworts (Jungermanniae) on the basis of the original hypothesis of Wegener on continental drift. The existence of an antique supercontinent (Pangaea) and its break-up to Gondwana and Laurasia, and successively to the present continents and large continental islands, is also possible to evaluate from geobotanical studies. In the case of liverworts, Schuster was successful to explain the present distribution of a series of highly disjunct ancient taxa of some very primitive Marchantiopsida. Only for rough information, a schematic model of Permian position of Gondwana continents is given in Fig. 1, with the range of Treubia genus (different taxa!).

From such a point of view - the continental drift - it is quite interesting to compare the different evolution and the fate of some groups of plants. There is limited information on the climatic conditions during those geological epoques estimated to have lasted about 200 m.y. World-wide fluctuations of droughts with interspersed variable fluvial periods, glacial advances concerning successively Southern and Northern hemisphere have had remarkable

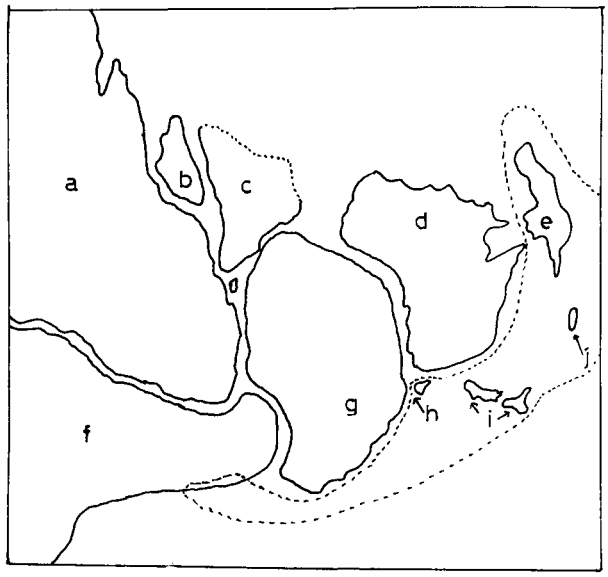

Fig. 1. Schematic model of Permian position of Gondwana continents (a - Africa, b - Madagascar, c - India, d - Australia, e - New Guinea, f - South America, g - Antarctica, h - Tasmania and j - New Caledonia). The present range of Treubia genus is given by a dotted line (simplified from Schuster, Ref. 1).

effect on the phylogeny of different developmental lines of existing plants. In a very simplified way it is possible to follow some of the numerous developing groups and remind those which were not able to survive. In a recent review, I have tried to present (Ref. 2) a scheme (see Fig. 2) from which

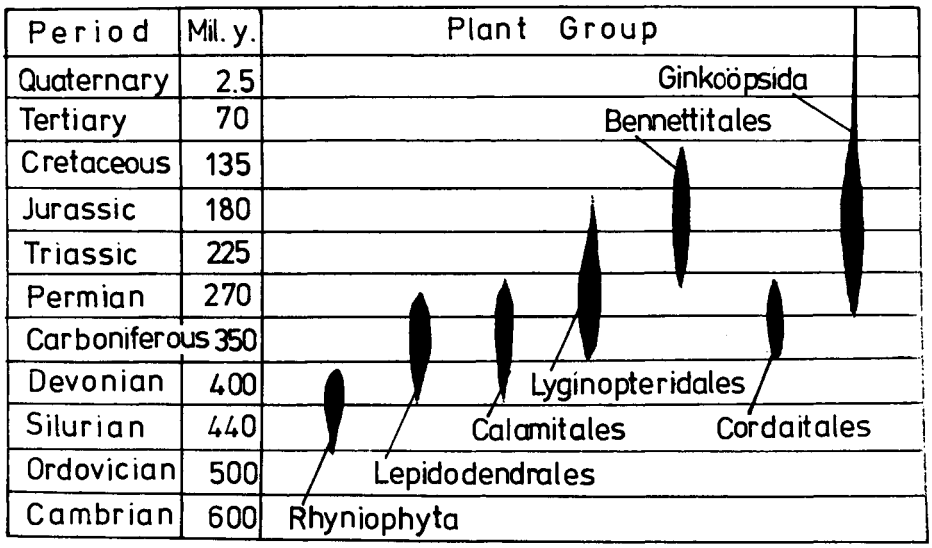

Fig. 2. The extinction of some evolutionary lines of land plants during geological periods.

the fate of a series of died-out groups of plants is presented. Their fossil remnants demonstrate us today that in the geological periods mentioned, they formed a rich and abundant flora covering the then unquiet continents. The liverworts, though seemingly a defenseless plant group, were able to survive these periods, and develop to a rich plant group (of course, with a lot of extinct taxa). For their survival, yet another limiting factor concerning their development can be presented: Leafy liverworts (Jungermanniae) are represented mostly by unisexual individuals (see, e.g. Schuster, Ref. 3), and since they were able to disperse by one-celled haploid spores, they established only unisexual populations. It is supposed that genetically viable species commonly develop more rapidly than organisms which propagate only as clones (Ref. 4). Therefore, asexual plant species seldom diversify, and this means a low rate of evolution.

Nevertheless, this group of organisms is clearly well adapted to some type of habitats, mostly wet and shadowy places, both in warm and cold climate where liverworts are able to be competitive to other forms of living organisms. I should like to stress that the advantage of their existence may also be a ground for their rich content of secondary products.

Chemical studies of the group of plants belonging to Marchantiopsida were performed during last few years only, partially also in our laboratories. As a class of mosses (Bryophyta), the liverworts have some characters distinguishing them from other mosses. Namely, in their cells they contain both morphologically and chemically interesting "oil bodies" or "oil droplets". According to our present knowledge, this bodies are formed mainly from secondary products of lipoid or mostly terpenoid nature. The isolation and structure elucidation of these components is the goal of the present active research. The results hitherto achieved are remarkable for these reasons: i) Their sesquiterpenoids are very often of unusual structure unknown in other classes of organisms - some examples of compounds of this type are represented in Fig. 3; ii) Similarly to some groups of organisms different in their development from so called higher plants, the compounds contained in liverworts being of the same structural type, differ in the sterical configuration, and are enantiomers; this fact has repeatedly been pointed out (see e.g. Ref. 5 - 8), and it is known from microorganisms, fungi, corals, etc. In Fig. 4, a few compounds originating from liverworts and belonging to enantiomeric types are presented; iii) Similarly to a series of lower developed plant groups, lunularic acid is a growth regulator of liverworts, and its function could be compared with the one of abscissic acid in higher plants (for a review, see e.g. Ref. 9). iv) A series of compounds of different structure was registered for liverworts, e.g. flavonoids, dihydrostilbene derivatives, simple alkaloids, fatty acid methyl esters, polysacharides, etc. In some families (Jungermanniaceae), an abundant presence of ent-kaurane type diterpenoids seems to be characteristic. But the class which might be of special interest, are sesquiterpenoids of drimane type and, among them, their dialdehyde and lactone derivatives, represented by polygodial (XIV), cinnamolide (XV) and dihydrocinnamolide (XVI): see Fig. 5. If we accept the view of some leading botanists - that liverworts represent an independent evolutionary line, and are not ancestors of higher (i.e. vascular) plants, then

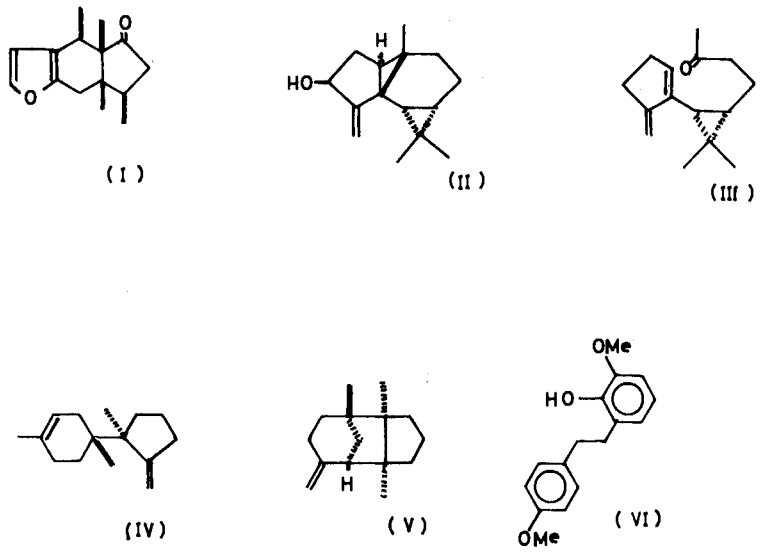

Fig. 3. Some sesquiterpenoids and other compounds of unusual structure but typical components of liverworts: Pinguisone (I), myliol (II), taylorinone (III), (+)-bazzanenone (IV), (-)-β--pompene (V), pellepiphilin (VI).

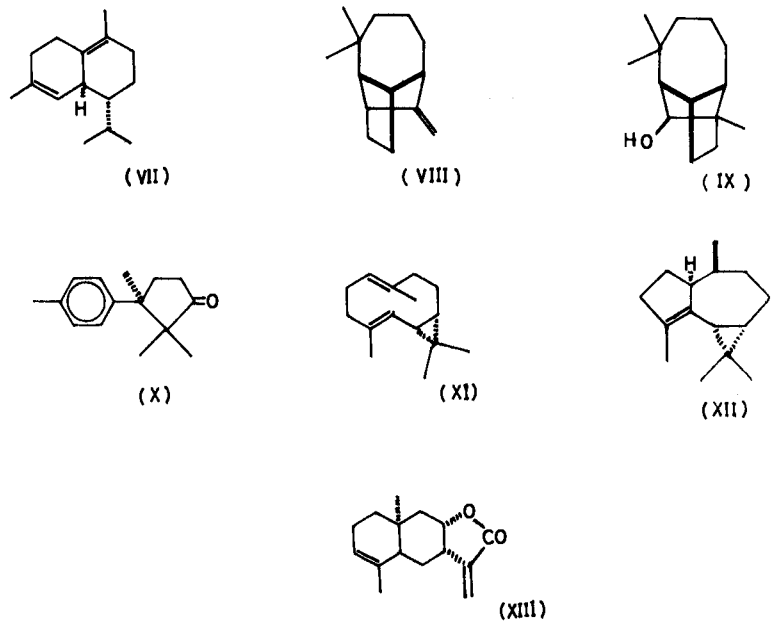

Fig. 4. Sesquiterpenoids detected in liverworts having an enantiomer structure to similar compounds from higher plants: -(δ)-cadinene (VII), (-)-longifolene (VIII), (-)-longiborneol (IX), (-)-α-cuparenone (X), ent-bicyclogermacrene (XI), ent--α-gurjunene (XII), diplophyllolide (XIII).

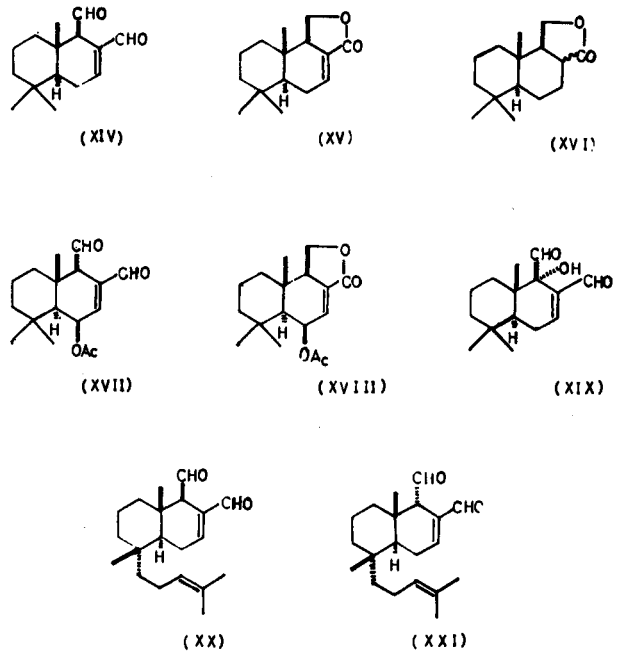

Fig. 5. Dialdehydes and lactones derived from drimane sesquiterpene type: Polygodial (XIV), cinnamolide (XV), dihydrocinnamolide (XVI), cinnamodial (XVII), cinnamosmolide (XVIII), and warburganal (XIX); derivatives of the diterpenoid nature sacculatal (XX) and isosacculatal (XXI).

the presence of this type of compounds represents a further chemical convergency. Polygodial (XIV) and a series of related dialdehyde derivatives and lactone derivatives were primarily found in Polygonaceae: In Polygonum hydropiper L. and in many Magnoliales /Winteraceae, in Drimys lanceolata (Poir.) Baill. polygodial; in Canellaceae namely Cinnamosma fragrans Bouillon cinnamodial (XVII), cinnamolide (XV) and cinnamosmolide (XVIII). Further, Warburgia ugadensis Sprague contains cinnamodial and warburganal (XIX)7; (more detailed references on these compounds are given in Ref. 2.). This list of compounds is remarkable from the point of view that the mentioned plant products, esp. with dialdehydic function, have a very pungent taste (as qualified by human taste). From the paper of Kubo et al. (Ref. 10) it can be taken that for herbivorous insect larvae the pungency is parallel to antifeeding effect. The concurrent existence of similar compounds in liverworts and some angiosperms can be then taken as advantageous for plants. In this respect it is useful to add that production of pungent derivatives is not limited to drimane skeleton, and that liverworts contain further similar products, i.e. sacculatal and isosacculatal (XX and XXI), both isomers characteristic as diterpenoids clearly derived from drimane type; they were found first in Trichocoleopsis sacculata (Ref. 11), and later in Pellia endiviifolia (Dicks.) Dum. (Benešová, not published results). Broad presence of such a type of derivatives with repellent function for herbivores could be taken

as a further proof for today more and more admitted opinion on the important function of the secondary plant product for survival. In the liverworts we registered more compounds of similar antifeeding activity. In collaboration with Prof. Wada (Sendai, Japan) it was possible to demonstrate an antifeeding activity for the prime very interesting liverwort metabolite isolated in our Laboratories, i.e. pinguisone (I). This type of derivatives was proved to be quite common in many liverworts. Sesquiterpene lactones belong, according to present knowledge, to potent feeding deterrents (Ref. 12, 13), and hence, their presence in some Marchantiopsida might have a similar function as the products occurring also in angiosperms (sesquiterpene lactones of the same type are very common in Compositae, Umbelliferae and Magnoliales). To this list of active compounds it is possible to add also alkaloids, encountered in Hepaticae not as too common; in Riccardia sinuata and R. incurvata (Ref. 14) we found two - up to now probably most simple - alkaloids of indole type, see Fig. 6.

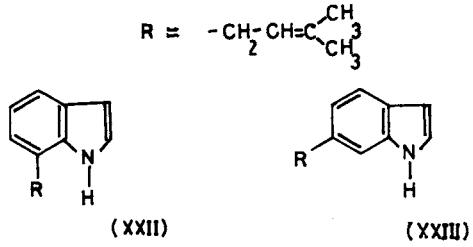

Fig. 6. The simplest indole alkaloids isolated from liverworts (XXII) and (XXIII).

As a summary of these data it can be presumed that liverworts are not so harmless plantlets, and their chemical "weapons" against coexisting predators from the animal kingdom are numerous. Not much is known about the antifungal and antimicrobial activities of Hepaticae, but the presence of phenolic derivatives (e.g. substituted dihydrostilbenes, Ref. 15, 16) is registered. The fungistatic and antimicrobial function of similar natural phenolic dihydrostilbenes and stilbenes, e.g. pinosylvin (Ref. 17), and of resveratrol (Ref. 18) was established. In some recent experiments in our Laboratories (Benešová and Chvojka, unpublished results) we were able to demonstrate a plantgrowth influencing activity of a series of liverwort metabolites which very probably execute an allelopathic action in the dense populations of Hepaticae.

Taking into account the presently much discussed role of secondary compounds as protective agents (from recent reviews see e.g. Ref. 2, 19, 29) we can see a good example in the data briefly reviewed for Marchantiopsida. This ancient evolutionary line of green plants having survived geological epoques could have profited to some extent from the rich array of their specific metabolites.

Of course, the measure of the protective value is difficult to demonstrate, and it would be an oversimplification to judge that just the inadequacy of

chemical equipment only was fatal for other ancient but today extinct taxa. If we consider, e.g., modern (i.e. living) lycopods or equisetoids, another developmental line of the plants characteristic by their large diversity and abundancy during Paleozoic times, it would be dubious to transfer mechanically the present knowledge of the phytochemical composition of present living members of both groups mentioned. There is minimally a large difference in their habitus: Many lycopods and Equisetoids having lived in the Carboniferous and Permian periods were trees but all extant members belonging to both groups are at present relatively small herbs. These modern members are characteristic by the presence of special types of alkaloids, polyphenols and horsetails, moreover by a high content of silica. Nevertheless, there is no possibility to check the chemical composition of died-out members representing in then times dominant flora, and the principal contributors to the vegetable deposit from the Carboniferous.

Today there is no doubt that protective properties of some secondary products get into function as soon as some developing forms of living organisms start to differenciate and willingly or unwillingly they get their status of "competitors" or "predators", and their "preys". As simply developed organisms as blue-green algae are, they also produce (once more - their present forms!) some kind of metabolites which are very potent allelopathic agents in their habitats (e.g., Anabaena spp., see Ref. 21, 22). The nature of active metabolites might be also rather peculiar, as it was shown in another very ancient evolutionary line as Pteridophyta are. During their phylogeny, they were able to produce some metabolites with the structure of insect molting hormones. Nothing is yet known of the possible function of phytoecdysones for the life functions of the plants themselves. They are sometimes accumulated in rather high concentrations; we were able to show the presence of ecdysterone and hydroxyecdysterone in the rhizoms of Polypodium vulgare L. (Ref. 23) in quantities over 1% of dry weight (about 1 000 000 times higher than was in some insect larvae!). It is probable that the biosynthesis of metabolites of this type, arising by some fortuitous mutations during the phylogeny and diversification of ancestral ferns, proved to be successful in the "coexistence" with herbivorous insect. In our extant ferns we are able to register only: i) The occurrence of phytoecdysones in Pteridophyta is quite frequent. ii) There are very few insect predators known feeding on present ferns. iii) The protection against them might be inter alia also due to the presence of insect hormones while their external unbalanced influence may disorder the ontogeny of individuals of the tentative predator species (see e.g., Review 24); very recently i has been proved that the content of phyto ecdysones in conifers is deletious for some insect larvae (Ref. 25).

In a paper (Ref. 2) published elsewhere I tried to summarize the most recent ideas on the role of "secondary compounds" and their interactions with other living organisms. This matter is now being studied intensively and in a contemporary investigation Seigler (Ref. 19) enumerated not less than 36 reviews on this topic. In the present part of this article I tried to present my views on the usefulness of tackling the problem of secondary metabolites not exclusively from the chemical point of view. E.g., thematically distant "continental drift", and its possible phytogeographical consequences might

be stimulatory, see, e.g., Schuster s study mentioned (Ref. 1) or Turner's deliberations (Ref. 26) on the origin and possible phylogeny of Compositae which in turn, has interesting connections to the content of their secondary products. According to Harborne (Ref. 27), ecological studies are very useful to solve questions concerning some of the functions of the secondary products, too. I suppose that just the interdisciplinary access and collaboration of specialists active in different branches of science could find the best ways of this old problem, and studies of plant - plant, plant - - fungus (or microorganism) and plant - animal interactions offer the most promising results.

During the past 15 years, much effort has been directed toward the elucidation of the many roles of allelopathy in plant - plant interaction; for a summary, see e.g., a recent paper by Rice (Ref. 27). Indisputable effects have been observed in ecology of plant communities, but allelopathy is without doubt a very complex phenomenon (see, e.g., Ref. 18), and only the future and more comprehensive research will make a better interpretation possible. The range of chemical compounds involved in allelopathic effects seems to be, according to our preliminary results, much broader than it was possible to show up to now. Thus a greater importance for plant species survival and its succession in populations is expected to be demonstrated.

The coexistence of fungi and plants represents a very interesting sphere of research, too. A common manifestation is a simple parasitism of a fungus, mostly moulds, bacteries, viruses, and others. Without effective defence systems, plants could be victims and could have been eradicated. But they developed different types of selfdefence (from many reviews, see a recent one by Swain, Ref. 18). To mention some examples of chemical nature: i) the cuticular waxes well developed at most of the Angiosperms, represent chemically quite simple products of biosynthesis, but in respect to cells of diverse microorganisms, a difficult permeable barrier, indeed. ii) Presently, very attractive studies concern the existence and the way of biosynthesis of phytoalexins (see Ingham, Ref. 28, 29 ; Stoessl et al., Ref. 30). In some respect being a parallel to the immunoglobulins of higher animals, they represent - according to the present knowledge - an intriguing defence system manifested by a production of "secondary products", having in fact a primary importance. In general, phytoalexins are produced according to taxonomic relations of the plant species; different but evolutionary related plant groups (e.g., families) produce identical or nearly related compounds. Presently, there exist detailed studies of formation of phytoalexins in a few (economically important) plant families, e.g. Leguminosae, Solanaceae, etc. It seems remarkable that Solanaceae produce phytoalexins which are compounds of sesquiterpenoid nature; this type of compounds is not known as a secondary product of healthy plants of this family (see Hegnauer, Ref. 31).

But in the case of non-pathogenic fungi there exist different conditions of the relation between fungus and host plant. In any case symbionts also need to be controlled, and held in limits by some general defence mechanisms. It is presumed that e.g. in ectomycorrhize of higher fungi with root systems of many trees, mono- and sesquiterpenes (Ref. 32) play an important role by

restricting the growth of mycorhizal fungi which in its beginning can be regarded as a parasitic process.

The coevolution of plant and animal kingdoms should be seen in its complex consequencies starting from the simplest fact that the autotrophic green plants are at the very outset of the food chain of all other living organisms. As a result it follows that any plant species has to face the danger of damage and/or full annihilation. Therefore it was necessary for them to develop many protective strategies to enhance the chance of survival. Some of such defences are of morphological nature (e.g., thorns, stinging hairs, thick cuticula, etc.), and all of them genetically controlled. A broad series of protective agents of chemical nature existing within the extant flora is genetically controlled, too. The value of any of the protective agents is variable owing to the adaptive properties of the herbivorous animals, and in the ecological conditions many of the protections are difficult to be evaluated or are even not discernible. A series of toxic substances has generally a protective value (e.g., alkaloids, cyanogens, glucosinolates, cardenolides, sesquiterpene lactones, etc.), and act more or less as feeding deterrents. This depends on active detoxication processes developed in animal kingdom, and variable in different species. Today copious nice examples are known where the originally highly toxic plant substance has been adapted by an insect specious as an efficient "weapon" in the struggle with its predators (for detailed information see, e.g., Rotshild, Ref. 33). However, the function of the secondary plant product in the life of the plant has, in reverse, also an attractive function for animals - in their function as pollinators as the most simple case.

Many factors, including the mentioned chemical equipment of plants, contributed to the balance of different biotypes on present nature, as a process of longdated evolution. This balance has always been and will at present remain a very labile one. It seems that the discussed interrelations of the members of different kingdom are disturbing the existing balance relatively slowly, but a new factor is presently becoming more important, i.e., the factor of human activity. Man's activity has been influencing the status of existing biosphera steadily during his existence on the Earth, but originally in a limited extent only. From the beginning of the industrial revolution in the last century, and in a much more important fashion during the present scientific-technical revolution, human influence rises enormously, and the biosphera is changing very quickly. The change concerns the ecosystems of all the parts of the world, and as a result, many animal and also plant species become extinct unceasingly. It would be outside the scope of this article to discuss the possible impact followed with anxiety by the scientific community, but the production of new chemicals and a lot of wastes deserve the prime attention. New pesticides of all kinds, herbicides inclusively, are important means to achieve better crops for the benefit of mankind, but secondary effects of such a use of pesticides are often disregarded. And these are "intentional" pollutions which together with the systematic pollutions originating in all kinds of industrial activity and urban wastes could definitely endanger the unique and irretrievable earth's biosphere. Towards the end of this presentation one last remark only: Man is, undoubtedly, a

representative of one of the highest evolutionary lines in the animal kingdom. Presently, his activity apparently exceeds what could be expected from a "single species activity" in the complex ecosystem of living organisms; it would be in the interest of the human community to use its exclusivity extremely rationally.

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HORMONE-RECEPTOR INTERACTIONS: A STUDY OF THE MOLECULAR MECHANISM OF RECEPTOR STIMULATION IN ISOLATED FAT CELLS BY THE PARTIAL AGONIST CORTICOTROPIN-(5-24)-ICOSAPEPTIDE

R. Schwyzer, G. Karlaganis and U. Lang

Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, CH-8093 Zürich, Switzerland

Abstract - Corticotropin-(5-24)-icosapeptide is a partial agonist for lipolysis and adenylate cyclase activation in isolated rat fat cells and plasma membranes, respectively. It is demonstrated that this partial agonist can occupy the same number of functional receptor sites as the full agonist, corticotropin-(1-24)-tetracosipeptide. It follows that partial agonism is - in this case - not due to partial occupation but to partial stimulation of a fully occupied receptor population. A chemical mechanism based on ACTH structure-activity relationships and the flexibility of the hormone molecule, and a biological mechanism based additionally on the activation of α- and β-type receptors by different portions of the hormone are proposed as possible explanations of full and partial agonism.

INTRODUCTION

The biological activity of hormones is usually described by two parameters, potency and intrinsic activity (Ref. 1). A hormone analogue with a smaller maximal response (intrinsic activity) than the parent hormone is called a partial agonist. In terms of receptor theory, the potency is commonly thought of as an apparent affinity in a reversible hormone-discriminator (hormone-receptor) complex. However, the origin of partial agonism is unclear. One view assumes a heterogeneous discriminator population, only a part of which can associate with or 'bind' the partial agonist and thus become stimulated: partial agonism would then be due to only partial occupancy of the discriminators available for the parent hormone even at high hormone analog concentrations. The other view assumes full occupancy of the same number of discriminators as becomes occupied by the parent hormone, but only partial stimulation of each receptor (see Fig. 1 for a description of the model for plasma membrane holoreceptors used here).

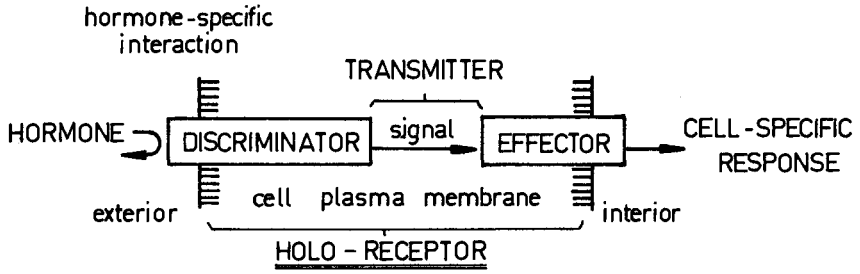

Fig. 1. A model for cell plasma membrane holoreceptors. The discriminator and effector subunits (proteins) are supposed to be subject to independent lateral movement in the plane of the membrane (Ref. 2 & 3).

We have recently described a partial corticotropin (ACTH) agonist for lipolysis in isolated rat fat cells (adipocytes) and adenylate cyclase activation in fat cell plasma membranes (Ref. 4). Corticotropin-(5-24)-icosapeptide ($ACTH_{5-24}$) elicits maximal effects on lipolysis and adenylate cyclase amounting to 63 % and 12 %, respectively, of those produced by the full agonist, corticotropin-(1-24)-tetracosipeptide ($ACTH_{1-24}$). Its intrinsic activities are thus $\alpha = 0.63$ and 0.12. $ACTH_{5-24}$ is a full agonist for steroidogenesis and a partial agonist for cyclicAMP accumulation with $\alpha = 0.45$ in isolated adrenal cortex cells (Ref. 5). It was also found to be a partial agonist ($\alpha = 0.2$) for adenylate cyclase activation in adrenal cortex cell plasma membranes (Ref. 6). CyclicAMP production and steroidogenesis, and cyclicAMP production and lipolysis are generally accepted to be coupled processes (Ref. 7 & 2) and their stimulation due to the interaction of the hormone with a β-type holoreceptor (Ref. 8), the effector of which is the enzyme adenylate cyclase (Ref. 7).

As we have recently been able to demonstrate a specific, reversible association ('binding') of a tritium-labeled $ACTH_{1-24}$ derivative with functional discriminators of fat cells and to distinguish it from non-functional binding (Ref. 9), we have now studied the apparent association constants and the number of functional binding sites (ACTH discriminators) of fat cells for $ACTH_{5-24}$ in order to resolve the question: Is partial agonism the consequence of partial occupancy or of partial stimulation at full occupancy?

Unfortunately, we have not been able to extend the study to isolated adrenal cortex cells for lack of a sufficient criterion of functional binding in this system.

MATERIALS AND METHODS

[2-Phenylalanine,4-(4,5-dehydro-4,5-ditritio)norvaline]corticotropin-(1-24)-tetracosipeptide (t_2ACTH_{1-24}), 7.42 Ci/mmol, was prepared according to Ref. 10 [23-(3,5-Dehydro-3,5-ditritio) tyrosine]corticotropin-(5-24)-icosipeptide (t_2ACTH_{5-24}), 8.82 ± 0.57 Ci/mmol was synthesized in a manner analogous to that of $ACTH_{5-24}$ (Ref. 11). Binding experiments with rat adipocytes were carried out at 37° as in Ref. 9. Lipolytic activity and activation of adenylate cyclase were determined as in Ref. 4.

RESULTS

The activity of t_2ACTH_{5-24} on lipolysis and on adenylate cyclase was identical with that of $ACTH_{5-24}$ over a relevant range of the log dose/response curves. As with $ACTH_{1-24}$ (Ref. 9), the maximal rates of lipolysis and of ATP → cyclicAMP conversion by adenylate cyclase were enhanced about 1.8 and 1.5 fold, respectively, in the presence of phenoxazones (10^{-6} to 10^{-5}M actinomycin or actinocine). The binding of t_2ACTH_{5-24} was, like that of t_2ACTH_{1-24}, complete in less than two minutes and rapidly reversed by excess $ACTH_{1-24}$, but remained unaffected by the potent lipolytic agent adrenaline. The binding experiments with t_2ACTH_{1-24} gave results that were practically identical with those obtained earlier (Ref. 9). They were verified by computer analysis of the binding data (direct plot) with the MINUIT program (H.R. Haller & E. Bürgisser, Laboratorium für Festkörperphysik der ETHZ and this laboratory). The essential parameters of binding and lipolysis are shown in the Table. The binding data for $ACTH_{1-24}$ were obtained through inhibition of t_2ACTH_{1-24} binding (Ref. 9).

The maximal binding capacities (B_{max}) for the full and the partial agonists are practically identical. In the presence of actinomycin D, they are enhanced to about the same extent as the intrinsic activities.

The apparent physical dissociation constants (K_{diss}) of the full agonists, $ACTH_{1-24}$ and t_2ACTH_{1-24}, have about the same numerical values as the corresponding apparent pharmacological (lipolytic) constants (EC_{50}). However in the case of the partial agonist, t_2ACTH_{5-24}, EC_{50} is about 10 times greater than K_{diss}: Although the lipolytic potency ($1/EC_{50}$) of t_2ACTH_{5-24} is about 80 times less than that of t_2ACTH_{1-24}, it binds only 4 - 7 times less strongly (towards $ACTH_{1-24}$, the differences are 10 times greater).

TABLE. Apparent dissociation constants (K_{diss}) and maximal binding capacities (B_{max}, in femtomoles per mg cell dry weight) from the binding experiments; hormone concentrations required for half-maximal stimulation (EC_{50}) and intrinsic activities (α) from the lipolysis assays. Exponents of ten in brackets.

	without actinomycin D				with 10^{-5}M actinomycin D			
	K_{diss}	B_{max}	EC_{50}	α	K_{diss}	B_{max}	EC_{50}	α
$ACTH_{1-24}$	1.4(-9)	30	0.9(-9)	1	1.5(-9)	50	2.1(-9)	1.8[a]
t_2ACTH_{1-24}	1.4(-8)	30	0.9(-8)	1	1.5(-8)	50	-	-
t_2ACTH_{5-24}	1.0(-7)	33	0.7(-6)	0.63[a]	0.6(-7)	48	1 (-6)	1.12[a]

[a]Based on the maximal response to $ACTH_{1-24}$ without actinomycin D.

DISCUSSION AND CONCLUSIONS

Characterization of the binding sites. The parallel enhancement of α and B_{max} by actinomycin D indicates that the binding sites characterized by the K_{diss} values of the Table are indeed functional discriminators (Ref. 9). The fact that the K_{diss} values obtained by the direct plot (MINUIT computer program) and the linearized plot (Scatchard) differ by less than 2 % may indicate that the assumption of a simple, monophasic binding model for the hormone/functional discriminator interaction is reasonable (Ref. 12).

Partial agonism. The partial agonist, t_2ACTH_{5-24}, can occupy the same number of discriminators as the full agonists, t_2ACTH_{1-24} and $ACTH_{1-24}$, and yet it can elicit only 63 % and 12 % of their stimulation of lipolysis and adenylate cyclase activity, respectively. This means that not partial occupancy of the discriminator population by t_2ACTH_{5-24}, but partial stimulation of the holoreceptors at full discriminator occupancy is the cause for the partial agonism of t_2ACTH_{5-24}.

Receptor threshold and receptor reserve. A very simple receptor theory would expect changes of K_{diss} to cause parallel changes of EC_{50}. This is not the case. The potency ($1/EC_{50}$) of $ACTH_{5-24}$ with respect to adenylate cyclase activation is about the same as that of $ACTH_{1-24}$ and other ACTH analogs (Ref. 4 & 6) despite the 70-fold reduction of the apparent association constant ($1/K_{diss}$). On the other hand, the potency for lipolysis is reduced about 10 times more strongly than the binding constant. Such phenomena can be described in terms of changes of receptor threshold and reserve and of stimulus-effect (discriminator-effector) coupling (Ref. 13), but are actually unexplained. (Perhaps the biological model proposed below might lend itself to better understanding of the complex situation.) Our data illustrate the fact that EC_{50} is not simply an apparent dissociation constant.

The role of the potentiator sequence in the function of ACTH. Our results support the idea that the N-terminal potentiator sequence of ACTH and α-MSH (α-melanotropin), Ser-Tyr-Ser-Met (which is without biological activity per se), enhances both the potency and the intrinsic activity of the adjacent message sequence, -Glu-His-Phe-Arg-Trp- (Ref. 14). They also prove for the first time that the potentiator sequence enhances the physical binding of the hormone to its functional discriminators (although to a lesser degree than potency). Both its potentiating and binding powers are dependent on the correct sequence (perhaps a correct balance and distribution in space of hydrogen-bonding, hydrophilic, and hydrophobic properties, Ref. 14): A change of Ser-Tyr-Ser-Met to Ser-Phe-Ser-Nva (as in t_2ACTH_{1-24}) reduces binding and potency 10-fold, complete omission 70- and 800-fold, respectively (Table). It must be borne in mind, however, that its effects on potency and binding may be caused not only by an additional interaction of the Ser-Tyr-Ser-Met moiety with a complementary structural element of the discriminator, but also by a conformational influence of the potentiator on the message sequence that induces the message moiety, -Glu-His-Phe-Arg-Trp-, to adopt more easily a conformation fitting the requirements of the discriminator. Such an influence could be for example the α-nucleating potential of Met^4, that would favor helix

formation of the message sequence when it comes into contact with a hydrophobic discriminator pocket (Ref. 14).

A CHEMICAL MODEL FOR β-TYPE RECEPTOR STIMULATION WITH ACTH AND FOR PARTIAL AGONISM

Partial stimulation of holo receptors at full discriminator occupancy (see Fig. 1) may - in an extreme view - mean either a reduced stimulation of each occupied discriminator (assuming a graded response) or a full stimulation of a reduced number of the fully occupied discriminators on the time average. A compromising view would admit - on the time average - a reduced number of partially stimulated discriminators among the occupied population.

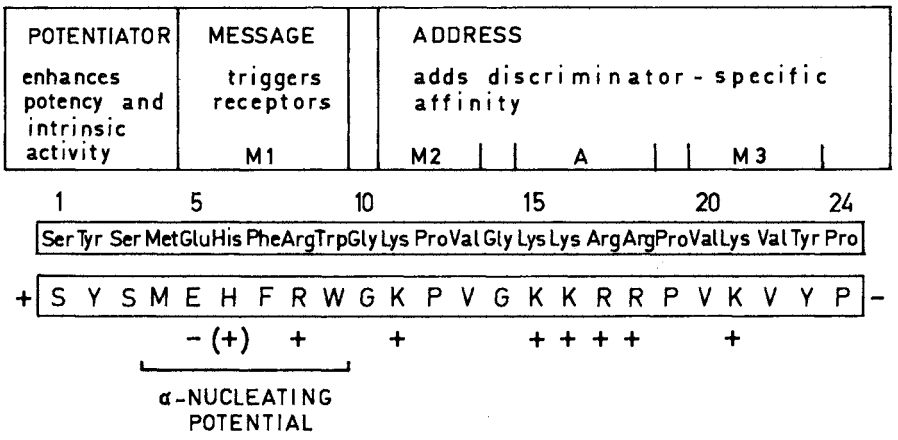

Fig. 2. The amino-acid sequence of corticotropin-(1-24)-tetracosipeptide ($ACTH_{1-24}$) and its subdivision into sequences with distinct biological functions (from structure-activity relationships, Ref. 14). Sequence with three-letter and one-letter symbols, charges at neutral pH, and the assignment of α-nucleating potential according to Ref. 19. Potentiator, message, and address with respect to the β-type adenylate cyclase/lipolysis and adenylate cyclase/steroidogenesis holoreceptors. In the address, an address proper (A) and two message regions for other receptor types can be distinguished: M2, a message for melanotropic activity in amphibian melanophores (Ref. 14 & 20); M3, a message for α-type receptors (Ref. 21), vide infra. Glycine residues 10, 14 and proline 19, 24 may serve as hinge or connecting elements between the functional units.

The model for partial agonism proposed here does not distinguish between these three possibilities. It is based on two well known properties of the $ACTH_{1-24}$ molecule: (i) that discrete sequences of adjacent amino-acid residues are responsible for discrete components of the biological activity (sychnologic organization of information, Fig. 2) and (ii) that $ACTH_{1-24}$ is conformationally flexible (a random coil in solution). It is further assumed that address, message, and potentiator of the $ACTH_{1-24}$ molecule express their part of the total biological activity by adapting to, and reacting as functional, three-dimensional entities with their structurally complementary correlates on the discriminator surface ('recognition pockets'), Ref. 15 & 16.

Because of the flexibility of the ACTH molecule, its reversible association with the probably (like an antibody) comparatively rigid discriminator molecule is assumed not to be of the 'all-or-non/on-or-off' type, but to lead to an ensemble of structurally different complexes, A, B, C..., that are in rapid equilibrium with each other:

$$\text{ACTH} + \text{discriminator} \quad \{A \rightleftharpoons B \rightleftharpoons C \rightleftharpoons D \ .....\} \qquad (1)$$

Although the internal equilibrium of the complex ensemble is not necessarily consecutive as in (1) (for example $D \rightleftharpoons A$ and other equilibria might also be possible) it is assumed that all states involving a discriminator-address interaction would be rather preferred because of the comparatively specific and strong interaction of this moiety, -Lys-Pro-Val-Gly-Lys- -Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro, with the discriminator (Ref. 14, 17 & 18); one of the reasons could be strong Coulomb interactions of the four adjacent basic residues, -KKRR-, with negative charges in the discriminator correlate, -kkrr-, see Fig. 3).

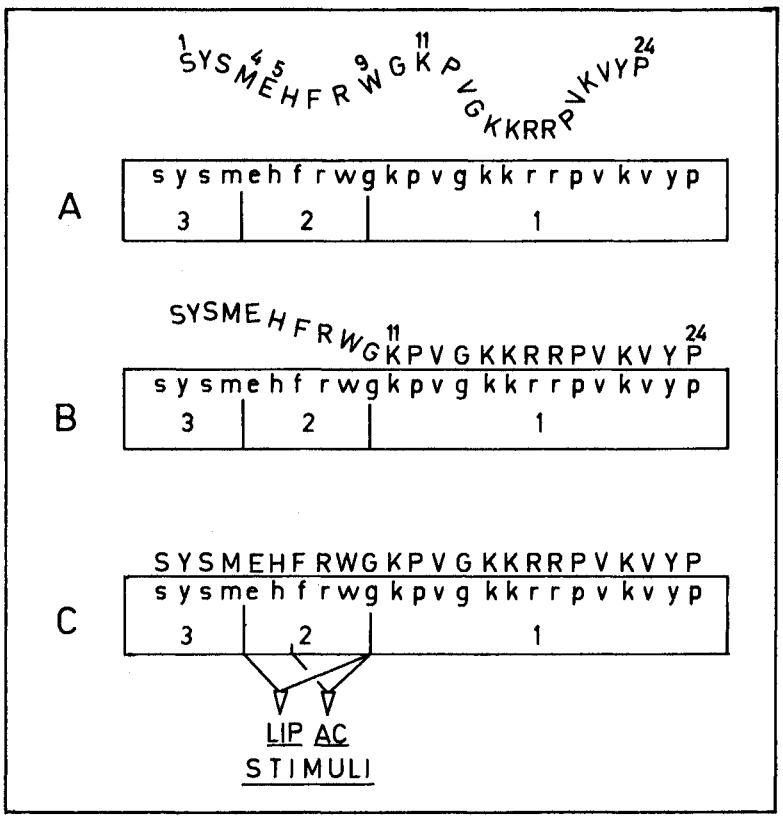

Fig. 3. A schematic representation of three states (A, B, C) of the hypothetical $ACTH_{1-24}$/discriminator complex ensemble, that are in internal equilibrium with each other (1). Amino-acid sequence of $ACTH_{1-24}$ in capital letters. Address, message, and potentiator recognition units of the discriminator (1, 2, and 3, respectively) are indicated with lower-case letters. These symbolize the topographical elements of the discriminator that recognize the complementary structural units of the hormone (not necessarily their individual amino-acids!), Ref. 15 & 16. The correct fit of the message unit to the complementary site of the discriminator in state C causes the adenylate cyclase (AC) and lipolytic (LIP) stimuli (Ref. 4) which are transmitted to the effector(s). States A and B are non-stimulatory. The position of the equilibrium B⇌C is strongly dependent on the correct structure and conformation of the message and potentiator units of the hormone analogues ($ACTH_{1-24}$, t_2ACTH_{1-24}, $Z\text{-}ACTH_{5-24}$, $ACTH_{5-24}$; Z = benzyloxycarbonyl).

Fig. 3 depicts in a very schematic manner three possible complexes within the complex ensemble. In A and B the address is partly or wholly bound, in C, message and potentiator are correctly interacting with the discriminator, and stimuli for the activation of adenylate cyclase and lipolysis (Ref. 4) are generated. (Other stimulatory complexes with perhaps only the message or only the message and the potentiator interacting with the discriminator are also possible, but of minor importance, vide supra.) The concentration of the stimulatory complexes - in particular of C - relative to the concentration of the unstimulatory complexes like A and B will determine the level of biological stimulation at full discriminator occupancy (intrinsic activity). The concentration of C relative to the other states within the complex ensemble is independent of hormone concentration: If the address is correct, it will depend only on the structures of the message and the potentiator (it might also be temperature-dependent).

In our experiments, omission of the potentiator reduced K_{diss} about 70 fold and rendered the compound ($ACTH_{5-24}$) a 63 % partial agonist. The reduction of K_{diss} may, as explained above, be partially due to a direct potentiator-discriminator interaction and partially to a conformational influence on the message. An influence further along the chain is difficult to envisage because of the pronounced flexibility, the intervening Gly and Pro residues, and the low α- and β-nucleating potential of the residues after Trp^9. In terms of our model, omission

of the potentiator would reduce the relative concentration of the stimulatory state C below that caused by the native hormone.

In the case of t_2ACTH_{1-24}, the reduction of K_{diss} is insufficient to produce noticeable partial agonism. A linear interpolation of the K_{diss} and the α values between $ACTH_{1-24}$ and t_2ACTH_{5-24} gives an expected α for t_2ACTH_{1-24} of 0.95; it is impossible to detect such a small deviation from 1 with the biological experiments currently used.

N^{α}-Benzyloxycarbonyl-$ACTH_{5-24}$ is a full agonist for lipolysis (Ref. 4). In terms of our model, this would mean that the relative concentration of C is restored to the value it has in the presence of full agonists. This is not unexpected in view of the strongly hydrophobic properties of both the benzyloxycarbonyl and the methionyl group. Furthermore, the α-amino group of Glu is now shielded, as in the peptide: Its charge vanishes and can no longer (potentially) disturb the Glu-His-Phe-Arg-Trp/discriminator interaction by repulsion or conformational restraints (Ref. 4).

Corticotropin-(1-10)-decapeptide and corticotropin-(5-10)-hexapeptide are (at high concentrations corresponding to their low potency) full agonists for cyclicAMP accumulation in adrenal cells (Ref. 14). $ACTH_{5-24}$ is a partial agonist (Ref. 5). If the requirements of the adrenal cortex discriminators are similar to those of the fat cell, this observation is easy to explain with our model (Fig. 3): states like A and B are non-existent, the hormone-discriminator interaction leads directly to stimulatory states (like C, only with the address residues, 11-24, missing). The (relative) concentration of the stimulatory state is now again dependent on hormone concentration because the strong influence of the address is lacking: where it is present, the address acts like a built-in competitive inhibitor.

A BIOLOGICAL MODEL OF PARTIAL AGONISM

The chemical model is based on the interaction of the flexible ACTH molecule with a β-type adenylate cyclase/lipolysis holoreceptor (Ref. 8). The biological model is based on the interaction of the pleiotropic (Ref. 8, 14 & 20) ACTH molecules with α- and β-type holoreceptors through two different message sequences (Fig. 4). The two models are not mutually exclusive, but could be synergistic.

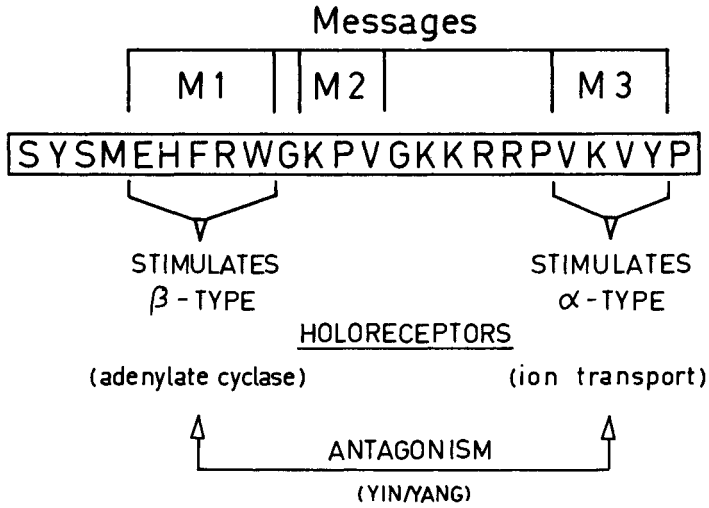

Fig. 4. A schematic representation of the action of $ACTH_{1-24}$ on α- and β-type holoreceptors through its message sequences M3 and M1, respectively. The two receptor types are assumed to act antagonistically. The M2 message stimulates melanophore receptors independently of the M1 message, but is not known to have a biological action on adipocytes per se.

Elliott, Draper, and Rizack (Ref. 21) have recently demonstrated that $ACTH_{1-24}$ stimulates not only lipolysis, but also Mg^{2+} accumulation by rat adipocyte plasma membrane vesicles. When studied separately, corticotropin-(1-20)-icosipeptide ($ACTH_{1-20}$) exhibited full lipolytic activity, but did not influence Mg^{2+} accumulation; on the other hand corticotropin-(11-24)--tetradecapeptide ($ACTH_{11-24}$, the inactive address sequence for β-type holoreceptors)

exhibited a full activity on Mg^{2+} accumulation, but none on lipolysis. Mg^{2+} accumulation was blocked by the α-adrenergic antagonist, phentolamine, much more than by the β-blocker, propranolol. This indicates that ACTH contains a second message sequence that can trigger α-type adrenergic holoreceptors: It most probably comprises the amino-acid residues 20-23(24), -Val-Lys-Val-Tyr(-Pro)-.

It is well known that α- and β-type receptors usually act antagonistically (Ref. 22). This might be true in our case, because $ACTH_{1-20}$ is lipolytically slightly, but significantly more potent than $ACTH_{1-24}$(Ref. 21).

The biological model for the partial agonism of $ACTH_{5-24}$ is thus very simple: a structural change impairing the interaction of Message 1 and its discriminator correlate (2 in Fig. 3) will have the observed effects of enhancing K_{diss} and reducing more strongly the potency for β-type stimulation. Because the Message 3 sequence is intact, its stimulation of the α--type receptor will be expected to be reduced only proportionally to K_{diss}: that means that the α-type potency will be - in our case - reduced about 10 times less strongly than β-type potency. This will alter the antagonistic balance in favor of the α-holoreceptor by a factor that should be the same at all concentrations of the partial agonist and be numerically equal to the intrinsic activity (α).

The two models represented here suggest a number of control experiments that will be able either to disprove them or to provide supporting evidence.

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MESSENGER RIBONUCLEOPROTEINS (INFORMOSOMES)

A. S. Spirin

Institute of Protein Research, Academy of Sciences of the USSR, Poustchino, Moscow Region, USSR

Abstract - Messenger ribonucleoproteins, first discovered in our laboratory (1964) as mRNA-containing particles of fish embryo cytoplasm and named informosomes, were later found to be universally occurred in eukaryotic cells. Messenger ribonucleoproteins of different intracellular localization such as free cytoplasmic informosomes, translatable messenger ribonucleoproteins in polyribosomes ("polyribosomal informosomes") and nuclear pre-mRNA-containing particles ("nuclear informosomes") are characterized by a number of features common for all of them. However, the transport from the nucleus into the cytoplasm as well as the transition from the free non-translatable state into the polyribosome-bound translatable state are accompanied by essential changes in the protein moiety of the particles.

The existence of free RNA-binding proteins in eukaryotic cells has also been shown in our laboratory. These proteins seem to represent a pool for the formation of messenger ribonucleoproteins (informosomes). It has recently been demonstrated that the eukaryotic translation factors and, in particular, both the elongation factors and some initiation factors are among the cytoplasmic RNA-binding proteins. It is speculated that the mRNA in eukaryotic cells at different stages of its life history carries on itself the proteins which are required either for its own biogenesis, processing and transport (nuclear informosomes), or for its existence in a temporarily inactive state (free cytoplasmic informosomes), or for its functioning as a template (polyribosomal informosomes): omnia mea mecum porto.

1. DISCOVERY OF mRNA-PROTEIN COMPLEXES

About 15 years ago, studying the cytoplasmic extracts of early fish embryos, we observed that a significant portion of their mRNA moved in the centrifuge separately from ribosomes, but at the same time had essentially higher sedimentation coefficients than the deproteinized mRNA (Ref. 1). The analysis of mRNA-containing zones in the CsCl density gradient showed that mRNA of the extract was complexed with protein (Ref. 2). These mRNA-containing particles of the cytoplasm differed from ribosomal particles in their buoyant density and a number of other properties. The discovered messenger ribonucleoproteins with an RNA:protein weight ratio of about 1:3 (buoyant density in CsCl about 1.4 g/cm^3) were named informosomes (Ref. 2, see also Ref. 3).

Later data were reported from G. P. Georgiev's group that DNA-like RNA of mouse and rat nuclei also existed in the form of ribonucleoproteins (Ref. 4 & 5). The buoyant density of the nuclear ribonucleoproteins in CsCl (1.4 g/cm^3) and the RNA:protein ratio (1:3) were similar to those of cytoplasmic informosomes (Ref. 6 & 7).

More later two American groups found that dissociation of animal polyribosomes resulted in the release of mRNA complexed with protein (Ref. 8 & 9). The released messenger ribonucleoproteins also had a low buoyant density in CsCl and, correspondingly, high proportion of protein.

Thus, three classes of messenger ribonucleoproteins of the informosome type were discovered in animal cells: (1) free non-translatable cytoplasmic particles (free informosomes); (2) polyribosome-bound translatable mRNA-protein complexes; (3) nuclear particles containing mRNA precursors. The latter two classes could be called polyribosomal and nuclear informosomes, respectively (Ref. 10).

Messenger ribonucleoproteins (informosomes) of all the three classes proved to have a universal occurrence in the animal world, including mammalia, birds, amphibia, fish, echinodermata, insects and others; in virus-infected cells informosomes were shown to contain viral mRNA (see reviews 10 & 11). During the last 5 years free cytoplasmic informosomes, polyribosome-bound messenger ribonucleoproteins and nuclear ribonucleoproteins of the informosome type were found also in the cells of higher plants (Ref. 12, 13 & 14). Free mRNA seems not to occur at all in eukaryotic cells: at all stages of its life history, beginning from early precursors in the nucleus, it exists in the form of the complexes with protein (Ref. 15).

Messenger ribonucleoproteins (informosomes) of different origin and different intracellular localization are characterized by a number of features common for all of them (11). These characteristic features are:

1. The presence of messenger, pre-messenger or messenger-like RNA as the principal component.
2. Relatively high and constant protein:RNA weight ratio of about 3:1 and the corresponding unique buoyant density in CsCl of about 1.4 g/cm^3; the density homogeneity.
3. Linear correlation between the sedimentation coefficients of the particles and the sizes of their RNA; sedimentation heterogeneity as a rule.
4. High sensitivity to ribonucleases, i.e. an exposed position of RNA.
5. Resistance to removal of Mg^{2+}, i.e. a non-ribosomal type of the structural organization.

The common characteristics allow to consider the different messenger ribonucleoproteins as particles of the same (informosomal) type possessing the group properties above-mentioned.

2. RNA-BINDING PROTEINS OF EUKARYOTIC CELLS

In addition to the mRNA-protein complexes (Ref. 10 & 11), the cytoplasmic extracts of animal cells have been shown to contain a fraction of high molecular-weight proteins with sedimentation coefficients from 6 to 10 S capable of forming complexes with exogenous RNA (Ref. 16, 17 & 18). The artificial complexes formed are characterized by a constant weight stoichiometry (protein:RNA = 3:1) and the corresponding buoyant density in CsCl of about 1.4 g/cm^3, i.e. they are similar to natural messenger ribonucleoproteins (informosomes). Such informosome-like particles are formed with any high molecular-weight RNA, be it homologous mRNA or alien ribosomal RNA. RNA-binding proteins, capable of forming similar complexes, have been found also in nuclear extracts of animal cells (Ref. 19). Cell extracts of higher plants have been shown to contain RNA-binding proteins as well (Ref. 20).

Though RNA-binding proteins of eukaryotic cell extracts are capable of forming complexes with any RNA, this interaction has been shown to possess a definite specificity. Thus, complexes of loach embryo RNA-binding protein with homologous mRNA have proved to be more stable than with *E.coli* ribosomal RNA (Ref. 21).

The binding constants of RNA-binding protein (from rat liver) with alien RNA (*E.coli* ribosomal RNA) were evaluated. Even in this case they were high, approximately in the range from 10^8 M^{-1} to 10^{13} M^{-1} (Ref. 22). It seems likely that the binding constants can be even higher in specific complexes when the two components are homologous.

The high binding constant of RNA-binding proteins with RNA (Ref. 22), the definite specificity of binding expressed in the greater stability of the complexes with homologous mRNA (Ref. 21) and the ability to form strictly stoichiometric ribonucleoprotein complexes similar to natural informosomes in buoyant density (1.4 g/cm^3) and in a number of other properties (Ref. 16, 23, 24 & 25) warrant the assumption that this fraction of cellular proteins represents a pool of free proteins for the *in vivo* formation of the messenger ribonucleoproteins (Ref. 10 & 11). This assumption was checked recently by a direct experiment where the highly purified fraction of frog oocyte RNA-binding proteins was isolated, radioactively labelled *in vitro* and then injected into the oocytes (Ref. 26). The incorporation of a significant portion of the injected RNA-binding proteins into free cytoplasmic informosomes was found *in vivo*. Control experiments showed that RNA-binding proteins were incorporated into informosomes as such, without preliminary degradation; that other protein fractions were not incorporated into informosomes; that the process of incorporation took place only *in vivo* and that it did not proceed during the incubation of the RNA-binding proteins in the homogenate or in the

extract of oocytes (Ref. 26). These experiments are the first direct demonstration that free RNA-binding proteins, at least partially, represent a pool of informosome-forming proteins of the cell.

3. INTERRELATION BETWEEN DIFFERENT CLASSES OF MESSENGER RIBONUCLEOPROTEINS

The life history of mRNA consists in synthesis of the precursor on the DNA template, processing, transport from the nucleus into the cytoplasm, and, finally, incorporation into polyribosomes. This suggests that nuclear ribonucleoproteins must be precursors of the cytoplasmic messenger ribonucleoproteins (Ref. 27). In the cytoplasm mRNA seems to appear first in the form of free informosomes and only then, at least partially, to undergo transition into translatable messenger ribonucleoproteins of polyribosomes (Ref. 28). The standing question is whether there is a difference between nuclear ribonucleoproteins, free informosomes and translatable messenger ribonucleoproteins? Do the particles change at their transport from the nucleus into the cytoplasm and from the free state into polyribosomes?

Comparative analyses of the protein compositions of all the three classes of the particles could give an answer to this. A contiguous question on the identity of free RNA-binding proteins and the protein component of the messenger ribonucleoproteins also requires a comparative analysis of the protein compositions. In recent years such attempts have been made in various laboratories, but unfortunately in most cases the purity of the preparations gives rise to doubt. The available data have been summarized in the recent review (Ref. 29).

The strength of all the literature evidence and also the results of our laboratory (Ref. 30) permit to assert that the messenger ribonucleoproteins of various cellular localization differ from each other in their protein compositions just as the protein composition of the particles is not identical to that of the free RNA-binding proteins. The difference between the nuclear and cytoplasmic particles is especially great: their sets of main polypeptide chains do not overlap at all and only a few minor polypeptides (in particular, the poly(A)-binding protein of about 75,000 daltons) can coincide. The main polypeptide components of nuclear ribonucleoproteins are represented by two close sizes of chains of about 40,000 daltons. The protein sets of cytoplasmic ribonucleoproteins vary depending on the species and tissue of animals, but, as a rule, contain main polypeptides of 50,000 and 75,000 daltons.

The following scheme of interrelation between the various classes of messenger ribonucleoproteins and between messenger ribonucleoproteins and RNA-binding proteins in the eukaryotic cell can be suggested. Newly synthesized nuclear pre-mRNA-protein complexes are a form where the post-transcriptional modification, processing and intranuclear transport of mRNA precursors take place. The transition from the nucleus into the cytoplasm is accompanied by a principal change of the protein composition: the main protein components of the nuclear particles remain in the nucleus while the mRNA interacts and forms complexes with a number of RNA-binding proteins of the cytoplasm, thus forming free cytoplasmic informosomes. Among the proteins of the informosomes there must be some protective, masking and repressory proteins ensuring the prolonged existence of mRNA (or at least a part of it) in the non-translatable state, separately from the ribosomes. In this case the transformation of non-translatable informosomes into translatable polyribosome-bound messenger ribonucleoproteins must be accompanied also by some kind of induced changes of the protein component of the particles, such as release or modification of repressory proteins and binding of activatory proteins, including, probably, some of the translation factors (see below). Free RNA-binding proteins are a pool of the proteins both for free informosomes and for active polyribosomal messenger ribonucleoproteins.

4. FUNCTIONS OF THE PROTEIN COMPONENT OF MESSENGER RIBONUCLEOPROTEINS

It is very likely that the nucleoprotein form must be somehow connected with those features of the mRNA life history in eukaryotes which distinguish it from that in prokaryotes. From this the following functions of the protein component of different classes of messenger ribonucleoproteins in the nucleus and in the cytoplasm can be suggested:

1. Specific processing and other post-transcriptional modifications of mRNA and their regulation.

2. Regulated intranuclear, nuclear-cytoplasmic and intracytoplasmic transport of mRNA.
3. Functional masking and, probably, physical protection of mRNA to ensure the preservation of the pre-synthesized long-living mRNA prior to translation.
4. Regulated initiation of translation.
5. Possible regulation of effectivity and rate of elongation.

Examples can be given when temporarily non-translatable mRNA is indeed accumulated in the cell as free informosomes. Thus, during loach embryo development, at the middle gastrula stage, intensive synthesis proceeds both of mRNA in the nucleus and of protein in polyribosomes, and the newly-synthesized mRNA enters the cytoplasm; however, it is not translated immediately (the products of its translation will be found only a few hours later), but is accumulated in the form of informosomes (Ref. 10). In the course of wheat embryo ripening less and less newly-synthesized mRNA is detected in polyribosomes, in parallel with a general drop of protein synthesis; this is accompanied by an increase in the amount of free cytoplasmic informosomes containing newly-synthesized mRNA, right up to the complete separation of non-translating ribosomes and non-translatable informosomes in dry seeds (Ref. 31). Other examples are given in the recent review (Ref. 29).

The existence of stored, masked or temporarily inactive mRNA in the form of informosomes suggests that some kind of special repressory components preventing the association with ribosomes and the initiation of translation must be present in the particles. Experimental evidence favouring the presence of specific (selective as regards mRNA) repressors of translation in free cytoplasmic informosomes have been obtained in some laboratories: the functional and structural analysis of 20 S informosomes from duck erythroblasts suggests the existence of repressory proteins selectively bound to globin mRNA (Ref. 32 & 33) while informosomes of chick embryo muscle and dormant Artemia salina gastrula have been shown to contain a special low molecular weight repressory RNA, denoted as tcRNA (translational control RNA) (Ref. 34-35 & 36, respectively). The transformation of non-translatable informosomes into polyribosome-bound messenger ribonucleoproteins must be somehow conjugated with the replacement of repressory components of the particles by activatory components, including activating proteins drawn from the pool of free cytoplasmic RNA-binding proteins. It is not excluded that initiation factors as well as the elongation factors, may be among such activatory proteins of messenger ribonucleoproteins.

5. EUKARYOTIC TRANSLATION FACTORS AS RNA-BINDING PROTEINS

Inasmuch as the repressory and activatory proteins of cytoplasmic messenger ribonucleoproteins are drawn apparently from the pool of free RNA-binding proteins, a direct check of the effect of the latter on translation deserves attention.

In our first experiments it has been shown that the removal of total RNA-binding proteins from the wheat embryo cell-free system of translation results in the inactivation of the system (Ref. 37). On the other hand the addition of a preparation of the total RNA-binding proteins from wheat embryos to the washed ribosomes supplied with only aminoacyl-tRNAs and natural mRNA makes the system active both in the initiation and elongation of polypeptide chains. From this the conclusion has been drawn that the eukaryotic initiation and elongation factors are RNA-binding proteins, and that, consequently, the total fraction of wheat embryo RNA-binding proteins contains a complete set of the initiation and elongation factors (Ref. 37).

In following experiments the capability of rabbit reticulocyte RNA-binding proteins to substitute for individual initiation and elongation factors in a purified cell-free eukaryotic system was studied. It was found that the total preparation of free RNA-binding proteins of rabbit reticulocytes contains both the elongation factors, EF-1 and EF-2, and a number of initiation factors, such as eIF-4C and eIF-5 as well as small amounts of eIF-1, eIF-3 and eIF-4B (Ref. 38).

The discovered RNA-binding ability of the translation factors can be directly connected with the existence of translatable messenger ribonucleoproteins in the eukaryotic cell. In this case it can be expected that whereas the eukaryotic translation factors are RNA-binding proteins their prokaryotic analogs must not be such. Indeed, a direct comparison of the RNA-binding ability of the purified elongation factors of eukaryotes (EF-1 and EF-2) and prokaryotes (EF-T and EF-G) has shown that both the eukaryotic elongation factors possess a high affinity to RNA and form complexes with it, while the prokaryotic

elongation factors display no capability to interact with RNA under the same conditions (Ref. 39). In other words, the RNA-binding capability proved to be an additional acquisition of just the eukaryotic, and not the prokaryotic, elongation factors.

6. OMNIA MEA MECUM PORTO

The observations described above have induced the formulation of a new concept proposed to explain such a feature of the eukaryotic cell as the existence of mRNA in the form of ribonucleoproteins at all stages of its life history. The essence of the hypothesis is that the mRNA in the eukaryotic cell carries on itself the proteins which are required for its own biogenesis, existence and functioning ("omnia mea mecum porto") (Ref. 40).

Thus, it is postulated that:

1. RNA-binding ability is characteristic of many eukaryotic proteins serving RNA and RNA-dependent processes.
2. The evolutionary acquisition of the RNA-binding function by proteins involved in mRNA biogenesis and its translation is directly connected with the necessity of concentration and compartmentation of these proteins near the sites of their functioning in the big volume of the eukaryotic cell.
3. It is the RNA-binding proteins of this kind that compose the protein moiety of messenger ribonucleoproteins of all the three classes.

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SELECTION OF mRNA INITIATION SITES FOR PROTEIN SYNTHESIS IN PROKARYOTES

M. Grunberg-Manago

Service Biochimie, Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France

Abstract - Two aspects of the selection mechanism of cistron initiation sites are discussed : 1) the role of initiation factors by studies on the localization site of interaction of initiation factors on the ribosome ; 2) the description of the different mutants in initiation factor IF_3 and in two regions of mRNA postulated to be involved in the recognition of the initiation sites of the different cistrons.

INTRODUCTION

I would like to discuss the series of events ensuring the recognition of particular regions of the mRNA chain specifying the place where decoding of the template RNA begins. During this step the initiator RNA, fMet-tRNA^{fMet}, providing the N-terminal amino-acyl residue of the nascent chain, is positioned opposite the initiation codon triplet present within the initiation signal. This step, called initiation, preceeds the formation of the first peptide bond.

It is established that the initiation triplet (AUG or GUG) is not necessarily located at the 5' end of the mRNA and that ribosomes select internal initiation codons in polycistronic mRNA. The initiation codons of fMet-tRNA^{fMet} (AUG or GUG) do not code specifically for this tRNA, but also for the Met-tRNA^{Met} or Val-tRNA^{Val} which position their aminoacid internally in the polypeptide chain.

It is evident that some other feature in mRNA should distinguish the correct initiation codon from the many other potential initiation triplets either in or out of phase in the mRNA.

In 1974 Shine and Dalgarno presented a hypothesis, which attributed the selection specificity, to the interaction between a polypurine region located in the vicinity of the 5' end of the initiation triplet and the 3' terminal sequence of the RNA of the small ribosomal subunit (1). Since then strong evidence has substantiated this suggestion and it is now believed that binding of mRNA to the 30S ribosomal subunit is primarily stabilized by two independent RNA-RNA interactions : 1) fMet-tRNA anticodon loop and initiator codon ; 2) the polypurine-rich portion of mRNA located in the vicinity of the 5' end of the initiation codon, pairing with the pyrimidine-rich 3' terminus of 16S RNA. The number of base pairs possibly involved in this last interaction varies from 3 to 9, the average being 4-5. The number of nucleotides separating the complementary region from the initiator triplet also varies with an average of 10 from middle to middle (2) (Table 1).

RESULTS

I would like to give only one recent evidence in favor of Shine and Dalgarno interaction being involved for the binding of ribosome to initiation site. The hypothesis predicts that if the 3' terminal region of the 16S tRNA is blocked, binding of the ribosome to the initiator region of the mRNA should be diminished or prevented while binding of the nucleotide AUG should not be affected. Taniguchi and Weissmann (4) have shown that the oligonucleotide A-G-A-G-G-A-G-G-U_{OH}, 8 bases of which are complementary to the sequence A-C-C-U-C-C-U-U-A_{OH}, at the 3' terminal region of the 16S RNA (ΔG = 16.9 kcal/mol) binds tightly to the 30S ribosomal subunit and is released as a complex with the 3' terminal 49-nucleotides of 16S rRNA after digestion with cloacin DF13.

Incubation of low salt-washed ribosomes (which retain initiation factors) and intact QβRNA in the presence of fMet-tRNA leads to the formation of a complex sedimenting at 70S. As in the case of all phage RNAs, ribosomes attach predominantly to the initiation site of the coat cistron, and to a lesser extent to that of the replicase cistron.

TABLE 1. Initiation sequences recognized by E.coli ribosomes (from 3)

mRNA	Ribosome binding sites [a]
R17 A	GAU UCC UAG GAG GUU UGA CCU AUG CGA GCU UUU AGU G
MS2 A	GAU UCC UAG GAG GUU UGA CCU GUG CGA GCU UUU AGU G
Qβ A	UCA CUG AGU AUA AGA GGA CAU AUG CCU AAA UUA CCG CGU
R17 coat	CC UCA ACC GGG GUU UGA AGC AUG GCU UCU AAC UUU
Qβ coat	AAA CUU UGG GUC AAU UUG AUC AUG GCA AAA UUA GAG ACU
f2, MS2 coat	CC UCA ACC GAG GUU UGA AGC AUG GCU UCC AAC UUU ACU
R17, MS2 replicase	AA ACA UGA GGA UUA CCC AUG UCG AAG ACA ACA AAG
Qβ replicase	AG UAA CUA AGG AUG AAA UGC AUG UCU AAG ACA G
f1 coat	UUU AAU GGA AAC UUC CUC AUG AAA AAG UCU UU
f1 gene 5	A AGG UAA UUC ACA AUG AUU AAA GUU GAA AU
f1 gene?	A AAA AAG GUA AUU CAA AUG AAA UU
T3 *in vitro*[b]	AAC AUG AGG UAA CAC CAA AUG AUU UUC ACU AAA GAG
T7 gene 0.3 site a	AAC UGC ACG AGG UAA CAC AAG AUG GCU AUG UCU AAC AUG
T7 gene 0.3 site b	GUA CGA GGA GGA UGA AGA GUA AUG U
λPR	ppp AUG UAC UAA GGA GGU UGU AUG GAA CAA CGC
λCI	TTG CGG TGA TAG ATT TAA CGT ATG AGC ACA AAA AAG
φX174 G	TTT CTG CTT AGG AGT TTA ATC ATG TTT CAG ACT TTT ATT
φX174 F	CCT ACT TGA GGA UAA AUU AUG UCU AAU AUU CAA ACU
φX174 D	ACC ACT AAT AGG TAA GAA ATC ATG AGT CAA GTT ACT
φX174 H	ACT TAA GTC AGG TGA TTT ATG TTT GGT GCT ATT
φX174 B	UAA AGG UCC AGG AGC UAA AGA AUG GAA CAA CUC ACU
φX174 J	ACG TGC GGA AGG AGT GAT GTA ATG TCT AAA GGT AAA
aroB	UUU UUU GGA UGG AGU GAA ACG AUG GCG AUU GCA AUU
trp leader	CAC GUA AAA AGG GUA UCG ACA AUG AAA GCA AUU UUC GUG
trpE	GAA CAA AAU UAG AGA AUA ACA AUG CAA ACA CAA AAA CCG
trpA	GAA AGC ACG AGG GGA AAU CUG AUG GAA CGC UAC GAA UCU
lac z	AAU UUC ACA CAG GAA ACA GCU AUG ACC AUG AUU ACG GAU
lac I	AGU CAA UUC AGG GUG GUG AAU GUG AAA CCA GUA ACG
galE	AUA AGC CUA AUG GAG CGA AUU AUG AGA GUU CUG GUU ACC
galT	TAT CCC GAT TAA GGA ACG ACC ATG ACG CAA TTT AAT CCC

a Underlining indicates contiguous bases complementary to the 3'- oligonucleotide of E.coli 16S rRNA. Dots indicate G.U base pairs. Initiator triplets are indicated by bold type.
b What was originally thought to be bacteriophage T7 has turned out to be T3.

Taniguchi and Weissmann also have shown that preincubation of low salt washed ribosomes with all the components of initiation complex and the above mentioned oligonucleotide leads to a 50 to 87 % inhibition of the initiation complex when the molar ratio of oligonucleotide to QβRNA varies from 2.5 to 10.

We repeated the experience of Taniguchi and Weissmann with the oligonucleotide they provided and high salt wash ribosome supplemented with purified initiation factors and found essentially the same results. The binding of H3 QβRNA is strongly dependent on the presence of initiation factors and fMet-tRNA and is inhibited in the presence of the oligonucleotide mentioned above (Table 2).

TABLE 2. Inhibition of QβRNA binding

Additions	Exp	% H3 QβRNA bound	% inhibition
Control	1	12	-
Control	2	10	-
- fMet-tRNA	1	1	-
+ 20 pmoles	1	6	50
oligo	2	3.7	63

We have also shown that the synthesis of the coat protein is inhibited by this oligonucleotide (Table 3).

TABLE 3. Inhibition of protein synthesis

Additions	cpm	% inhibition
Control	44.600	-
Oligo (in pmol)		
5	36.600	19
10	30.700	31
20	26.300	42

(buffer-Mg-GTP, leucovorin ATP PEP py. kin. tRNA)
70S ribosome 5.7 pmoles ; QβRNA 4 pmole ;
initiation factors (IF_1 + IF_2 + IF_3) 3 pmoles each ;
C^{14} aa mix (0.06 mM of each aa). sp. act. 195 cpm $pmole^{-1}$.

The selection of the initiation codon is not limited to these two interactions. The secondary structure of mRNA controlling the availability of the Shine and Dalgarno sequence as well as that of the codon is an important factor (2). In MS_2RNA there are 5 more potential sites for initiation which are not used. Different proteins are also involved namely initiation factors (called IF_1, IF_2, IF_3) and ribosomal proteins S_1 and S_{12} (2).

I would like today to focus on two aspects of the selection mechanism. 1) the role of initiation factors ; 2) the description of different mutants in the two regions of mRNA postulated to be involved in the recognition of the different cistrons and in the initiation factor IF_3.

Localization of initiation factor IF_3 on the ribosome

As an approach towards understanding how initiation factors participate in the initiation step, efforts were directed on localizing the site of interaction of IF_2 and IF_3 on the ribosome (2).

Using different cross-linking reagents it is possible to cross-link IF_3 and IF_2 to both 30S proteins and 16S RNA. The major proteins cross-linked to IF_3 were identified by specific immunoprecipitation.

Upon irradiation with 365 nm light in the presence of a mononucleotide (5) the major cross-linked proteins were S_{12}, S_{13}, S_{19} and S_{11} and S_7 when using tartaryl diazide (6). This enabled us to localize IF_3 on the right forehead of the 30S ribosomal subunit near the 3' end of mRNA (2) (Fig. 1).

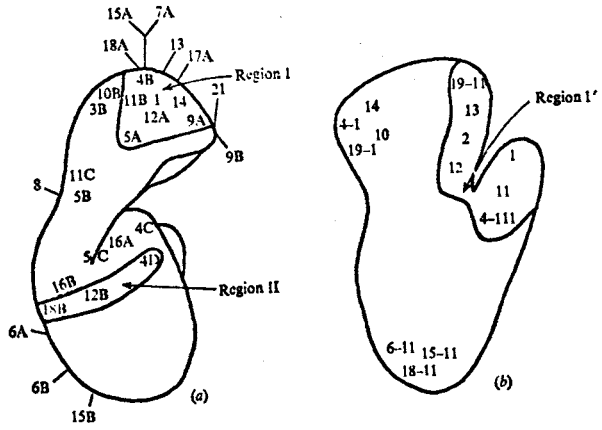

Fig. 1. Models for the 30S subunit (2) : (a) Stöffler and Tischendorf ; (b) Lake and Kahan. The numbers correspond to those of the proteins ; letters differentiate multiple antigenic sites when observed (from 2).

As for cross-linking initiation factors to 16S RNA, after periodate oxidation and reduction of 30S ribosome, it has been found that all three initiation factors as well as S_1 can be cross-linked to the 3' end of 16S RNA (2). Moreover, using the 365 nm irradiation technique

we were able to show that IF_3 cross-links to two different sites on the 16S RNA one being near the 3' terminus (B. Ehresman, J.P. Ebel, B. Cooperman, J. Dondon, M. Grunberg-Manago, unpublished results).

Another approach to the understanding of the role of IF_3 and S_1 and their relation to the 3'OH end of 16S RNA is to use 30S ribosomal subunits where the 3' end of 16 RNA was either only cleaved or removed after colicin E3 treatment (7). Colicin treatment results in a nucleotide cleavage at about the 50th nucleotide from the 3' terminus of the 16S RNA.

When the ribosomes were separated by centrifugation at 53.000 rpm no IF_3 binding to those 30S(-E3) particles was observed (7) (Fig. 2), whereas it has been found that S_1 can bind to those 30S(-E3) particles (8).

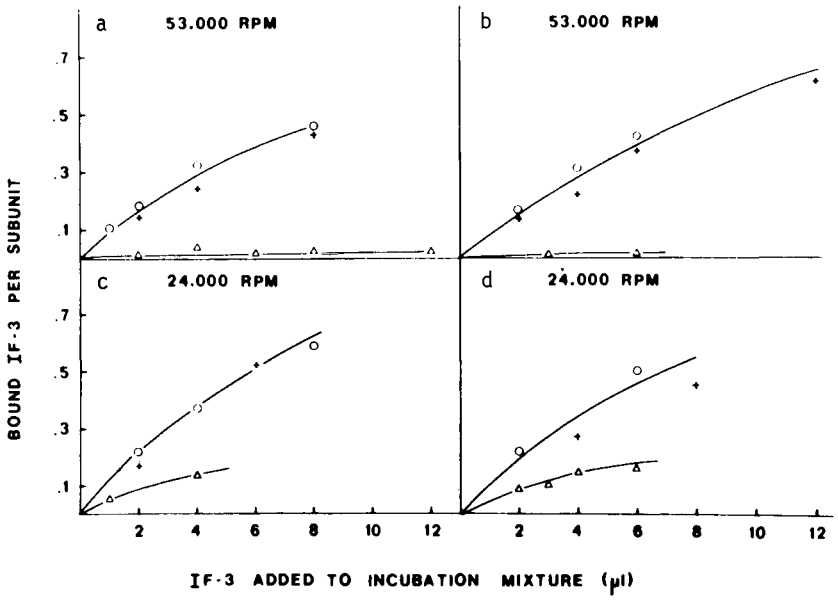

Fig. 2. Stoichiometry of IF_3 binding to various types of MRE600 30S subunits. 54 pmol 30S subunits were incubated for 15 min at 37°C with ^{14}C labelled IF_3 (23.7 pmol/µl).

Fig. 2a. Centrifugation at 53.000 rev/min for 2.5 h. (o) Stoichiometry on control 30S : uncleaved 30S subunits which went through the procedure to remove the colicine E3 fragment. (+) Stoichiometry on NH_4Cl cores, lacking S_2, S_2, S_3, S_9, S_{10}, S_{14}, S_{20}, S_{21}. (Δ) Stoichiometry on 30S subunits lacking the 3' end of 16S RNA and S_1 (30S(-E3)).

Fig. 2b. Centrifugation as in Fig. 2a. (o) Stoichiometry on normal 30S subunits. (+) Stoichiometry on colicin E3 cleaved 30S subunits. (Δ) Stoichiometry on 30S subunits lacking only the 3' end of 16S RNA. 30S(-E3) were incubated with 6-fold excess S_1 (0°C, 10 min) before addition of IF_3.

Fig. 2c. Centrifugation at 24.000 rev/min for 12 h. Otherwise as in Fig. 2a.

Fig. 2d. Centrifigation as in Fig. 2c. Otherwise as in Fig. 2b (from 7).

However when the experiments were repeated at lower rotor speed (24.000 instead of 53.000 rpm) (Fig. 2c and 2d), a striking observation emerged : a 10-fold increase in the apparent affinity of IF_3 for 30S(-E3) (Fig. 2c). On the other hand, the affinity of IF_3 for control 30S, cleaved 30S, and normal subunits was practically unchanged (compare Figs. 2a+b and 2c+d).

The demonstrable pressure dependence of the IF_3-30S(-E3) complex indicates that the molar volume of such an interaction is much larger than when IF_3 binds to either control, cleaved or normal 30S subunits. One explanation is that IF_3 binding to 30S(-E3) results in the trapping of excess solvent in a groove normally occupied by the missing RNA. This would confirm the localization of IF_3 near the 3' OH end of 16S RNA, at the Shine and Dalgarno interaction site.

In order to localize IF_3 in connection with the initiation triplet, a series of affinity labelling experiments were performed with chemically reactive AUG analogs bearing a reactive group (4 bromo-acetamido-phenylphospho) covalently bound either at the 5' or at 3' end of the triplet (O. Pongs, G. Stöffler, H.U. Petersen and M. Grunberg-Manago, unpublished results)

In initiation complexes, 5' reactive AUG was cross-linked to factor IF_3 and to five ribosomal proteins ; no cross-linking to IF_3 occurred with 3' reactive AUG. This localizes IF_3 in the 5' vicinity of the AUG triplet, therefore in the mRNA region postulated by Shine and Dalgarno which is again consistent with the postulated role for IF_3 function.

IF_3 has another activity ; it prevents the association of the 30S to the 50S ribosomal subunit. It was postulated that this interaction occurs between 16S RNA and 23S RNA involving the site on 16S RNA near the one implicated in mRNA binding (9). IF_3 stabilizes mRNA-16S RNA interaction and thus prevents 16S RNA-23S RNA interaction (Fig. 3).

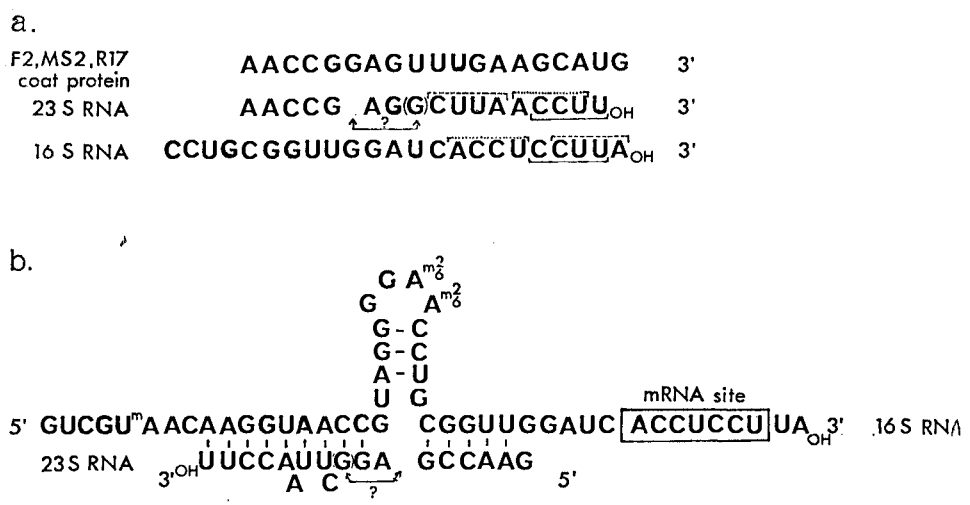

Fig. 3. Possible base-pairing between 3'-OH termini of 16S RNA and 23S or mRNA. (a) The nucleotide sequences of the 3'-end of the 16S RNA and 23S RNA are compared with those of the bacteriophage RNA ribosomal binding site associated with the coat-protein cistron. (b) Hydrogen-bonding schemes are depicted for the 16S and 23S RNA interaction (from 9).

In summary our present knowledge of initiation codon selection is consistent with the existence of the two RNA-RNA interactions. Protein factors are involved in both these interactions.

The different protein factors are more important for ribosome binding at initiation sites when Shine and Dalgarno base pairing is weak (10) ; this is evidenced by a more stringent factor requirement for the binding at the coat protein and replicase mRNA binding sites (3-4 bases pairing) than at the maturation site (7 base pair) (Table 4).

TABLE 4. Effect of S_1 and factors on recognition of R17 initiator regions (from 10)

Expt. No.	Ribosomes	Factors	Ratio A:coat:replicase
I	30S	IF2,IF3	1:2.0:0.5
	30S	-	1:0.2:0.05
	30S(-S1)	IF2,IF3	1:0.3:0.04
	30S(-S1)	-	1:0.4:0.03
	30S	Crude	1:7.1:2.7
	30S	IF2,IF3	1:3.6:0.8
	30S	-	1:0.2:0.2
	30S(-S1)	IF2,IF3	1:0.8:0.2
	30S(-S1)	-	1:0.1:0.06

IF_1 and IF_2 stabilize codon-anticodon interaction while IF_3 is only required with mRNA containing the Shine and Dalgarno polypurine region ; it is not necessary when an AUG triplet is used as mRNA (2) (Table 5).

TABLE 5. Stimulation of fMet-tRNA binding to 30S subunits by initiation factors

Additions	poly (A,G,U)	AUG
IF2	511	553
IF2 + IF3	3.167	956
IF2 + IF1	2.597	2.923
IF1 + IF2 + IF3	5.517	2.102

Mixtures contained in 100 µl : 50 mM Tris-HCl, pH 7.4 ; 100 mM NH_4Cl ; 5 mM Mg^{2+} acetate ; 7 mM 2-mercaptoethanol ; 1 mM GTP ; 0.1 A_{260} units poly (A,G,U) or 0,5 A_{260} units AUG ; 1.0 A_{260} units (^{3}H)fMet-tRNA, sp. act. 3.000 $pmole^{-1}$; 0.35 A_{260} units 30S subunits ; and initiation factors as indicated : 15 pmole IF_1 ; 10 pmole IF_2 and 10 pmole IF_3. Mixtures were incubated 10 min at 37°C, diluted with cold buffer and filtered through Millipore HA nitrocellulose filters ; the filters were dried and immersed in scintillation fluid for counting. The results are reported as counts per min, following the subtraction of blanks (minus all factors) of 855 cpm for AUG and 2.677 cpm for poly (A,U,G) (Hershey,J.W.B., Dondon,J., Grunberg-Manago,M., unpublished experiments).

Studies with different mutants

It is obvious that information as to how essential are the two RNA-RNA interactions and the different proteins involved in initiation in vivo can only be obtained with the help of specific mutants. Mutants in the two regions of mRNA involved in initiation have now been isolated and studied. The first series of mutants are those affecting the gene 0.3 of bacteriophage T7 (11).

This gene codes for an early mRNA of about 600 nucleotides long that specifies two proteins : 1) the 0.3 protein of about 100 amino acids long, made in large amount during T7 infection, and responsible for overcoming host restriction and 2) a protein referred to as 0.4 protein of unknown function. The initiation site on the 0.3 mRNA has been identified by sequencing the 0.3 protein and the mRNA which is protected from nuclease digestion by ribosome during initiation complex formation.

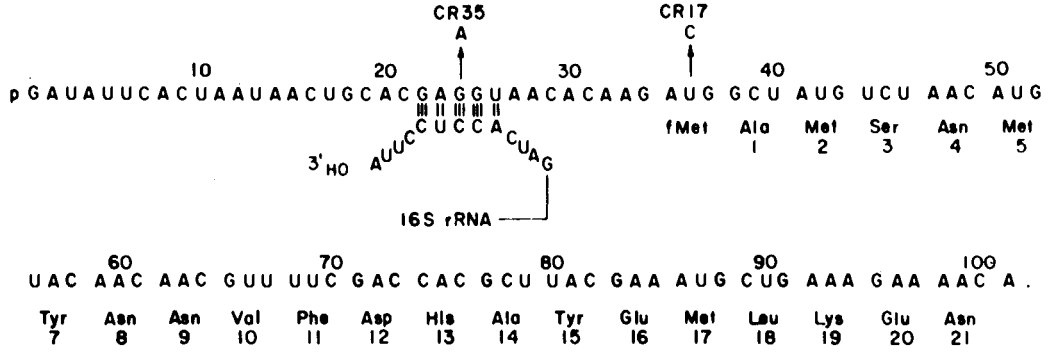

Fig. 4. Nucleotide and amino acid sequences near the beginning of the 0.3 protein. The amino acid sequence was determined from the purified 0.3 protein which lacks the initial fMet (unpublished data). The nucleotide sequence at the 3' end of the 16S ribosomal RNA is also given, to show the potential pairing between the 0.3 RNA and the ribosomal RNA. The nucleotide changes caused by the CR17 and CR35 mutations are indicated. The CR35a-1 suppressor mutation changes nucleotide 23 of CR35 from A to G, producing the sequence GGAG for nucleotides 22-25 (from 11).

The Shine and Dalgarno sequence has been identified as GAGGU corresponding to a 5 base complement between the 0.3 RNA of T7 and the 16S RNA of E.coli (Fig. 4).

Two mutants in the 0.3 gene having a greatly reduced rate of synthesis for 0.3 protein but normal rate for the 0.4 protein were isolated (CR17 and CR35).

The nucleotide sequence of each mutant was found to differ from the wild type by a single base. In CR17 a U to C transition occurs at nucleotide 36, changing the initiator AUG triplet to ACG. In CR35 a G to A transition occurs at nucleotide 24 in the center of the Shine and Dalgarno sequence interrupting the five base sequence (nucleotides 22-26) that is complementary to the sequence at the 3' end of the 16S ribosomal RNA. It is evident that a single mutation either in the Shine and Dalgarno sequence or in the anticodon drastically affects the rate of protein synthesis.

Two spontaneous phenotypic revertants were also isolated. CR17-1 and CR35a-1 ; sequence analysis showed that they both retained their original mutation but had acquired a second mutation.

The additional mutation in CR35a^{-1} was an A to G transition at nucleotide 23 which created a new four base complementarity with bases near the 3' end of 16S ribosomal RNA. The base change occuring in the other mutant CR17-1 is neither in the initiation codon nor in the Shine and Dalgarno sequence. Studier et al. (11) investigated the ability of the 0.3 RNA of wild type and mutants to bind to ribosomes. The protected site for wild type or the mutants CR17 in the codon, was found to contain at least nucleotides 17-50 of the 0.3 mRNA and not to extend past nucleotides 52. The protected site of CR35 (Shine and Dalgarno mutant) has shifted about 12-15 bases from wild type to position containing nucleotides 34-62 and not extending beyond 27-65 ; this 12-15 base shift suggests that ribosomes may bind in a position that initiates at the in-phase AUG at nucleotide 50-52, 15 bases from the AUG that initiates in the wild type. It may be significant that this AUG is followed by an A, which, as will be seen further, stabilizes codon-anticodon interaction.

The CR35 mutation not only causes a great decrease in the rate of synthesis of 0.3 protein but also shifts the position of the ribosome binding site observed in vitro. The CR35a-1 suppressor mutation shifts the ribosome protection site back to the wild type position. The CR35a-1 suppressor mutation restores both the wild type rate of synthesis and the wild type position of ribosome binding. This suggests that pairing between the mRNA and the 16S RNA may be important both in selecting the site in mRNA at which ribosome binds and in determining the efficiency of initiation of protein chains.

The CR17 mutant with a change in the AUG initiation codon to ACG causes a drastic decrease in the rate of synthesis of 0.3 protein both in vivo and in a cell free protein synthetizing system, but ribosomes in vitro bind at the same site and with the same efficiency as does wild type 0.3 RNA. It is possible that it is the Shine and Dalgarno interaction that results in ribosome binding at this site even in the absence of an AUG.

The other mutants I would like to describe exhibit changes in the initiation codon for the Qβ coat protein. Taniguchi et al. (1977) (12) obtained mutants of the Qβ coat protein cistron in which the third position of the initiator AUG and the next residue (the first position of the succeeding alanine codon) were altered. They compared the potential base pairing between the initiator tRNA anticodon region and the relevant QβRNA sequence with the relative efficiency of in vitro ribosome attachment to the same four initiator regions.

The efficiency of ribosome binding increased when the nucleotide following an AUG initiation codon was changed to A, presumably because AUGA can form an additional base pair with the anticodon loop of fMet-tRNA (Table 6).

TABLE 6. mRNA.tRNA interaction at Qβ coat protein initiator region[a] (from 12)

	mRNA sequences	Relative ribosome binding
wild-type	c**AUG** G	1
mutants	C**AUG** A	2.8
	C**AUA** G	< 0.1
	C**AUA** A	0.33

anticodon loop sequence of $tRNA^{fMet}$: 3' $AUACU_5$'

a. Mutant QβRNAs and ribosome binding data. Underlining indicates possible base pairing between the mRNA and the anticodon loop sequence of $tRNA^{fMet}$. The "initiator triplet" appears in bold type.

In the mutant isolated by Weissmann and Taniguchi a change in the initiation codon for the Qβ coat protein from AUG to AUA completely abolished ribosome binding in the absence of the additional A in the next nucleotide ; this is in opposition with what happens in the CR17 mutation where AUG is changed to ACG. The different strength of the Shine and Dalgarno base pairing in the 0.3 mRNA (5 base pairs) could possibly explain this opposition and would suggest that the interaction of AUG with fMet-tRNA is more important for binding at sites that have a relatively unfavorable interaction with 16S RNA.

It can be inferred that initiation complex formation is more or less dependent on either one of the two RNA-RNA interactions depending on the nature of the mRNA : when the mRNA-rRNA interaction is weak, mRNA binding to ribosomes has to be mainly stabilized by IF_2-dependent codon-anticodon interaction., IF_3 being drastically required ; conversely when mRNA-rRNA interaction is strong, the interaction between initiator codon and anticodon can be weak (two out of three base-pairs) and initiation factors are less essential.

In order to definitely understand the role of IF_3 in vivo it is necessary to have conditional mutants in IF_3. A thermosensitive mutant with a thermolabile IF_3 has been isolated (13). E.coli thermosensitive mutants were first selected after nitrosoguanidine mutagenesis and enrichment for thermosensitivity by the tritiated amino acid "suicide method", and then screened in vitro for thermolabile initiation factors (13).

The effects of preincubating the extracts of the different mutants at 40° was compared using a first test which is dependent on initiation factor activity and a second which is not. The first test measures the incorporation, into TCA precipitable products, of (^{14}C) valine in response to poly (U,G) at 5 mM Mg^{2+}, using precharged aminoacyl-tRNAs ; the second test measures (^{14}C) phenylalanine incorporation in response to poly U at 10 mM Mg^{2+}, using precharged (^{14}C) phenylalanyl-tRNA. Both tests should be insensitive to amino acid tRNA ligase modifications in the extracts. In these tests some mutants did not behave like the parental strain and among them strain C18 was further studied (13) (Table 7).

A series of experiments show that the thermolability of the extract was due to a defect in initiator factor (Fig. 5) further characterized as IF_3. Genetic data show that the Ts mutation is located near the 38 min on the E.coli map and is 68 % cotransducible with the aro D marker.

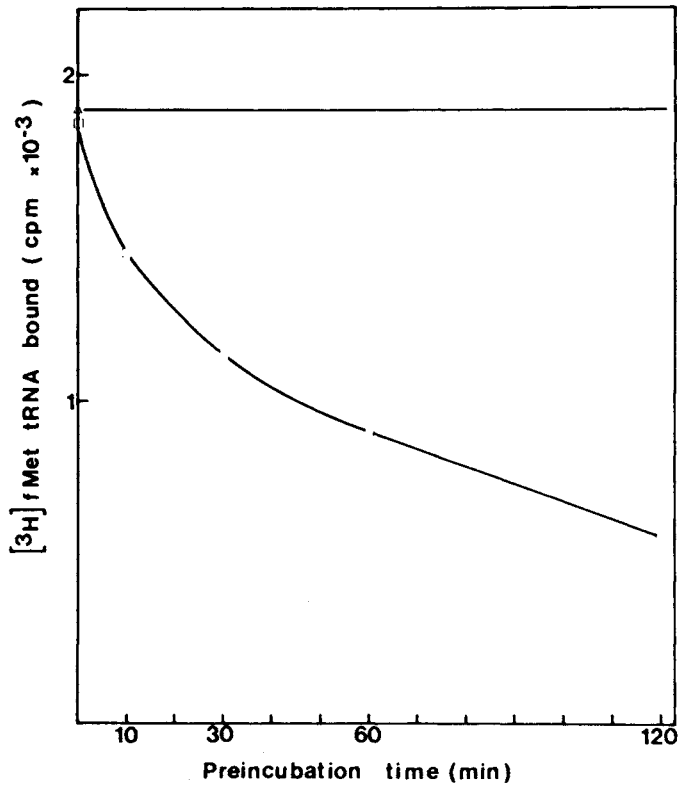

Fig. 5. Inactivation kinetics of crude IF fractions from mutant and parental strains. Preincubation was carried out at 43°C. □ crude IF fraction from C18 ; △ crude IF fraction from D1.

TABLE 7. Screening of thermosensitive mutants.

strains	% activity after preincubation at 40° (^{14}C) phenylalanine	(^{14}C) valine	$\frac{(^{14}C)\ \text{phenylalanine}}{(^{14}C)\ \text{valine}}$
C 18	158	71	0.44
C 90	135	72	0.53
C 259	77	40	0.51
C 38	70	37	0.52
C 178	82	96	1.17
D 1	102	74	0.72

Different hybrid phage lines prepared in vitro and containing the E.coli region near the 38 min were used to infect mutant strain. Several phages confering thermoresistant phenotype were thus selected (Table 8) and it was shown that they contain the structural gene for IF_3 (14).

TABLE 8. Thermolability of fMet-$tRNA^{fMet}$ binding activity (from 13)

Strains	Activity left % 50°	Activity left % 55°
AB 1361	21.1	13.6
AB 1361-2	56.1	24.1
AB 1361-7	59.3	23.5
AB 1365	67.9	23.2

AB 1361 thermosensitive mutant strain
AB 1365 wild type
AB 1361-2 infected with a hybrid λ phage p2
AB 1361-7 infected with a hybrid λ phage p7

Several other phages were prepared and enabled us to map precisely the gene for IF_3 on the E.coli map (15) (Fig. 6).

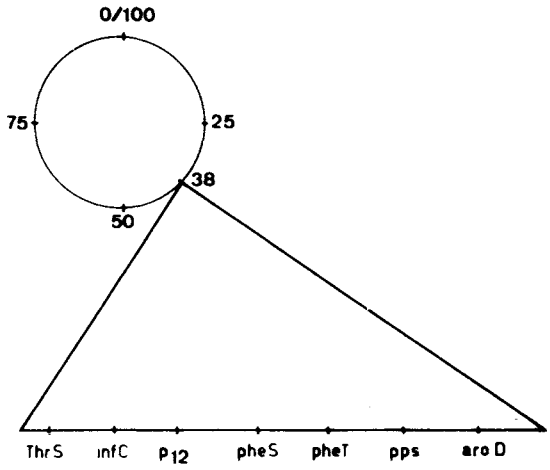

Fig. 6. Mapping of initiation factor IF_3 gene (infC) in E.coli
thrS threonyl-tRNA synthetase
infC IF_3
P12 protein PM 12.000
pheS subunit α phenylalanyl synthetase PM 38.000
pheT subunit β phenyl-tRNA synthetase PM 94.000 (from 15)

We are now in the process of cloning the IF_3 gene on a plasmid and of studying the regulation of its synthesis and function in vivo.

CONCLUSION

Our present knowledge of the selection of initiation codon is consistent with the view of two RNA-RNA interactions :

Shine and Dalgarno base pairing
codon and anticodon base pairing

protein factors IF_3 and S_1 being involved in the first one, IF_1 and IF_2 in the second.

Initiation factors do not in themselves display specificity for particular mRNAs ; nevertheless, the amount of IF present can affect relative mRNA translation. This may be clearly grasped if mRNAs are considered to be divided into classes based on their affinity for ribosomes, depending largely on the stability of the Shine and Dalgarno interaction.

When ribosomes are present in excess of mRNA then all species will be efficiently translated. When, however, mRNAs are in excess of available ribosomes, competition between them will take place in initiation and those species making the most stable interaction will be preferentially translated.

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THE RIBOSOMAL PEPTIDYL TRANSFERASE CENTER. POSSIBLE BASES FOR THE COMMON ACTION OF PEPTIDE BOND FORMATION INHIBITORS OF BACTERIAL AND EUKARYOTIC RIBOSOMES

D. Vázquez and J. P. G. Ballesta

Instituto de Bioquímica de Macromoléculas, Centro de Biología Molecular, C.S.I.C. and U.A.M. Canto Blanco, Madrid-34, Spain

Abstract - Mainly protein L16 and to a lesser extent L2, L15, L27 of the bacterial ribosome and proteins L21/23, L26, L27, L28, L29 and L36 of rat liver ribosomes have been postulated as being involved in the ribosomal peptidyl transferase center. However no single protein has been found individually responsible from this activity and when bacterial ribosomes are treated with proteinase K, all these proteins that might be involved in peptidyl transferase activity are lost at faster rate than the activity itself. Furthermore other studies suggest that there is no identity between any of the individual proteins of the bacterial and eukaryotic ribosomes that were postulated as being involved in the peptidyl transferase activity, although there are a number of antibiotics (sparsomycin, blasticidin S, gougerotin, amicetin and anthelmycin) which are equally active in blocking peptidyl transferase activity by bacterial and eukaryotic ribosomes. Furthermore the treatment with a number of chemical reagents that modify the proteins have very little or no effect on the peptidyl transferase of the bacterial ribosome. On the other hand there is increasing evidence supporting the relevant role of the ribosomal RNA in the peptidyl transferase center. Thus the replacement of Mg^{2+} by Ba^{++} induces the breakage of the ribosomal RNA with a concomitant decrease in the peptidyl transferase activity. Furthermore ribosome treatment with a number of reagents that chemically modify the ribosomal RNA also affect drastically the peptidyl transferase activity. Therefore we propose that antibiotics which specifically inhibit peptide bond formation by bacterial and eukaryotic ribosomes (sparsomycin, gougerotin, blasticidin S, amicetin and anthelmycin) might recognize some moiety common in the RNA of the larger ribosomal subunit.

PROTEIN INVOLVEMENT IN THE RIBOSOMAL PEPTIDYL TRANSFERASE ACTIVITY

Peptide bond formation in protein synthesis is catalyzed by the larger subunit of prokaryotic (Ref. 1) and eukaryotic (Ref. 2) ribosomes. The catalytic center in both cases was termed peptidyl transferase in accordance with standard enzyme nomenclature (Ref. 3). It was initially assumed that ribosomal peptidyl transferase, being an enzyme activity, might be associated with some ribosomal protein(s) of the larger ribosomal subunit. It was indeed shown by different techniques that some proteins might be required for the active conformation of the ribosomal peptidyl transferase center but no individual protein has been found responsible for this activity. Indeed it was shown in early studies that a series of protein deficient particles (β-, γ-cores) can be prepared from 50S subunits by isopycnic centrifugation in CsCl solutions of decreasing Mg^{++} concentrations (Ref. 1). The cores contain 23S and 5S RNA but lack increasing numbers of 50S proteins. The β-cores, which lack proteins L1, L7, L12, L25 and have reduced amounts of L6, L16 and L31 (Ref. 4) possess good activity for catalysis of peptide bond formation (Ref. 1). In contrast, the γ-cores, which lack proteins L1, L6, L7, L10, L12, L15, L16, L25, L31 and L33 and have reduced amounts of L5, L8, L9, L11, L18, L20, L27, L28 and L30 (Ref. 4) are devoid of these activities (Ref. 1). Restoration of activity can be achieved by readdition to the γ-cores of the proteins separated in the conversion β-, γ-cores (Ref. 5).

In further studies we have treated radioactive ribosomes from *Escherichia coli* with increasing concentrations of NH_4Cl in the presence of 50% ethanol at 37°C (Ref.6). The treated particles were tested for activity in several functional assays directly or indirectly related to the peptidyl transferase center, such as "fragment

reaction", binding of (U)CACCA-Leu-Ac and (U)CACCA-Leu, and binding of chloramphenicol, lincomycin and erythromycin (Figure 1). Peptidyl transferase activity is unaffected by the treatment up to 1.5 M ammonium chloride and even the activity is slightly stimulated at lower salt concentrations. The capacities for binding the acetylated fragment, chloramphenicol and erythromycin are lost in parallel while lincomycin binding is slightly more resistant to the treatment (Figure 1).

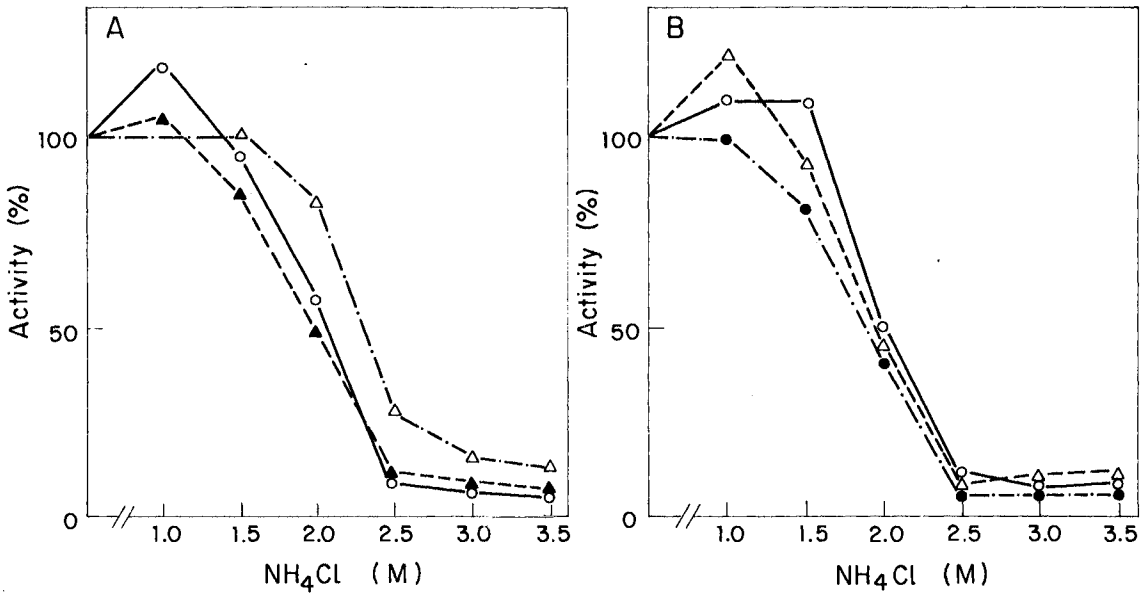

Fig. 1. Activity of 70S ribosomes treated with increasing concentrations of NH_4Cl. (A) Peptidyl transferase activity (O—O), binding of (U)C-A-C-C-A-Leu-Ac (▲—▲), binding of (U)C-A-C-C-A-Leu (△—△). (B) Binding of chloramphenicol (●—●), binding of erythromycin (△—△), binding of lincomycin (O—O).

The proteins present in the ribosomes treated with ethanol-NH_4Cl were extracted and separated by two-dimensional gel electrophoresis. The estimation of the proteins present was performed using radioactive ribosomes labelled with tritium and ^{14}C. The only protein whose release pattern parallels the lost of peptidyl transferase activity is protein L16 (Figure 2). In the cases of all the other proteins either they are released well before the peptidyl transferase activity is lost or are still attached to the particle after the activity is lost. These results clearly suggest an important role of protein L16 of the bacterial ribosome in the peptidyl transferase activity (Ref. 6).

This important role of L16 is also supported by reconstitution experiments of peptidyl transferase (Ref. 7), chloramphenicol binding (Ref. 8), and erythromycin binding (Ref. 9) activities. The ability of L16 to reconstitute the peptidyl transferase activity is abolished by ethoxyformic anhydride treatment of the protein indicating the involvement of his histidine residue in that activity (Ref. 10). The possible involvement of protein L16 in the peptidyl transferase center was also suggested by affinity labelling experiments with some chloramphenicol (Ref. 11), and Phe-tRNA radioactive (Ref. 12) analogues since L16 is one of the proteins labelled by these antibiotics when they bind to the ribosomes. Furthermore 47S particles accumulated in E. coli mutants lack only proteins L16, L28 and L33 and are devoid of peptidyl transferase activity and do not bind chloramphenicol (Ref. 13).

However the use of different techniques and experimental approaches suggested in some cases the involvement of other ribosomal proteins in the peptidyl transferase activity besides or instead protein L16. Thus bacterial ribosomes treated with 2-methoxy-5-nitrotropone are inactivated for polypeptide synthesis, aminoacyl-tRNA binding, and elongation-factor-dependent GTPase but catalyze peptide bond formation (Ref. 14). When proteins from treated 50S subunits were analyzed by two-dimensional polyacrylamide gel electrophoresis, proteins L3,

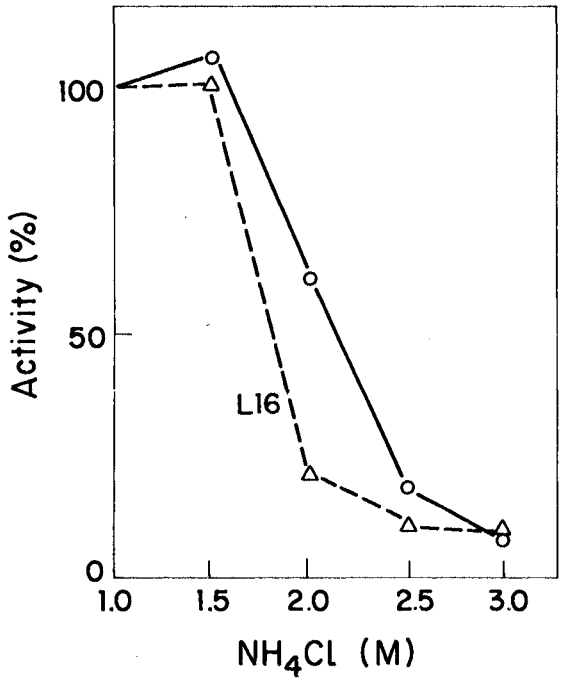

Fig. 2. Release of protein L16 from 60S ribosomes by increasing concentrations of NH_4Cl in the presence of 50% ethanol. ^3H-labelled ribosomes were treated and the proteins in the core particles were separated by two-dimensional gel electrophoresis together with a fixed amount of ^{14}C-labelled proteins from untreated ribosomes as internal control. The ratio of $^3H/^{14}C$ in each spot of the gel plates prepared from untreated particles was taken as 100%. The patterns of release of protein L16 (Δ--Δ) and of loss of peptidyl transferase activity are represented (0—0).

and L15 were found not to be affected by the treatment, proteins L1, L2, L4, L10 L13, L16, L17, L18, L22 and L24 showed spots of much less intensity than the controls and the other protein spots were not detected (Ref. 14). These results together with the reconstitution experiments of γ-cores (Refs. 1,4) pointed to protein L15 as possibly involved in the peptidyl transferase center of the ribosome. The involvement of protein L15 in the peptidyl transferase center has also been suggested by affinity labelling with p-nitrophenyl-carbamyl-Phe-tRNA (Ref. 12). Affinity labelling experiments with a number of chloramphenicol (Refs. 10, 13) and Phe-tRNA (Refs. 12, 16) analogues suggested the possible involvement of proteins L2, L27, L24, L13 and L14 on the peptidyl transferase center.

Although protein L11 was initially postulated as either a part of the active center or even as the actual peptidyl transferase itself (Ref. 4) it was soon realized that this is not the case since 50S-derived cores deprived of L11 are as active as control 50S subunits in promoting peptide bond formation (Refs. 17-19).

However, γ-cores which lack proteins L1, L6, L7, L10, L12, L15, L16, L25, L31 and L33 as indicated above (Ref. 4) bind (3H)puromycin (Ref. 20) (an analogue of the substrate that binds to the acceptor site of the peptidyl transferase center) and CACCA-Leu-Ac (Ref. 4) (a substrate that binds to the donor site of the peptidyl transferase center). Indeed there are no significant differences in the total extent of (3H)puromycin binding to 50S ribosomal subunits and γ-cores (Table 1). Therefore proteins L15 and L16 do not appear to be an absolute requirement for substrate binding to the ribosomal peptidyl transferase center.

The main difficulty of assigning specific functional roles to certain ribosomal proteins on the bases of reconstitution experiments is that the lack of the same protein in different ribosomal cores can affect the conformation of such particles diversely and consequently a given response to the addition of the

TABLE 1. (^{3}H)Puromycin binding to 50S ribosomal subunits and derived cores

Conditions	(^{3}H)Puromycin bound (pMoles/tube)
Expt. 1	
50S	1.80
γ-cores	2.10
$SP_{50-\gamma}$	0.87
γ-cores + $SP_{50-\gamma}$	2.44
Expt. 2	
50S	2.24
γ-cores	2.04
$SP_{50-\gamma}$	0.11

(^{3}H)Puromycin binding to 50S subunits, 50S-derived γ-cores and the split protein fraction released in the obtention of γ-cores from 50S subunits ($SP_{50-\gamma}$) was studied by equilibrium dialysis (Fernández-Muñoz and Vázquez, 1973). Concentration of (3H)-puromycin was 10^{-6} M. 50S subunits were added when indicated at 12.5 mg/ml. γ-cores (15 mg/ml) and the corresponding amount of the $SP_{50-\gamma}$ fraction were added when required. Equilibrium dialysis was carried out for 10 hours at 4°C. In Experiment 1 the indicated particles or protein fractions were preincubated, prior to the equilibrium dialysis, in 20 mM Tris-HCl buffer pH 8.0 containing 20 mM Mg, 0.1 M NH_4 and 3 mM 2-mercaptoethanol for 90 min at 50°C. No such preincubation was performed in Experiment 2. Experiments 1 and 2 were carried out simultaneously with the same batch of ribosomes.

protein in question is not necessarily the same. We have attempted to overcome such difficulties by studying ribosome activities of 50S ribosomal subunits disassembled by 2 M LiCl reconstituted in the presence of an excess of ribosomal proteins previously inactivated by treatment with fluorescein isothyanate (Ref. 21). The reconstituted particles might show alterations in some functional activities resulting from the incorporation of the inactive ribosomal proteins added exogenously. Thus proteins L16 and L24 are apparently involved both in the GTP hydrolysis dependent on elongation factor G an in peptidyl transferase activity whereas the modified protein L11 does not affect the peptidyl transferase and the modified L27 protein does not affect either of both activities (Ref. 21) (Table 2).

TABLE 2. Activity of 50S ribosomal subunits treated with LiCl in the presence of different modified ribosomal proteins

Treated protein	Fragment reaction	Fragment binding	EF G-dependent GTPase hydrolysis of GTP	
			$-SP_0$	$+SP_0$
	counts min^{-1} (pmol ribosome)$^{-1}$		mol/mol ribosome	
L11	30.5	48.6	9.0	22.1
L13	31.3	49.0	20.9	26.0
L16	18.9	29.0	15.0	14.5
L24	9.1	25.0	9.1	2.9
L25	11.4	19.6	3.3	6.9
L27	41.0	64.9	23.4	31.7
LiCl-treated 50S subunits	42.0	61.3	35.3	35.7
Untreated 50S subunits	73.8	115.5	56.0	63.4

50S subunits were treated with 2 M LiCl in the presence of a 40-fold molar excess of proteins treated with fluorescein isothiocyanate. After reconstitution and reactivation aliquots were taken for the activity tests. The data shown represent the average of three determinations using two different preparations of particles.

Therefore it is likely that L16 and perhaps some other ribosomal proteins might have a role in the overall structure of the particle rather than in the specific peptidyl transferase center.

The above conclusion is strenghthened by the results obtained when the peptidyl transferase activity was studied in ribosomes or 50S ribosomal subunits treated with proteinase K for increasing periods of time. Comparison of the amounts of proteins present in the treated particles with the residual activity indicated that only proteins L3 and L14 are released at a similar rate to that at which peptidyl transferase activity is lost (Ref. 22) (Figure 3). Therefore none of the ribosomal proteins that following other experimental methods were suggested as being possibly involved in the peptidyl transferase activity appear to be good candidates by following the proteinase K treatment. Control experiments showed that proteinase K treatment does not affect the pattern of the ribosomal RNA (Ref. 22).

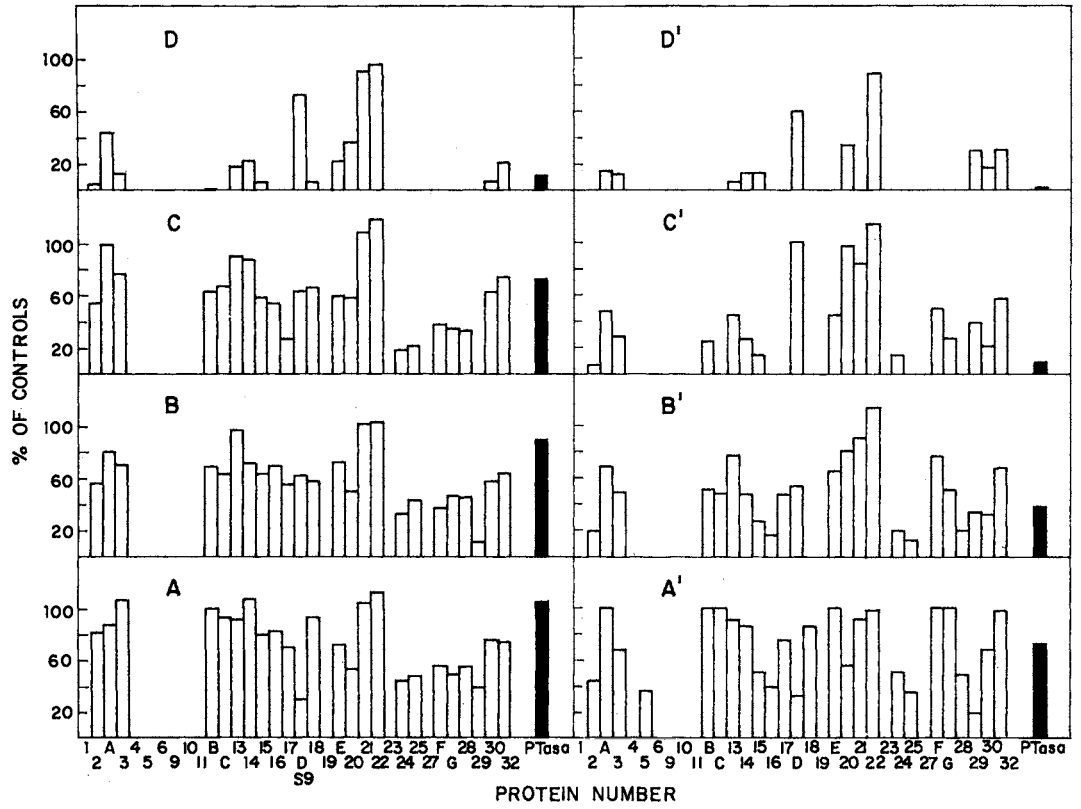

Fig. 3. Amounts of protein present in treated particles. A, B, C and D: 70S ribosomes treated with proteinase K for 30 min, 1 h, 2 h and 5 h respectively. A', B', C' and D', 50S subunits treated with proteinase K for 10 min, 30 min, 1 h and 2 h respectively. The shaded column represents the peptidyl transferase activity of the particles.

In ribosomes from eukaryotic cells the studies on the proteins implicated in ribosomal functions are starting now and the data available are rather scarce. Studies were initiated in our laboratory using as model systems rat liver and yeast ribosomes. Several proteins from the large ribosomal subunit have been localized as possibly implicated in the peptidyl transferase center of rat liver ribosomes by treatment with chemical reagents (Ref. 23) and high salt (Ref. 24). Affinity labelling techniques using derivatives of aminoacyl-tRNA have been also used by others in rat liver (Refs. 25, 26) and by ourselves in Saccharomyces cerevisiae (Ref. 27). It is interesting to note that two proteins from the large subunit of rat liver ribosomes, namely proteins L21 and L36, are pointed as possibly related to peptidyl transferase by more than one of the systems used so far (Table 3).

The lack of an equivalent between ribosomal proteins from different systems makes it impossible to relate the findings obtained in rat liver and Saccharomyces cerevisiae or even in the same systems when the techniques used

by the different groups to identified the proteins are different.

TABLE 3. Proteins from rat liver ribosomes related to peptidyl transferase activity by different techniques

Techniques	Ribosomal proteins that might be involved in peptidyl transferase activity
Chemical treatment	L3, L5, L8, L13/15, L21/23, L26 (Ref. 23).
High salt washing	L21, L24, L27, L28, L36 (Ref. 24).
Affinity labelling	L13, L21/23/26, L32/33, L36 (Ref. 25) L27, L29 (Ref. 26).

RNA INVOLVEMENT IN THE RIBOSOMAL PEPTIDYL TRANSFERASE ACTIVITY

Degradation of ribosomal 23S RNA by replacing Mg^{2+} by Ba^{2+} in the ribosomal structure. Mg^{2+} ions play a critical role in the stabilization of the ribosomal structure through its interaction with the rRNA (Ref. 28). The substitution of Mg^{2+} by monovalent, divalent or polyvalent cations induces an alteration in the ribosomal structure with a concomitant irreversible inactivation of ribosome polymeration activities (Refs. 29-31). This loss of activity has been interpreted as a result of the conformational changes that the replacement of Mg^{2+} induces in the ribosome, probably as a consequence of the modification of the rRNA tertiary structure (Ref. 32). However our results show that the irreversible inactivation of the 50S subunit upon replacement of Mg^{2+} by Ba^{2+} is in fact due to degradation of the 23S RNA.

Replacement of the bound Mg^{2+} by Ba^{2+} in the ribosome irreversibly inactivates the particles. Table 4 shows how this inactivation affects all the partial reactions tested, (i.e. fragment reaction, (U)CACCA-(3H)Leu-Ac binding and EF G-dependent GTP hydrolysis) although methanol partially restores the elongation factor dependent activity. However, contrary to what occurs in L7 and L12 deficient cores (Ref. 33), in 2-methoxy 5-nitrotropone (Ref. 12) and ethidium bromide (Ref. 34) treated ribosomes, the addition of proteins L7 and L12 does not restore GTPase activity to $BaCl_2$-particles, indicating that the loss of this activity is not due to the lack of the two proteins. In fact two dimensional gel electrophoresis of the treated particles revealed that all the proteins were present in the particles in amounts similar to those in controls (not shown).

TABLE 4. Activity of 50S ribosomal subunits treated with $BaCl_2$

50S subunits	Fragment reaction cpm/pmol ribosomes	Fragment binding cpm/pmol ribosomes	EF G-dependent GTP hydrolysis molecules/ribosome		
			no addition	+Methanol	+L7/L12
Untreated	214	574	--	--	--
Control	191	404	54.3	89.3	70.7
$BaCl_2$	0	2	3.4	31.0	5.3

Untreated subunits were taken directly from the particle preparation stored at -20°C in 50% glycerol. $BaCl_2$ samples were treated in presence of 1 mM $BaCl_2$. Control particles were treated exactly as the $BaCl_2$ samples but in the absence of barium salt. The particles were incubated for 30 min at 50°C before the assay.

Weiss et al. (Ref. 31) reported that after $BaCl_2$ treatment there was no alteration in the sedimentation properties of the rRNA in a sucrose gradient. However, since this technique is not very sensitive to small alterations in the rRNA structure we decided to study the rRNA by polyacrylamide gel electrophoresis. As Figure 4 clearly shows the 23S RNA became degraded upon $BaCl_2$ treatment.

Degradation of the rRNA upon unfolding or disassembly of the ribosome is probably due to the action of an endogenous RNAse activity that Ceri and Maeba (Ref. 35) have reported to be associated to a protein of the 50S ribosome subunit. These authors reported that this hydrolytic activity can be inhibited by a variety of compounds, among them short chain alcohols like methanol. We have confirmed this protective effect of methanol during LiCl disassembly of

ribosomes (Ref. 36) and assumed that a similar effect could be displayed in the case of the $BaCl_2$ treatment if the irreversibly inactivation is actually due to a degradation of the 23S RNA. Therefore we repeated the experiments with 15% methanol present during the $BaCl_2$ treatment. Table 5 clearly shows that the peptidyl transferase activity as well as the polymerizing activity of the 50S subunits treated in these conditions is protected by the presence of methanol. In this respect it is interesting to note that in agreement with other reports (Ref. 36) the polyphenylalanine synthetic activity has lower structural requirements than the activity in the "fragment reaction". Only in this second case is a preincubation of the particles required in order to reactivate them. The reasons for these discrepancies in activities that should be closely related have been discussed elsewhere (Ref. 36).

The electrophoregrams shown in Figure 5 confirm that methanol acts by protecting the 23S RNA. While the rRNA from $BaCl_2$ 50S subunits appears totally degraded, the rRNA from the particles treated in the presence of methanol still shows a substantial 23S RNA peak.

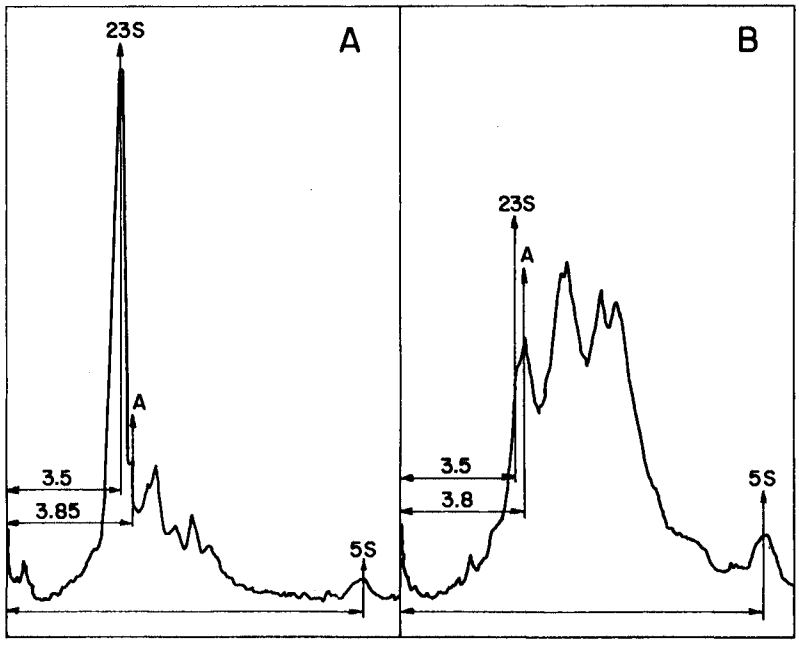

Fig. 4. Gel electrophoresis of ribosomal RNA from $BaCl_2$ treated 50S subunits. Ribosomes (about 250 µg) in 30 µl of electrophoresis buffer (0.09 M Tris-base, 0.09 M boric acid, 0.0025 M EDTA, 0.2% SDS) adjusted to pH 8.3 were directly applied to the gels. Panel A: Control 50S subunits. Panel B: $BaCl_2$ treated 50S subunits. Numbers in the figure indicate distances (cm) to the origin.

Although it is clear from these results that methanol protects the 50S subunits from inactivation by $BaCl_2$ treatment, the alcohol might in some way hinder the substitution of the Mg^{2+} ions by Ba^{2+} thus avoiding the real inactivation due to the replacement of cations. In order to test this possibility the Mg^{2+} content of the particles was measured. The amount of Mg^{2+} present in the ribosomes, either in the presence or in the absence of methanol, being the ratio of Mg^{2+} to RNA phosphate 0.014 and 0.0043 respectively, is well below the limit of 0.025 required for inactivation according to Weiss et al. (Ref. 31).

The results presented above strongly suggest that the irreversible inactivation suffered by the 50S subunit after replacement of Mg^{2+} by Ba^{2+} is due to the degradation of the 23S RNA. It is possible to suppress the inactivation and the RNA degradation at the same time by inhibition of the RNAse activity responsible for both effects.

We feel that the irreversible inactivation is a consequence of the hydrolysis of the 23S RNA by an endogenous RNAse (Ref. 36) that is activated upon

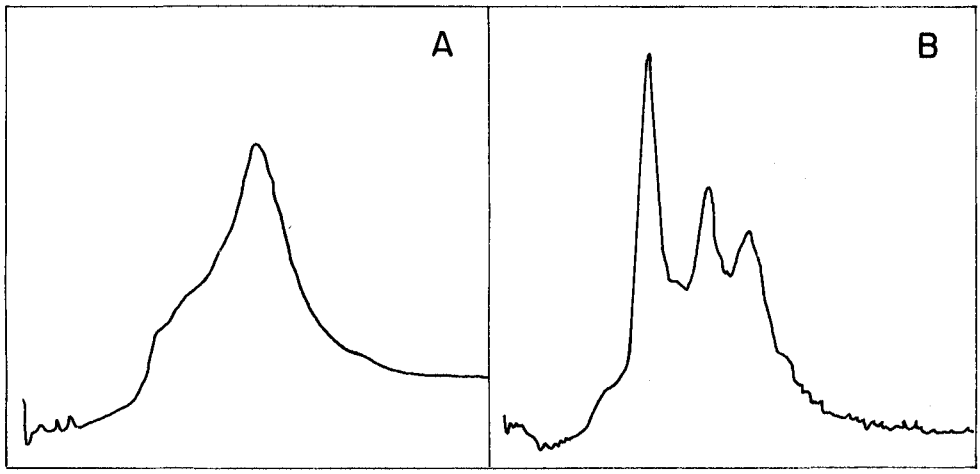

Fig. 5. Ribosomal RNA from 50S subunits treated with $BaCl_2$ in the absence (Panel A) and in the presence (Panel B) of methanol.

TABLE 5. Activity of 50S subunits treated with $BaCl_2$. Effect of methanol

Treatment	Incorporation of (^{14}C)-phenylalanine (pmol)		Fragment reaction (cpm/pmol ribosomes)	
	Without pre-incubation	With pre-incubation	Without pre-incubation	With pre-incubation
Control	10.7	10.9	139	146
$BaCl_2$	0.17	0.85	0.6	0
$BaCl_2$, Methanol	4.4	7.0	4.7	85

50S ribosome subunits were treated with $BaCl_2$ as in Table 3. Methanol was present when required at 15% final concentration. Before the activity tests the particles, in the same ionic conditions required for the assay, were preincubated at 40°C for 30 min. 30S ribosome subunits were present in equimolar amounts in the (^{14}C)phenylalanine incorporation assays.

unfolding of the ribosomal structure caused by the replacement of Mg^{2+} ions. Our results seem to indicate that the unfolding itself, is a reversible process since the $BaCl_2$-treated particles can be reactivated when the degradation of 23S RNA is inhibited by methanol. It is interesting to note that Weiss and Morris (Ref. 29) studying the replacement of Mg^{2+} by polyamines found that the 50S subunits are inactivated with two different kinetics: a fast one corresponding to the replacement of the Mg^{2+} by the polyamine and a second, slow process that could well involve a hydrolytic action of the endogenous RNAse. If our hypothesis is correct, the inactivation occurring in the first type of process would by reversible while the inactivation due to the RNA hydrolysis would obviously be irreversible.

Sensitivity of peptidyl transferase to r-RNA modification. Chemical reagents that affect RNA inactivate peptidyl transferase and polyphenylalanine synthesis at similar rates, while the protein reagents have only slight effects on the former activity under conditions that drastically inactivate the latter (Ref. 37) (Table 6). The simplest interpretation of these results is that RNA contribution to peptidyl transferase activity is more important than that of ribosomal proteins. The high sensitivity of peptidyl transferase to RNA modification can be explained by disruption of the binding sites for the substrates. Proteins might not be a physical part of either substrate binding or peptidyl transferase activity and might have a role in the conformation of the peptidyl transferase center.

Indeed 23S RNA has been labelled by affinity labelling studies with acceptor (Ref. 38) (puromycin) and donor (Ref. 39) substrate derivatives. In further experiments on the affinity labelling with the puromycin derivative the sequence GUC*CGp of 23S RNA has been isolated where C* represents C modified by

TABLE 6. Sensitivity of peptidyl transferase to chemical reagents

Reagent	Concentration mg/mg ribosomes	Treatment time	Specificity	Activity of treated particles (% of untreated controls)	
				Peptidyltransferase	Synthesis of polyphenylalanine
Perphtalic acid	1.5	10 min	RNA	35	10
Bisulphite	2.5	6 h	RNA	28	25
Nitrous acid	1.1	4 h	RNA	40	37
Kethoxal	5.1	30 min	RNA + Protein	38	30
2-Methoxy-5-nitrotropone	0.4	6 h	Protein	80	1
N-ethyl-maleimide	0.025	18 h	Protein	106	48
1,5-difluoro-2,4-dinitrobencene	0.020	90 min	Protein	75	3
5,5'-dithiobis (2-nitrobenzoic)	0.029	2 h	Protein	97	36
N-bromosuccinimide	0.014	30 min	Protein	67	11
Succinic anhydrine	0.1	1 h	Protein	108	3
Maleic anhydrine	0.025	1 h	Protein	90	24

the affinity label (Ref. 40).

PEPTIDE BOND FORMATION AND SUBSTRATE BINDING INDUCED BY SPARSOMYCIN

The catalytic mechanism of peptide bond formation is not well understood. It appears that peptide bond formation takes place spontaneously at a very high rate once the substrates are bound correctly to the peptidyl transferase center. Therefore substrate binding to the acceptor or to the donor site of the peptidyl transferase center as well as peptide bond formation has been frequently studied as a test of the peptidyl transferase center. A cooperative effect on substrate- and antibiotic binding to the A- and P-sites has been repeatedly observed. Thus peptidyl-tRNA bound to the P-site enhances chloramphenicol and thiamphenicol binding (Ref. 41) and sparsomycin enhances substrate binding to the P-site (Refs. 42-45). Conversely peptidyl-tRNA binding to the P-site appears to enhance sparsomycin binding to the A-site since this antibiotic inhibits chloramphenicol binding to polysomes but not to ribosomes (Ref. 46). Since there is ample evidence showing that sparsomycin, chloramphenicol and thiamphenicol act on the acceptor site of the peptidyl transferase center (Ref. 47) the above results suggest a cooperative effect on the binding to the acceptor and the donor site. Therefore the sparsomycin-induced substrate binding to the acceptor site has been frequently studied as a reaction to study peptidyl transferase activity. However, in certain cases the peptidyl transferase activity in the "fragment reaction" assay does not parallel the sparsomycin-induced substrate binding activity to the donor site of the peptidyl transferase center. Indeed treatment with proteinase K of bacterial ribosomes results in a progresive inactivation of the particles when tested for the peptidyl transferase activity but inactivation of sparsomycin-induced fragment binding is more drastic than inactivation of the fragment reaction assay (Figure 6) (Ref. 22). Basically a similar finding was observed with rat liver ribosomes since their derived cores obtained by treatment with 1 M NH_4Cl and 50% ethanol are lacking a few proteins and mantain some 80% of the activity in the fragment reaction assay (Figure 7) whereas their activity in the sparsomycin-induced fragment binding is drastically affected (Ref. 24) (Table 7).

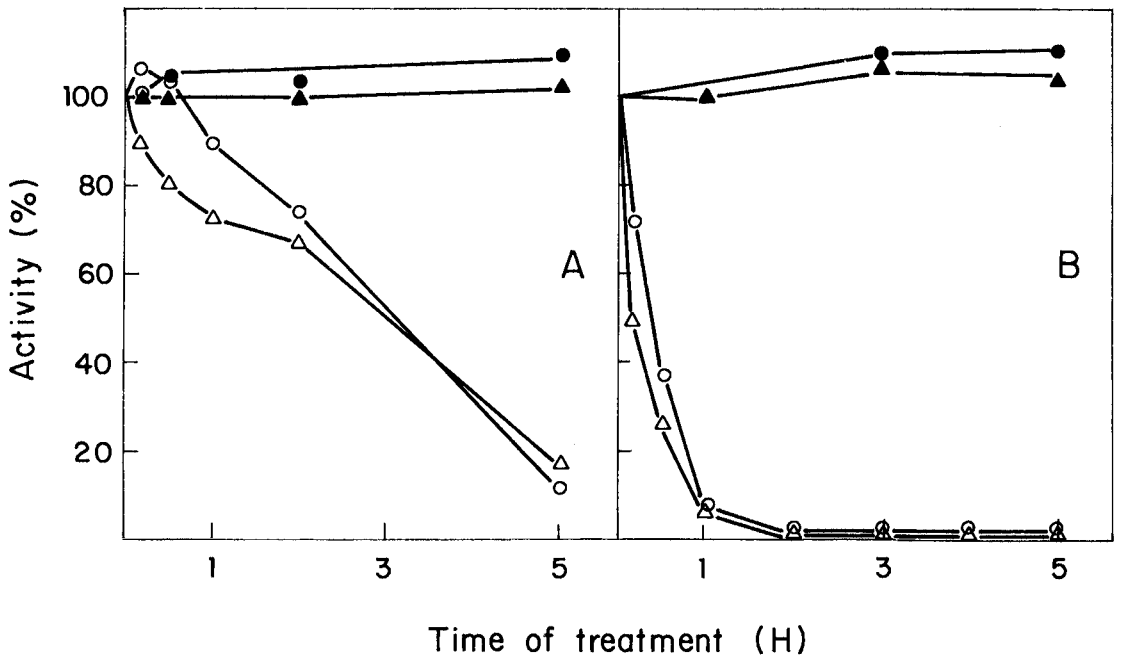

Fig. 6. Activity of proteinase K-treated particles. Particles, either 70S ribosomes (A) or 50S subunits (B), were tested for peptidyl transferase activity in the "fragment reaction" assay (O—O) and sparsomycin-dependent (U)CACCA-(^{3}H)Leu-Ac binding (Δ—Δ). Control particles, treated in the same conditions but in the absence of protease were also tested (●—●, peptidyl transferase; ▲—▲ sparsomycin-dependent (U)CACCA-(^{3}H)Leu-Ac binding).

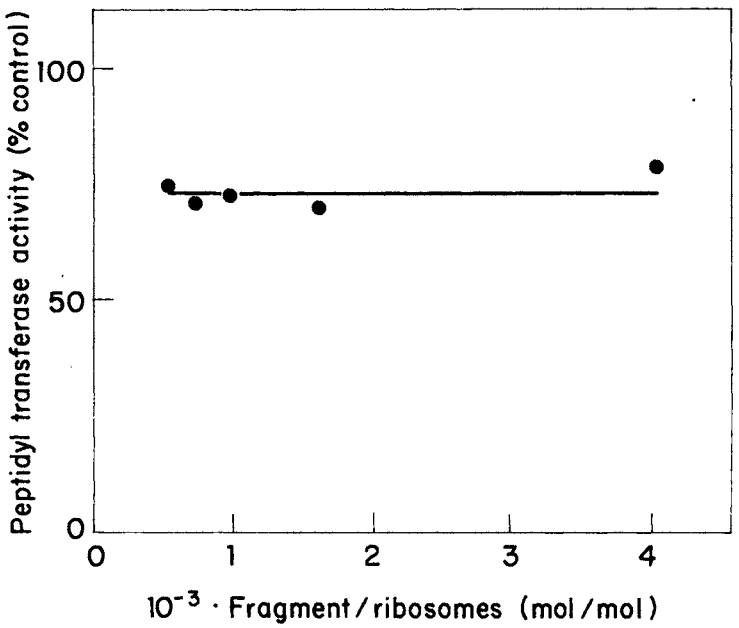

Fig. 7. Peptidyl transferase activity of P_{37}-cores from rat liver ribosomes. The activity is represented as a percentage of the untreated control particles as a function of the ratio of (U)CACCA-(3H)Leu-Ac fragment to particles in the reaction mixture.

TABLE 7. Binding of (U)C-A-C-C-A-(^{3}H)Leu-Ac to P_{37}-cores particles in the presence of sparsomycin

Particle	Fragment bound with ribosomes	
	20 pmol	40 pmol
	counts/min	
80S ribosomes	3301	5141
P_{37}-cores	762	1237

Assay carried out by filtration in the ionic conditions of the "fragment reaction" in the presence of 10 μM sparsomycin and 20 or 40 pmol ribosomes.

Since peptide bond formation is less affected than the binding of the substrate, a direct effect of the protease on the binding site of the fragment must be excluded as responsible for this difference. Therefore the mechanism by which sparsomycin stimulates the binding of the fragment must be preferentially affected in the cores. If this is so, and considering that the inhibitory effect of sparsomycin on peptide bond formation is unaffected in the proteinase K-treated particles (Ref. 22) the substrate binding cannot be stimulated by the same mechanism involved in sparsomycin inhibition of peptide bond formation. By interacting with the A-site of the ribosome the drug probably causes a direct inhibition of peptide bond formation and indirectly stabilizes the binding of the substrate in the P-site, perhaps through a change of conformation of the ribosomal structure or their RNA moiety.

THE NATURE OF THE RECOGNITION SITE(s) FOR ANTIBIOTICS THAT INTERFERE WITH THE PEPTIDYL TRANSFERASE OF BOTH BACTERIAL AND EUKARYOTIC RIBOSOMES

Inhibitors of peptidyl transferase can be classified, according to their specificity, into those affecting systems of (a) the prokaryotic ribosome, (b) the eukaryotic ribosome and (c) both the prokaryotic and the eukaryotic ribosome (Ref. 47). This clearly shows that the peptidyl transferase centers from the prokaryotic and the eukaryotic ribosome have a number of common structural features but differ in others. It is very interesting indeed that the different inhibitors of peptide bond formation acting in both bacterial and eukaryotic ribosomes (puromycin, sparsomycin, gougerotin, amicetin, blasticidin S,

actinobolin and anthelmycin) are precisely those interacting with some moiety with the acceptor site suggesting that there is a similar structure on this site on bacterial and eukaryotic ribosomes. We propose that a sequence of ribosomal RNA which be common to bacterial and eukaryotic ribosomes might be directly involved in this acceptor site of the peptidyl transferase center since (a) none of the proteins postulated as being involved in the peptidyl transferase center of bacterial ribosomes have been found so far in eukaryotic ribosomes, (b) labelling of a defined sequence of the ribosomal RNA by an analogue of puromycin has been described (Refs. 38, 40) and (c) all the peptidyl transferase inhibitors acting on both prokaryotic and eukaryotic ribosomes (gougerotin, sparsomycin, blasticidin S, anthelmycin, amicetin and actinobolin) prevent puromycin interaction with the ribosome (Refs. 47, 48).

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AMINOACYL-tRNA SYNTHETASES: APPROACHES TO THE STRUCTURE AND FUNCTION OF THE ACTIVE CENTERS

D. G. Knorre and L. L. Kisselev*

Institute of Organic Chemistry, Siberian Division of the Academy of Sciences of the USSR, Novosibirsk 90, USSR
**Institute of Molecular Biology of the Academy of Sciences of the USSR, Moscow V-334, USSR*

Abstract - Aminoacyl-tRNA synthetases (EC 6.1.1) play a key role in decoding of genetic information. The main chemical approaches used in the ARSases* investigation are discussed below and illustrated by some results including those obtained in our laboratories with beef pancreas Trp-RSase and E.coli Phe-RSase. The role of the different groups of the aminoacids and ATP was established by investigating either substrate or inhibitor properties of the analogs lacking the respective groups or bearing some modifications of these groups. Some information about the essential SH-groups, histidine and lysine residues was obtained using chemical modifying agents. Affinity modification of enzymes with tRNA and ATP analogs carrying chemically reactive groups was used as an approach for investigation of the structure and function of their active centers.

INTRODUCTION

Aminoacyl-tRNA synthetases (EC 6.1.1., ARSases*) (Refs 1 & 2) relate amino acids to respective codons by attaching the formers to tRNA's bearing complementary anticodons

$$aa^* \leftrightarrow ARSase \leftrightarrow tRNA \leftrightarrow codon$$

The reaction catalyzed by ARSase proceeds via intermediate formation of the enzyme aminoacyl adenylate complex E•aa~pA

$$E + aa + ppA(ATP) \rightleftharpoons E{\bullet}aa{\sim}pA + pyrophosphate$$

$$E{\bullet}aa{\sim}pA + tRNA \rightleftharpoons aa\text{-}tRNA + pA + E$$

Therefore, ARSase deals with three substrates, three products and at least one intermediate. At least four types of sites are necessary to recognize these components: a) aminoacyl site for recognition of the starting amino acid and aminoacyl moiety of aa~pA and aa-tRNA; b) adenylate site for recognition of AMP and of adenylate moiety of ATP and of aa~pA; c) pyrophosphate site for recognition of pyrophosphate itself and pyrophosphate segment of ATP; d) tRNA site (Fig. 1).

Besides aminoacyl adenylate enzyme complexes the direct covalent binding of amino acid to enzyme in the form ameable to be transferred to tRNA was found for beef pancreas Trp-RSase (Refs 3-5).

The data suggesting the existence of the additional centers with the affinity to nucleotides and to some of their analogs were obtained recently in our laboratories. It was found that $\gamma(\beta)$-p-azidoanilides of ATP, GTP, CTP, ADP, GDP being inhibitors of the reactions catalyzed by Trp-RSase from beef pacreas at low concentration enhance the reaction rate up to 20-30% (Ref. 6).

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*Abbreviations used: aa - amino acid or aminoacyl residue; ARSase - aminoacyl-tRNA synthetase; aa-RSase - ARSase specific to aa (e.g. Phe-RSase, Trp-RSase); aa~pA - aminoacyladenylate.

A great number of ARSases were found to be functional dimers of either α_2, or $\alpha_2\beta_2$ structure (Ref. 1). The repeated sequences were found in some enzymes represented by a single polypeptide chain thus suggesting the existence of two similar domains in these enzymes (Ref. 7).

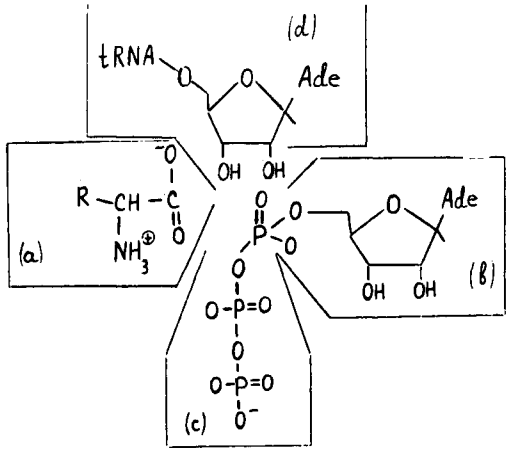


Fig. 1. The main types of groups recognized by ARSases: amino acid (a); ATP (b)+(c); aminoacyl adenylate (a)+(b); pyrophosphate (c); tRNA (d); aminoacyl-tRNA (a)+(d); AMP (b).

The total reaction catalyzed by the enzyme is usually assayed by measuring the covalent binding of radioactive amino acid to tRNA. The first step of the reaction is commonly assayed by measuring the accumulation of [^{32}P]ATP in the presence of [^{32}P]pyrophosphate due to reversibility of this step. The formation of the complexes of ARSases with tRNA, aa-tRNA and aa∼pA may be easily detected using the nitrocellulose filter technique as well as gel filtration. The complexes with other substrates and their analogs may be detected by equilibrium dialysis as well as by fluorimetric titration of the enzyme by the ligand.

The binding of tRNA to one of the subunits decreased significantly the affinity of the other subunit to tRNA for E.coli Tyr-RSase (Ref. 8), E.coli Phe-RSase (Ref. 9), E.coli (Ref. 10) and beef pancreas Trp-RSases (Ref. 11). Strong negative cooperativity is also suggested for aminoacyl adenylate formation (Ref. 12).

Up-to-now the single sequenced ARSase is Trp-RSase from Bacillus stearothermophilus (Ref. 13). The electron-density map of Tyr-RSase at 2.7 Å resolution is described (Ref. 14). Therefore all our knowledge on the active centers of ARSases is based on the chemical and biochemical data.

The main chemical approaches used in the ARSases investigation are discussed below and illustrated by some results including those obtained in our laboratories with beef pancreas Trp-RSase and E.coli Phe-RSase.

SUBSTRATE GROUPS ESSENTIAL FOR THE INTERACTION WITH AMINOACYL-tRNA SYNTHETASES

The role of the different groups of the substrates may be established by measuring either substrate or inhibitor properties of the analogs lacking the respective groups or bearing some modification of these groups. The significance of the group under consideration may be quantitatively described by either K_m'/K_m or K_i/K_m value, K_m being the Michaelis constant of the unmodified substrate.

α-Amino group was found to be essential for the interaction of α-aminoacids with ARSases. The role of this group was discussed recently (Ref. 15) in connection with the results obtained with N^{α}-acylated tryptophanes. K_i/K_m values for N^{α}-formyl tryptophane and N^{α}-acetyltryptophane were found to be 230 and 125 respectively. The similar effect was demonstrated by comparison of the inhibitor properties of Phe∼pA analog phenylalaninyl adenylate and its N^{α}-formyl derivative, K_i'/K_i being 280 (Ref. 16). These data suggest

the existence of some anionic group in the amino acid site of the enzyme. The carboxylic group seems to be less essential (for review see ref. 17).

Usually rather small changes in the specific group of amino acid are permitted without severe damage of the affinity to the enzyme. Thus, phenylalanines monofluorinated in the benzene ring (Ref. 18) and tryptophanes monofluorinated in the indole ring (Ref. 19) were found to be substrates of the respective ARSases. K'_m/K_m values are presented in Fig. 2. It is seen that the smaller is the distance of the fluorine atom to NH_3^+ group in phenylalanine, the lower is the affinity of the respective analog. This may be due to repulsion between the lone electron pairs of F atom and anionic center of the enzyme. In tryptophane the similar decrease in the affinity is seen with the approach of F atom to NH group of the indole ring. This may be explained assuming H-bond formation between NH group of tryptophane and some lone pair bearing atom X of the enzyme. The repulsion between this lone pair and the lone pairs of fluorine may decrease the affinity of the substrate.

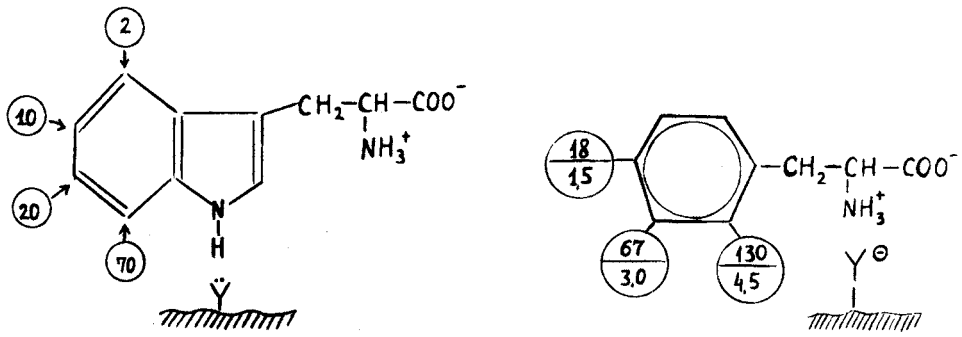


Fig. 2. The decrease of the affinities of the substrates to respective ARSases (beef pancreas Trp-RSase, E.coli MRE 600 Phe-RSase, yeast Phe-RSase) due to substitution of one H for F. K'_m/K_m values are indicated in the circles. In phenylalanine the upper values are those for E.coli, and the lower ones for yeast enzyme.

The most systematic investigation of the ATP analog interaction with ARSases was performed by Freist et al. (Refs 20 & 21). 19 analogs were studied with five yeast ARSases. Complete loss of either substrate or inhibitor properties was found when either C^8 was changed for N or H atom was substituted for Br, N_3, SH or NH_2 in this fragment. In most cases introduction of one CH_3 group in 6-NH_2 resulted in complete loss of the affinity of ATP to enzymes. Methylation of either 2'-OH or 3'-OH group also completely destroyed even inhibitor properties of the analog. Some positions demonstrated quite different significance with different ARSases. The examples are given in the Table 1.

TABLE 1. K'_m/K_m (K_i/K_m) ratios for the ATP analogs with various yeast ARSases (Refs 20 & 21)

Change in the structure	ARSases				
	Phe	Ser	Val	Ile	Tyr
$N_9-C_8 \rightarrow C_8-N_9$	1	33	30	37	- *
$C_2-H \rightarrow C_2-Cl$	0.5	0.83	10	-	-
2'-OH → 2'-H (dATP)	1	3.3	-	-	-

*(-) means the absence of any affinity.

The attachment of the aromatic residues to γ-phosphate via amide bond results in the analog with only slightly decreased affinity to ARSases. Thus, for both γ-anilide (Ref. 22) and γ-p-azidoanilide of ATP (Ref. 23) K_i/K_m value was found to be near 2 for E.coli Phe-RSase. The possibility of

significant changes in the triphosphate moiety of ATP with significant increase in the affinity to beef pancreas Trp-RSase (Ref. 24) and without severe damage of the affinity to Leu-RSase from the cytoplasm of Euglena gracilis (Ref. 25) was demonstrated recently with the reactive ATP analogs.

The role of the AMP moiety of aminoacyl adenylate was studied using modified aminoalkyl adenylate. It was found with phenylalaninyl adenylate that K'_i/K_i value in the Phe-tRNA formation catalyzed by E.coli Phe-RSase is 14 for analogs with C_8-H substituted for C_8-Br, and 220 for analog with cis-diol group oxydized by periodate both with and without subsequent reduction of the aldehyde groups (Ref. 16).

The general features of the tRNA structure essential for tRNA-ARSase interaction are discussed in the recent paper of A. Rich and P. Schimmel (Ref. 26). The main conclusion drawn from the tertiary structure of tRNA and the review of the data dealing with the structural influences on the acceptor ability of tRNA is that the major part of the binding site for most or all ARSases is along and around the diagonal side of the L-shaped tRNA molecule.

Chemical modification of the bases of anticodon was demonstrated to damage severely the acceptor activity for a set of tRNA's (see refs 1 & 27). The catalysis of the hydrogen exchange at C-5 of the uridine in position 8 in E.coli $tRNA^{Ile}$ and $tRNA_2^{Tyr}$ by cognate ARSase was demonstrated thus indicating the close contact of this uridine with the enzyme (Ref. 28). Oligonucleotide A-U-G representing position 8-10 of $tRNA^{Ile}$ was found to bind with Ile-RSase and to be a potent inhibitor of the Ile-tRNA formation (Ref. 29). The oligonucleotides protected by respective ARSases against alkylation with nitrogen mustard derivative were found in E.coli $tRNA^{Phe}$ to be A-G-A-G-Cp in the D-stem (Ref. 30) and in yeast $tRNA^{Trp}$ A-G-G-T in T-stem (Ref. 31). Both results are in agreement with the general concept of the diagonal interaction.

CHEMICAL MODIFICATION OF ARSases

Some information about the groups present in the active centers of ARSases was obtained using chemical reagents specific for one type of the side radicals of the amino acid residues of the enzymes.

The role of SH groups was studied using alkylation with N-ethyl maleimide, reaction with either p-hydroxy or p-chloromercuribenzoate and the reaction with 5,5'-dithio-bis-(2-nitrobenzoate). In most cases it was found that formation of aminoacyl adenylate is damaged to a less extent than the formation of aminoacyl-tRNA (E.coli Ile-RSase (Ref. 32), Leu-RSase (Ref. 33) and Met-RSase (Ref. 34), rat liver Trp-RSase (Ref. 35), yeast Phe-RSase (Ref. 36)). In some of these cases it was also shown, that the tRNA binding properties of the enzyme remain unchanged (Refs 33, 36 & 37) and that aminoacyl adenylate protects strongly the enzyme against inactivation. These data suggest that SH group is essential in the transfer of the aminoacyl residue to tRNA possibly via intermediate thioester bond formation between amino acid and the enzyme (Ref. 36). The peptide containing essential cysteine in E.coli Ile-RSase was sequenced (Ref. 38). The data are presented in the next section in the Table 5. At the same time ARSases with low sensitivity to SH-group modifying reagents are known, too.

Modification of the histidine residues of E.coli Phe-RSase was performed using reaction with diethyl pyrocarbonate and photooxidation in the presence of rose Bengal (Ref. 39). The enzyme modified in the presence of phenylalanine and ATP still catalyzes the ATP-[^{32}P]pyrophosphate exchange but the tRNA-aminoacylating activity is completely abolished. This is not due to damage of the affinity of Phe-RSase towards tRNA. Therefore, it is concluded that histidine residue does not participate in tRNA binding but is essential for transfer of amino acid residue to tRNA.

This is not the case with beef pancreas Trp-RSase. Modification of this enzyme with diethyl pyrocarbonate results in parallel loss of both tRNA aminoacylating and tRNA binding activities. In this case the protonated imidazole groups of the histidine residues may be involved in the interaction with the negatively charged groups of tRNA (Ref. 40).

The role of the positively charged residues of ARSases in the interaction with negatively charged ATP and tRNA up-to-now is investigated only in few cases and only with the lysine modifying reagents. Using pyridoxal-5'-phosphate (Ref. 41) it was demonstrated that one lysine residue essential for aminoacylation catalyzed by ARSases exists in E.coli Val-, Ile- and Leu-

RSases. With Ile-RSase it was shown that enzyme may be protected against inactivation by tRNA.

Modification of the lysine residues of E.coli Phe-RSase with 2,4-pentanedion at pH 7.0 in the conditions were arginine residues remain untouched results in the decrease of the tRNA aminoacylating and the tRNA binding activities with conservation of the catalysis of ATP-[^{32}P]pyrophosphate exchange. tRNA rather than the low molecular weight substrates protect the enzyme against inactivation (Ref. 42).

Acetylation of three different lysine NH_2-groups in Tyr-RSase from Bacillus stearothermophilus was found to be partially protected by cognate tRNA (Ref. 43). The essential observation made in this paper is that one of these residues is protected simultaneously in both subunits in spite of the presence of only one tRNA in the complex with ARSase. This fact may be regarded as indicating that tRNA is in contact with both subunits thus giving a reasonable explanation for the negative cooperativity phenomenon.

The peptides containing the lysine residues protected by tRNA against acetylation were sequenced. They are presented in the Table 5.

AFFINITY LABELLING OF ARSases

A set of reagents for affinity labelling of ARSases is described including ATP, amino acid and tRNA derivatives. The reagents are presented in the Tables 2-4. The affinities of the reagents as compared with that of substrate are given as the ratios of the dissociation constants K'_d/K_d for tRNA derivatives and as K_i/K_m ratios for the ATP and amino acid derivatives.

TABLE 2. The reactive derivatives of tRNA and the ratios of the dissociation constants of the reactive analog and substrate K'_d/K_d

Reactive group and the point of attachment	Enzyme	K'_d/K_d	Ref.
p-NO_2-C_6H_4-O-CO attached to α-amino group of aminoacyl-tRNA	E.coli Met-RSase	data not given	(47)
Br-CH_2-CO attached to α-amino group of aminoacyl-tRNA	E.coli Ile-RSase	data not given	(48)
($ClCH_2CH_2$)$_2$NC_6H_4(CH_2)$_3$CO-(chlorambucilyl) attached to α-amino group of aminoacyl-tRNA	E.coli Phe-RSase	0.26	(49)
	Beef pancreas Trp-RSase	1.15	(11)
N_3-C_6H_4-NHCOCH$_2$- attached to 4-thiouridine ^{4}sU-8	E.coli Phe-RSase	0.95	(50)
	E.coli Met-RSase	1.6	(51)
N_3-C_6H_4-CO-NH-N= attached to $NaIO_4$ oxidized 3'-end of tRNA	E.coli Met-RSase	0.57	(51)

It should be mentioned that direct attachment of tRNA (Refs 44 & 45) and ATP (Ref. 46) by irradiation of the respective complexes with ARSases was performed. In the former case however no data concerning the points of modification of ARSases were reported.

Up-to-now only few data about the sequences around the amino acid residues modified by affinity labelling are reported. The results together with the data obtained by chemical modification described in the preceding section are presented in the Table 5.

The main assumption of the affinity labelling approach is that the reagent interacts specifically with the same site of the enzyme as the natural substrate does. Therefore, one may expect that the cooperativity relations existing between the active centers should be revealed in the affinity labelling processes.

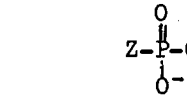
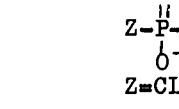
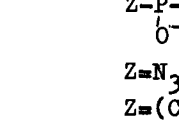
This was really found in several cases in our experiments with the affinity labelling of ARSases.

TABLE 3. The reactive amino acid derivatives (aminoacyl methylene halides) and K_i/K_m ratios

Analog	Enzyme	K_i/K_m	Ref.
Valyl-CH_2Cl	beef liver Val-RSase	100	(52)
	E.coli Val-RSase	140	(53)
Isoleucyl-CH_2Br	E.coli Ile-RSase	120	(54)
Phenylalanyl-CH_2Cl	E.coli Phe-RSase	25	(55)

First of all we have demonstrated with both E.coli Phe-RSase (Ref. 56) and beef pancreas Trp-RSase (Ref. 11) that the cognate chlorambucilyl-aminoacyl-tRNA's (chb-aa-tRNA) binds only to one of two identical subunits of the enzymes. This is in agreement with the negative cooperativity revealed by the binding experiments. It is worth mention that the alkylation of the enzyme with the tRNA analog is sensitive to the presence of either amino acid or ATP the reaction rate being lowered significantly in the presence of one of these substrates. In the presence of both substrates probably forming aminoacyl adenylate the enzyme is completely protected against alkylation, although chb-aa-tRNA still binds strongly to enzyme (Refs 11, 49 & 57). These data suggest that some relations exist between the chlorambucilyl-aminoacyl residue area of the E·chb-aa-tRNA complex and the amino acid and ATP sites of the enzyme. In accordance with this result covalent attachment of chb-aa-tRNA results in ~ 50% decrease in the ATP-[^{32}P]pyrophosphate exchange rate thus indicating that one of two identical centers dealing with aminoacyl adenylate formation does not operate in the modified enzyme (Ref. 11).

TABLE 4. The reactive ATP analogs and their K_i/K_m ratios

Analog	Leu-RSase, Euglena gracilis (Ref. 25)	Trp-RSase beef pancreas (Ref. 24)	Phe-RSase E.coli
			
Z=$ClCH_2CH_2$-O-	28	0.125	-
Z=$BrCH_2CH_2$-	-	0.045	-
Z=$(CH_3)_3$-C_6H_2-CO-O-	32	0.165	-
			
Z=CL-CH_2	14.7	0.02	-
Z=Br-CH_2CH_2-	-	0.08	-
Z=$(CH_3)_3$-C_6H_2-CO-O-	-	0.075	-
			
Z=N_3-C_6H_4-NH	-	5 (11)	2 (23)
Z=$(CH_3)_3$-C_6H_2-CO-O	93	no inhibition	-

The switching off one of the ATP sites in Trp-RSase with covalently attached chb-Trp-tRNA may be demonstrated by subsequent modification of this enzyme with the photoreactive ATP analog, ATP γ-p-azidoanilide. Prior to describe these experiments it should be mentioned that due to high reactivity of nitrene radicals formed by the irradiation of azido groups some unspeci-

fic modification may be expected. Therefore we investigated the level of the affinity labelling with this analog in the presence of ATP. It is seen (Fig. 3) that the excess ATP protects against binding of two moles of the analog (Ref. 58).

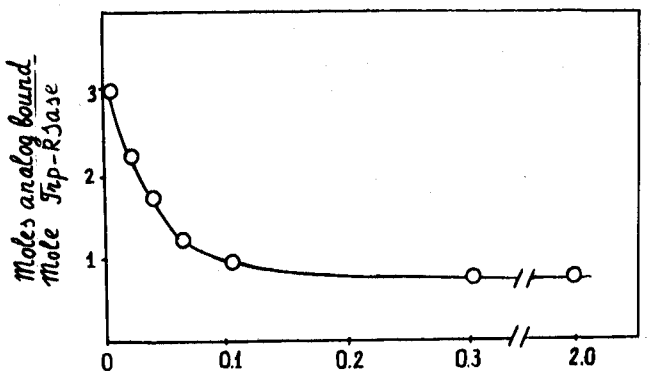


Fig. 3. The dependence of the number of ATP γ-p-azidoanilide covalently bound to Trp-RSase on the ATP concentration.

Using this approach it was demonstrated that the irradiation of the native enzyme with photoreactive ATP analog results in the labelling of two ATP sites whereas in the chb-Trp-tRNA modified enzyme only one ATP site is modified (Ref. 11).

TABLE 5. Peptides found in the active centers of ARSases

ARSase	Site of modification	Peptide	Ref.
Ile-RSase *E.coli*	Essential Cys residue modified by N-ethylmaleimide	Ile-Glu-Ser-Met-Val-Ala-Asp-Arg-Pro-Asn-Trp-Cys-Ile-Ser-Arg	(38)
	Cys residue affinity labelled with Ile-CH_2Cl	Exactly the same	(54)
	Peptide modified at unknown residue X by photochemical cross-linking with ATP	Lys-Val-Ala-Gly-Asx-X	(46)
Met-RSase *E.coli*	Lys residue* acylated by affinity labelling with p-nitrophenyl carbamoyl derivative of Met-tRNA	Phe-Thr-Tyr-Gln-Lys-Leu-His-Asn	(47)
Tyr-RSase *B.stearothermophilus*	Peptides containing lysine protected by tRNA against acetylation with Ac_2O	Arg-Ile-Val-Thr-Gly-Met-(Lys-Thr)-Arg-Tyr	(43)
		Gly-Leu-Thr-Ile-Pro-Leu-Val-Thr-Lys-Ala-Asp-Gly-Thr-Lys-Phe	
		Gly-Lys-Thr-Glu-Ser-Gly-Thr-Ile-Trp	

* The functional role of this Lys residue is not shown.

The above experiments permitted us to obtain two new forms of Trp-RSase with one switched off subunit. The modification with chb-Trp-tRNA alone results in the enzyme with one subunit completely lacking enzymatic activity and the other subunit active in the aminoacyl adenylate formation. The double affinity labelling with chb-Trp-tRNA and then with ATP γ-p-azidoanilide results in completely inactive enzyme. However the subsequent alkaline treatment results in the splitting off tRNA moiety and reactivation of one of the subunits in both ATP-[^{32}P]pyrophosphate exchange and tRNA aminoacylation.

As it was already mentioned when used in the dark ATP γ-p-azidoanilide being competetive to ATP inhibitor at high concentrations exhibits some activator properties at low concentration. Similar behaviour exhibit GTP γ-p-azidoanilide and β-p-azidoanilides of ADP and GDP as well as GMP and some other nucleotides. The dependence of the enzymatic activity on the ATP γ-p-azidoanilides concentration is presented in Fig. 4.

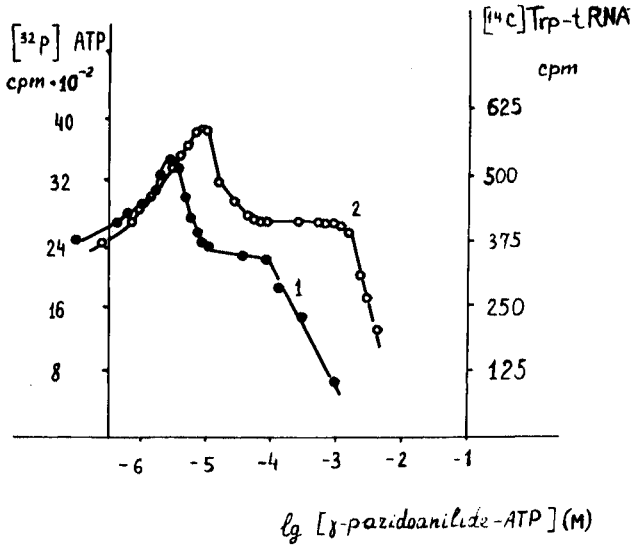


Fig. 4. The dependence of the initial rate of the ATP-[^{32}P]pyrophosphate exchange (1) and of the tRNA^{Trp} aminoacylation (2) catalyzed by beef pancreas Trp-RSase as a function of ATP γ-p-azidoanilide concentration (at constant substrate concentrations).

It seemed reasonable to suggest that some additional noncatalytic nucleotide binding centers exist. A set of nucleotides and derivatives may interact with these centers including ATP γ-p-azidoanilide. These compounds behave as effectors of the reactions catalyzed by ARSase. If this is the case, ATP γ-p-azidoanilide should modify both catalytic ATP site and noncatalytic nucleotide binding center.

In Fig. 5 the kinetics of photoaddition of ATP γ-p-azidoanilide to beef pancreas Trp-RSase is presented in the absence of any other ligands, in the presence of excess GMP and in the presence of the excess of both ATP and GMP. It is seen that GMP really protects two sites in the enzyme against the reaction with the analog and that the action of ATP and GMP is additive. The latter result means that really two types of the centers exist (Ref. 6).

In order to modify the enzyme only in noncatalytic center we performed photoaffinity labelling with GDP β-p-azidoanilide. The enzyme remains active in tRNA aminoacylation after the modification thus indicating that the catalytic sites are not damaged. The enzyme may be partially protected against reaction with analog by GMP, but not by ATP. These results demonstrate that GDP derivative selectively modifies noncatalytic nucleotide binding center. The ATP-[^{32}P]pyrophosphate exchange was studied using this enzyme in the presence of one of the effectors, ADP β-p-azidoanilide. The kinetic curves

of the exchange without effector and at different effector concentration are presented in Fig. 6.

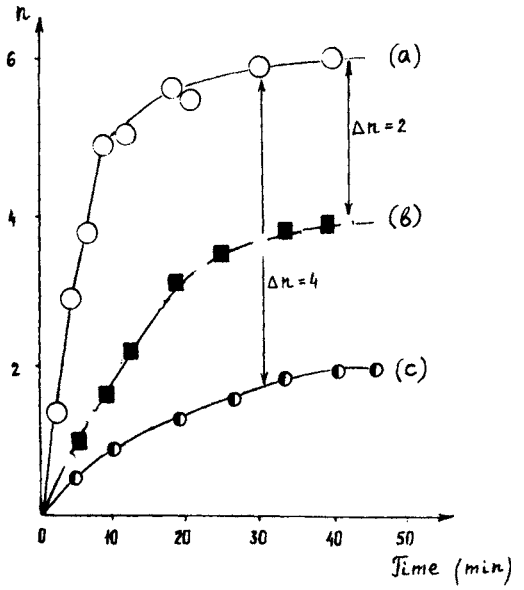


Fig. 5. The kinetics of the attachment of ATP γ-p-azidoanilide to Trp-RSase under irradiation at $\lambda > 300$ nm: (a) Trp-RSase + analog; (b) Trp-RSase + analog + GMP; (c) Trp-RSase + analog + GMP + ATP. n - the number of the analog molecules covalently bound per one enzyme molecule.

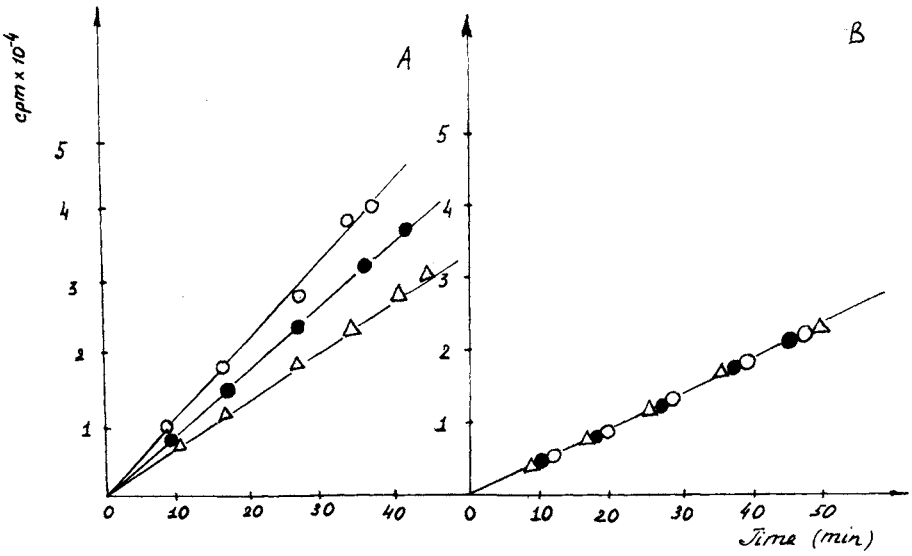


Fig. 6. The kinetics of the [^{32}P]ATP accumulation due to exchange with [^{32}P]pyrophosphate in the [^{32}P]ATP exchange catalyzed by native beef pancreas Trp-RSase (A) and the enzyme modified with GDP β-p-azidoanilide (B) at various concentrations of the effector. Δ - in the absence of GDP β-p-azidoanilide; ● - in the presence of $2 \cdot 10^{-6}$ M of GDP β-p-azidoanilide; O - in the presence of $3 \cdot 10^{-6}$ M of GDP β-p-azidoanilide.

It is seen that at concentrations of the effector exhibiting essential activation on the reaction catalyzed by the native enzyme there is no influence on the reaction catalyzed by enzyme with modified noncatalytic centers. Therefore we may conclude that this Tpr-RSase derivative is a new form of the eyzyme with the effector sites switched off due to modification.

The results presented in the last section clearly demonstrate that affinity labelling may be used as an approach to study the interaction between various types of the active sites of the multisubstrate enzymes as well as a method to prepare the enzyme derivatives in which some types of the centers are switched off by chemical modification. These derivatives may be extremely useful to study the mechanism of the reactions catalyzed by ARSases in the simplified conditions. The kinetic characteristics of these derivatives are under investigation.

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DIVERSITY OF SUGAR PUCKER IN THE NUCLEIC ACIDS

A. Rich, A. H.-J. Wang and G. J. Quigley

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

Abstract - Both the ribose and the deoxyribose sugars of polynucleotide chains exist in two major conformations. These conformations result from changes in the pucker of the sugar ring and are associated with different distances between successive phosphate groups along the chain. We consider examples of these leading to conformations in polynucleotide structures which allow them to accommodate other molecules interposed between the bases. Such structural adaptations are found in oligonucleotides as well as in the three-dimensional structure of yeast phenylalanine transfer RNA.

INTRODUCTION

One of the widely appreciated features of protein structures is the fact that the polypeptide chain can fold into different conformations. This includes tightly folded conformations such as found in the α helix or more extended conformations as is found in the β sheet. It is less widely appreciated that the polynucleotide chain also has conformational flexibility which results in significant modifications of the chain extension. Many of these differences are associated with different puckers of the five-membered furanose ring which is a component of both the DNA and RNA chains. Here we illustrate changes in the sugar ring conformation in a variety of structures, taking examples in oligonucleotides as well as in polynucleotides. The property of polynucleotide sugar rings to alter their pucker should be looked upon as a degree of conformational freedom. It is important to have this kind of flexibility in order to form complex structures beyond the simple double helix.

RING PUCKER IN THE DOUBLE HELIX

Double helices can be formed with both DNA and RNA molecules. These double helices have similar features in that the bases are in the center of the molecule with the familiar Watson-Crick pairing, with guanine-cytosine pairs and either adenine-thymine pairs in DNA or adenine-uracil in RNA. The sugar-phosphate chains are on the outside of the double helix. Although the two types of double helices are similar, they differ in one fundamental feature; the pucker of the five-membered furanose ring is different in ribose and deoxyribose (1). The differences in ring pucker are illustrated in Figure 1. The furanose ring is viewed edge on in a plane which is defined by the ring oxygen O1' and carbons C1' and C4'. C1' has attached to it the ring nitrogen (N) of the purine or pyrimidine. C4' has attached to it the additional carbon atom C5' as indicated in the diagram. Both of these constituents, the base (N) and C5', are located on the upper side of the ring in Figure 1. It is clear that the principal difference in the conformation in the sugar ring is whether the carbon atom C2' or C3' is above the plane of the ring. Atoms which are above the plane of the ring in Figure 1 are considered to be in the *endo* conformation (2). Thus the conformation C2' *endo* has C2' on the upper side of the ring and this correspondingly forces C3' to be on the lower side of the ring. In the other conformation, C3' is *endo* and C2' is on the lower side of the ring. The major conformation of a deoxyribose chain is one in which the furanose ring has a C2' *endo* conformation. In contrast, the normal ribose chain conformation is C3' *endo*. The principal reason for the difference is associated with the additional space occupied by the oxygen atom attached to C2' in ribose which is absent in the deoxy series where only a hydrogen atom is attached to C2'. It should be emphasized that even though these are the normal conformations of these sugars in their respective double helices, the energy barriers involved in changing sugar conformation are not very great (3).

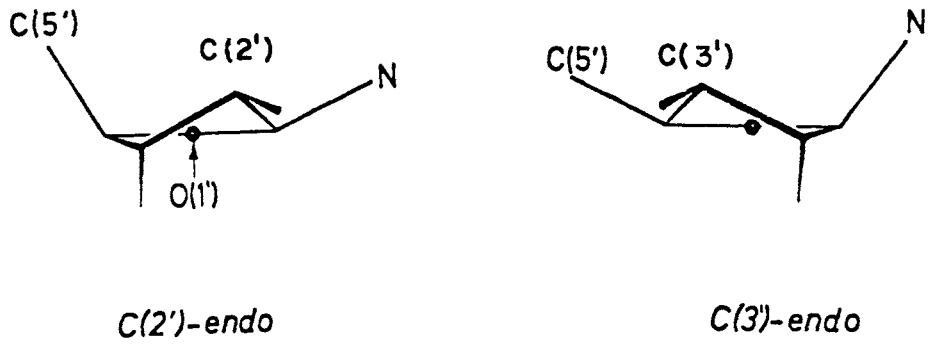

Figure 1: Diagram illustrating two different conformations of the ribofuranose ring. The plane of the ring is defined by three atoms: C1' to which is attached the glycosidic bond indicated by N, O1' and C4' which is attached to the atom C5'. In this diagram we are looking edge on at the plane defined by these three atoms. The remaining two atoms in the ring, C2' and C3' are located either above or below the plane of the ring. Atoms located above the plane of the ring are in the *endo* position. On the left C2' is in the *endo* position and on the right C3' is in the *endo* position. Although two examples of ring pucker are shown in this diagram, there are actually a number of intermediate states in which the displacement of C2' or C3' is not as great as that illustrated here. The detailed nomenclature for furanose ring pucker is complex (2); here we elect to use only the simplified *endo* conformations.

Because of the C2' *endo* conformation of deoxyribose, the form of the DNA double helix is such that the base pairs are found on the helix axis in the familiar B form of the DNA. The bases occupy the axis of the molecule so that the familiar double helical DNA has a solid, rod-like appearance with bases in the center and two helical grooves running down the molecule. These are the major and minor grooves which occupy the space between the deoxyribose-phosphate chains. In contrast, the C3' *endo* conformation of ribose makes significant modifications in the RNA double helix. The base pairs are no longer perpendicular to the helix axis, but are tilted at about 15-18°. Furthermore, they are set back away from the helix axis. In fact there is a clear space in the center of the molecule of approximately 3 Angstroms in diameter. The RNA double helix thus looks more like a molecular ribbon wrapped around an imaginary cylinder 3 Angstroms in diameter in which there is now a very deep major groove and a comparatively shallow minor groove on the outside of the helix.

These forms of the familiar double helices are not invariant. Reducing the water content of the medium by adding alcohol readily converts the familiar B form of DNA into the A form in which deoxyribose adopts the C3' *endo* conformation which is normally found in RNA double helices. The DNA double helix then changes its conformation and looks more like double helical RNA. It is noteworthy that in the conversion from the B to the A form of DNA, there is over an Angstrom difference in the phosphate-phosphate distance along each polynucleotide chain.

The reason for the change in phosphate-phosphate distance can be seen schematically in Figure 2 which shows the conformation of two forms of an adenosine nucleotide in a ribose polynucleotide chain. In the upper figure the ribose is in the C3' *endo* conformation and the phosphorus-phosphorus distance is near 5.9 Angstroms. In the lower part of Figure 2, the ribose is in a C2' *endo* conformation (normally found in DNA) and the phosphorus-phosphorus distance is near 7 Angstroms. The change in distance is largely associated with a change in the pucker of the ring, although there are also small changes in the other dihedral angles in the backbone. The polynucleotide chain thus has a degree of conformational freedom which allows it to change the degree of extension in a significant way. This flexibility is used in a variety of ways in polynucleotide structures.

POLYNUCLEOTIDE CHAINS MAY TURN CORNERS WITH CHANGES IN RING PUCKER

There are many examples in which conformation of the sugar ring is changed. In some cases, the changes in pucker are associated with a change in

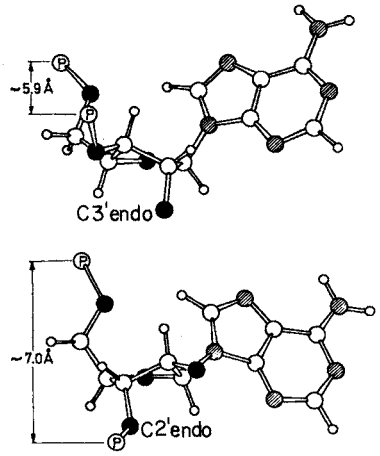

Figure 2: Illustration of two different conformations of adenylic acid. In the upper diagram with the C3' *endo* conformation, the two phosphate groups are both above the plane of the ribose ring and are approximately 5.9 Angstroms apart. In the lower diagram the C2' *endo* conformation, the phosphate attached to O3' is located below the plane of the ribose ring and the phosphates are now approximately 7 Angstroms apart. These two conformations are associated with considerable differences in the extension of the sugar phosphate chain.

the direction of a polynucleotide chain. An example of this is seen in Figure 3 which shows the crystal structure of adenylyl-(3',5')-uridine (ApU)

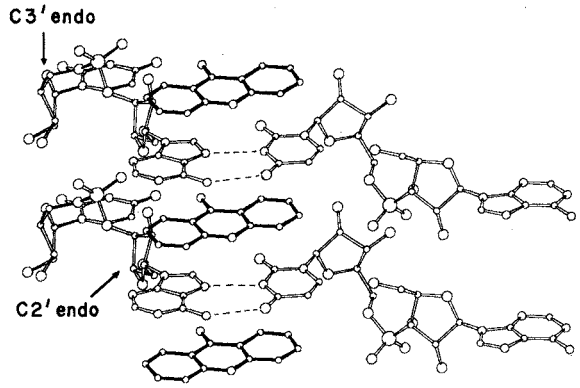

Figure 3: The crystal structure of the complex of ApU and 9-amino acridine. The polynucleotide chain of ApU is in an extended conformation and the chain turns a sharp corner. This can be seen by the different orientation of the two ribose residues in the oligonucleotide. The change in direction is associated with a change in ring pucker.

which formed a crystalline complex with 9-amino acridine (4). In this structure there are stacked columns of planar molecules in which the 9-amino acridine molecule alternates with an adenine-uracil base pair hydrogen bonding through the ring nitrogen N7 of adenine. Uracil is at one end of the chain and the backbone has an extended conformation in which the molecule actually turns a corner so that its adenine residue is then hydrogen bonded to still another uracil residue (Figure 3). In the course of making this turn, one of the sugar residues adopts the unusual C2' *endo* conformation as shown in the diagram. As seen at the right of the diagram, the uridine ribose O1' is found at the bottom while it is found at the top of the adenosine ribose. This illustrates the extent to which the chain has radically changed direction in this structure. A somewhat similar conformation is seen in the structure of adenylyl-(3',5')-adenosine (ApA) which crystallizes with proflavin in a simi-

lar manner (5). However, it is not obligatory for the ribose in RNA to change pucker in coiled regions of the molecule. The examples cited above illustrate the fact that changes in pucker may be seen in some nucleotide structure determinations.

Tables I and II cite the structure of oligonucleotides which have crystallized by themselves (Table I) or together with intercalators (Table II).

TABLE I

OLIGONUCLEOTIDES

		5' end	3' end	Reference
DNA fragments	pdTpdT	C2' *endo*	C2' *endo*	15
	d-(pApTpApT)	C3' *endo* (A)	C2' *endo* (T)	16
RNA fragments	ApU	C3' *endo*	C3' *endo*	17
	GpC	C3' *endo*	C3' *endo*	18
	GpC	C3' *endo*	C3' *endo*	19
	ApA^+	C3' *endo*	C3' *endo*	6
	A^+pA^+	C3' *endo*	C3' *endo*	6
	UpA	C3' *endo*	C3' *endo*	20, 21

TABLE II

INTERCALATOR COMPLEXES

	Nucleotide	Intercalator	Sugar Pucker 5' end	Sugar Pucker 3' end	Reference
DNA fragments	dCpG	Pt-terpyridine$^+$	C3' *endo*	C2' *endo*	7
RNA fragments	CpG	Acridine orange$^+$	C3' *endo*	C2' *endo*	8
	rIodo UpA	Ethidium$^+$	C3' *endo*	C2' *endo*	9
	rIodo CpG	Ethidium$^+$	C3' *endo*	C2' *endo*	10
	rIodo CpG	9-Aminoacridine$^+$	C3' *endo*	C3' *endo*	11
	rIodo CpG	Acridine orange$^+$	C3' *endo*	C2' *endo*	12a
	rIodo CpG	Ellipticine$^+$	C3' *endo*	C2' *endo*	12b
	CpG	Proflavine$^+$	C3' *endo*	C3' *endo*	13
	rIodo CpG	Proflavine$^+$	C3' *endo*	C3' *endo*	12b
Non-helical structures	ApU	9-Aminoacridine$^+$	C2' *endo*	C3' *endo*	4
	ApA	Proflavine$^+$	C2' *endo*	C3' *endo*	5

They are listed with a description of the sugar conformation in various parts of the backbone. Although we have cited two examples in which oligonucleotides change pucker where there is a change in the direction of the polynucleotide chain, there are several oligonucleotides in which there has been no change in the pucker even though the chain is in a fairly extended conformation. An example is seen in the structure of the trinucleotide ApApA (6).

INTERCALATION CHANGES DOUBLE HELIX CONFORMATION

One of the conformational changes frequently encountered in double helical nucleic acids is that associated with the binding of a planar intercalator molecule which lodges between the base pairs. It does this without substantial disruption of the helix. Intercalation has two important effects: it introduces a gap between adjacent base pairs which are then separated by 6.8 Angstroms instead of the normal 3.4 Angstroms. This is due to the planarity of the intercalator which usually has unsaturated rings with π electrons and is 3.4 Angstroms thick. In addition there is an unwinding of the double helix.

A series of structures have been solved involving intercalators, mostly in the ribonucleotide series but with one in the deoxynucleotide series (Table II). The structure of a double helical fragment of DNA together with an intercalator is shown in Figure 4. Here we see the structure of deoxy CpG which accommodates a terpyridine-platinum intercalator in the midst of a double helical fragment (7). As seen in the diagram, the 5' end of the double helical segment adopts the unusual C3' *endo* conformation while the 3' maintains the C2' *endo* conformation which is normal for double helical DNA.

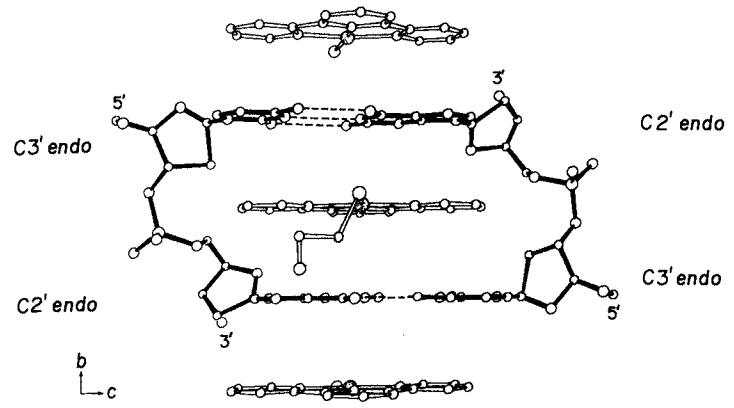

Figure 4: The structure of the deoxy CpG-platinum terpyridine intercalator complex. It can be seen that the DNA double helix has a planar intercalator lodged between the base pairs. This is associated with an unusual ring pucker on the 5' side of the oligonucleotide segment. This conformation allows an extension of the polynucleotide chain.

Figure 5 shows the structure of the ribose dinucleoside phosphate CpG together with the intercalator acridine orange (8). It can be seen that the 3'

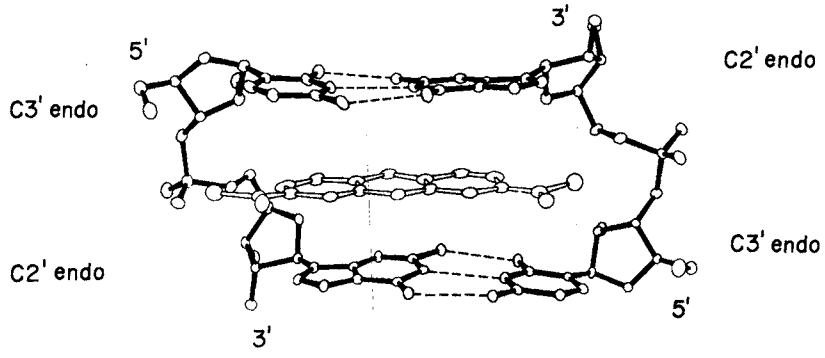

Figure 5: The structure of the CpG-acridine orange intercalator complex. It can be seen that there is an unusual conformation at the 3' end of the oligonucleotide chain surrounding the intercalator. This mixed pucker conformation is associated with an extension of the polynucleotide chain.

end of the double helical RNA fragment has adopted the unusual C2' *endo* conformation while the 5' end maintains the normal C3' *endo* conformation. A large

number of intercalator structures have been solved in the ribonucleotide series as listed in Table II. Sobell and his colleagues were the first to point out that intercalation is associated with a modification of the pucker of the ribonucleotide chain on the 3' side of the intercalator (9-12). Although intercalation is generally associated with conformations similar to those seen in Figure 5 a number of alternative conformations are listed at the bottom of Table I. These are usually associated with the intercalators proflavin (5, 12a, 13) and 9-amino acridine both of which have the property of forming hydrogen bonds between the intercalator and the phosphate of the dinucleoside phosphate. In both cases other modes of pucker are found. For example, in the complex of proflavin with the ribose CpG fragments, both residues have the normal C3' *endo* conformation (13).

For "simple" intercalators in which there is no hydrogen bonding to the phosphate residue, it is possible to make an interesting generalization about the way double helical DNA and RNA accommodate intercalator addition. This is illustrated schematically in Figure 6. At the left the DNA double helix is shown diagrammatically with the normal C2' *endo* conformation in all residues,

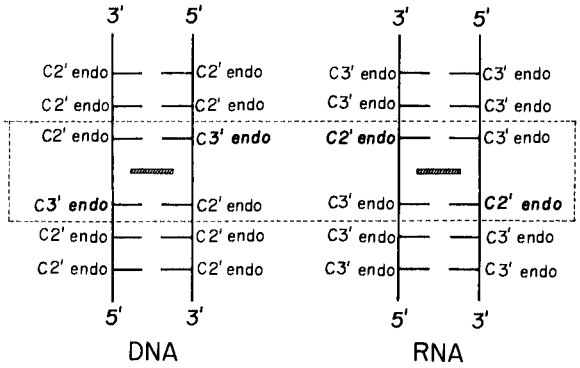

Figure 6: A schematic diagram illustrating the manner in which either DNA or RNA double helices change the pucker of the sugar residues in the region immediately surrounding a simple intercalator. Although the changes are different in the two types of double helices, the conformation in the region surrounding the intercalator enclosed by the dashed rectangle is the same in both cases.

except for those on the 5' side of the intercalator where the unusual C3' *endo* conformation is adopted in a manner analogous to that which is illustrated in Figure 4. On the right the diagram shows the way in which double helical RNA accommodates an intercalator. All of the residues are in the normal C3' *endo* conformation except for those residues on the 3' side of the intercalator which adopt the unusual C2' *endo* conformation. However, it can be seen that in the region immediately surrounding the intercalator (enclosed in the dashed line) the conformation of both the RNA and DNA chain are similar. Thus both molecules elongate to accommodate an intercalator by adopting a similar conformation. These conformation changes, as described by Sobell (9-12) explain the nearest neighbor exclusion. If a DNA or RNA double helix is saturated with an intercalator, the most that can be accommodated is one intercalator for every two base pairs. The reason for this is probably associated with the necessity for mixed pucker on either side of the intercalator.

CONFORMATIONAL FLEXIBILITY IN YEAST $tRNA^{Phe}$

Examples of changes in the type of sugar pucker can be seen in the three-dimensional structure of yeast phenylalanine transfer RNA ($tRNA^{Phe}$). Although most of the seventy-six nucleotides in this transfer RNA molecule adopt the C3' *endo* conformation which is normal for a ribonucleotide chain, several residues are found to adopt the less common C2' *endo* conformation. This is frequently associated with an interesting type of structural accommodation which is similar to those interactions described above in the oligonucleotide structures. Figure 7 is a diagram which shows secondary and tertiary hydrogen bonding found in the yeast $tRNA^{Phe}$. This schematic diagram is useful in interpreting Figures 8 through 11 which illustrate conformational changes in various parts of the molecule (14).

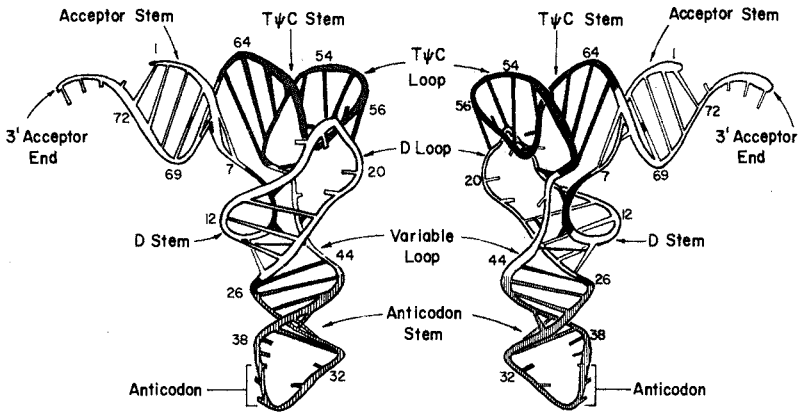

Figure 7: A schematic diagram showing two sides of yeast $tRNA^{Phe}$. The ribose-phosphate backbone is depicted as a coiled tube, and the numbers refer to nucleotide residues in sequence. Shading is different in different parts of the molecules, with residues 8 and 9 in black. Tertiary hydrogen bonding between bases is shown as solid black rungs, which indicate either one, two or three hydrogen bonds. Those bases that are not involved in hydrogen bonding to other bases are shown as shortened rods attached to the coiled backbone.

CHANGES IN POLYNUCLEOTIDE DIRECTION

The principal sites using C2' *endo* conformation in yeast $tRNA^{Phe}$ are listed in Table III. Nine examples are cited. These occur in two principal situations. In five cases (residues 7, 17, 18, 48 and 60) the C2' *endo* conformation is

TABLE III

C2' *Endo* Conformations in Yeast $tRNA^{Phe}$

Residue Number	Description
Intercalation:	
9	8 - 9 intercalation by C13
19	18 -19 intercalation by G57
46	45 - 46 intercalation by A9
58	57 - 58 intercalation by G18
Change of Direction:	
7	Extended segment at bend in chain, juncture between acceptor stem and 8 - 9 connection
17	Looping out of backbone at residues at 16 and 17
18	Looping out of backbone
48	Looping out of variable loop backbone at U47; juncture between variable loop and T stem
60	Extended segment at bend in chain, bases 59 and 60 excluded from T stem and loop stacking

adopted when the polynucleotide chain undergoes a distinct change in direction. For example, nucleotides 1 through 7 are involved in the double helical acceptor stem but residues 8 and 9 are extended and provide a linker region which attaches one end of the acceptor stem with the beginning of the D stem. In this region, there are several conformational changes, one of which is associated with the C2' *endo* conformation of ribose 7 where the polynucleotide

chain changes direction. Other examples are found in regions where the backbone loops out away from the center where the bases are stacked. The dihydrouracil residues 16 and 17 are extended out away from the remainder of the molecule and their bases are not stacked with other bases in tRNA. The backbone has a "bulge" or looping-out at that point (Figure 7). In order to accommodate this extended conformation, residues 17 and 18 adopt the C2' *endo* conformation. In addition ribose 47 in the variable loop is extended and its base thrusts away from the center of the molecule. This is associated with a C2' *endo* conformation in ribose 48. Still another example is found in the T loop where bases 59 and 60 are excluded from the stacking of the other bases in the T stem and loop (Figure 7). This exclusion involves a C2' *endo* conformation in ribose 60. All of these conformational changes are thus associated with an abrupt change in the direction of the polynucleotide chain. This directional change is accommodated by adoption of a C2' *endo* conformation and there is a substantial change in the phosphorus-phosphorus distance along the polynucleotide chain.

It should be pointed out, however, that there are many other examples in the loop regions of $tRNA^{Phe}$ in which the polynucleotide chain changes direction but the C2' *endo* conformation is not used. Thus, the change in pucker is a structural accommodation which may be adopted in the molecule, especially where the chain is extended, but it is not generally used when the polynucleotide chain undergoes a change in direction.

INTERCALATION IN YEAST $tRNA^{Phe}$

There are two parts of the molecule in which extensive intercalation is found involving pairs of nucleotides. These are in the central region with intercalations involving nucleotides 8 and 9 as well as in the corner of the molecule where the T and D loops interact. Figure 8 shows the conformation adopted by the sugars of residues G45 and m^7G46 which has the adenine ring of A9 intercalated between them. Residues 45 and 46 are not involved in a double

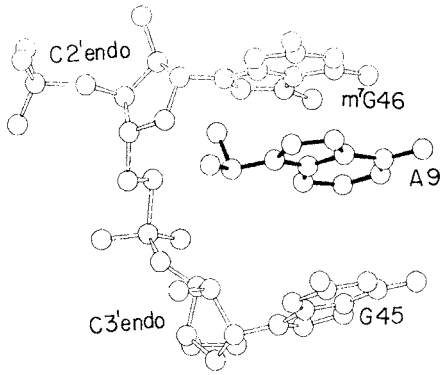

Figure 8: Intercalation in yeast $tRNA^{Phe}$. Adenine of residue 9 intercalates between the bases of 45 and 46. This intercalation is accommodated by a change in the ribose pucker at the 3' end of the segment. The ribose of m^7G46 is in the unusual C2' *endo* conformation.

helix; nonetheless, the conformation of m^7G46 is C2' *endo* in a manner analogous to the conformational changes which are seen for double helical ribonucleotide fragments surrounding intercalators. The C2' *endo* conformation is adopted at the 3' end of the intercalator region in Figure 8. Another example is shown in Figure 9 where residues U8 and A9 are found in the extended segment which connects the acceptor stem with the D stem. It can be seen that A9 at the 3' end of the segment has adopted the C2' *endo* conformation even though it is not in a complementary double helical structure.

Figure 9 shows that intercalated between U8 and A9 are the bases m^7G46 and C13 both of which are hydrogen bonded to G22. The base pair C13·G22 is part of the D stem. In Figure 9 it can be seen that the nucleotides U8 and A9 accommodate the additional distance associated with intercalation by adopting the C2' *endo* conformation in residue A9.

By comparing Figures 8 and 9 it can be seen that they are both portions of the same structure in which there are two interacting segments of the polynucleotide chain, both of which intercalate around each other. The polynucleotide

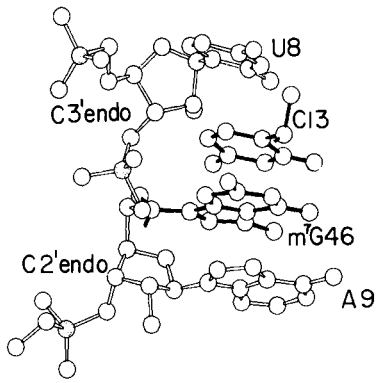

Figure 9: The nucleotides U8 and A9 have intercalated between them the bases C13 and m^7G46. These bases are both hydrogen bonded to G22 in the tertiary structure of yeast $tRNA^{Phe}$. This intercalation is accommodated by a change in the conformation of ribose of A9.

chains are interleaved between each other so that each intercalates into the opposite member of the pair.

A similar pair of interleaved structures are found near the corner of the molecule where the bases of nucleotides 18 and 19 interact with the bases of 57 and 58. In Figure 10 it can be seen that m^1A58 and G57 are spread apart with residue G18 intercalated between them. This intercalation is associated with a C2' *endo* conformation found in m^1A58. In this case it also adopts the

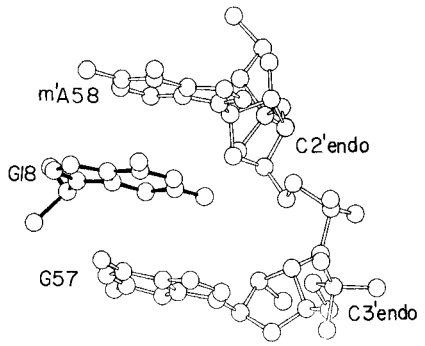

Figure 10: Diagram showing the intercalation of guanine 18 between G57 and m^1A57. The ribose of m^1A58 is in the C2' *endo* conformation to accommodate the intercalation.

C2' *endo* conformation at the 3' end of the oligonucleotide segment which surrounds the intercalating guanosine of G18. This is quite similar to the double helical RNA intercalator models which are described in Table II, as the bases G57 and m^1A58 are hydrogen bonded to other residues, although not in Watson-Crick pairs.

An interesting variant is seen in Figure 11 which shows the intercalation of G57 between the bases of G18 and G19. This intercalating interaction is one of the important stacking interactions which stabilize the corner of the tRNA molecule and helps maintain the interaction of the T loop and D loop. However, in this example, the less common C2' *endo* conformation is found in both riboses of G18 and G19. By analogy with the simple intercalator structures one would expect a C2' *endo* conformation to be found in G19 but not in G18. Here it is likely that the unusual C2' *endo* conformation of G18 is not associated so much with the intercalation of G57 but is probably related to the fact that residues 16 and 17 are excluded from the molecule as described above so that their bases do not stack with the others. Another interesting feature associated with this is the fact that the bases of G18 and G19 are not only separated from each by a distances of 6.8 Angstroms, which would be necessary for the intercalation of G57, but they are also translated relative to each other so the bases are no longer strictly on top of each other. This translation of G18 relative to G19 in the plane of the bases, can only be accommo-

dated if both residues are in the C2' *endo* conformation. It is likely that this feature is also related to the fact that the bases of residues 16 and 17

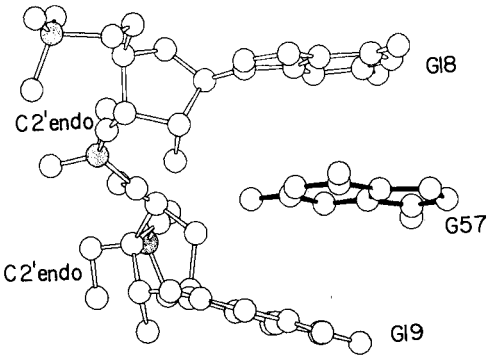

Figure 11: The intercalation of G57 between G18 and G19 is illustrated. Unlike the previous examples, the riboses of G18 and G19 are both in the unusual C2' *endo* conformation. The C2' conformation of G18 is probably associated with the unusual conformation of residues 16 and 17 immediately adjoining G18.

are excluded from stacking with the remainder of the bases in the tRNA structure. Some tRNA molecules contain only one nucleotide in this region which is excluded from the stacking, instead of the two seen in yeast $tRNA^{Phe}$. It is possible that in these cases, with only one residue, that G18 may have the normal C3' *endo* conformation since the translation of G18 relative to G19 in the plane of bases may not be required.

DISCUSSION

In the above we have discussed the conformational flexibility found in the polynucleotide chains. This flexibility is inherent in the fact that there is a furanose ring in the chain which can adopt two different conformations which are associated with a varied extensibility of the chain. Structural studies on simple oligonucleotides reveal that conformational changes occur in regular double helical structures associated with intercalation as well as in other structures where polynucleotide chains undergo an abrupt change in direction. In the three-dimensional structure of yeast $tRNA^{Phe}$ examples are shown on both types of conformational changes. Changes in pucker due to intercalation in general follow trends which are seen in intercalation in simple double helical RNA structures even though the tRNA examples do not involve simple RNA double helices. This implies that the adoption of a C2' *endo* conformation together with an associated extension of the polynucleotide chain is not solely limited to double helical intercalation, but may in fact be of a more general nature as shown by the examples cited above.

Nucleic acid molecules have considerable conformational flexibility. We have only a hint of this flexibility in studying the structure of the double helix itself. However, when one begins to study the interaction of double helical structures with other molecules, especially intercalators, we see that there is a method for accommodating them involving changes in extensibility. In complex globular polynucleotides such as the transfer RNA molecule, a variety of changes in sugar pucker are observed. The molecule adopts unusual conformations associated either with chain extension, changes in direction of the polynucleotide chains or with the accommodation of other bases which intercalate into the chain even though the chains are not involved in a double helical array. As the structure of more complex polynucleotide structures is solved, it is our expectation that this will be found to be a completely general feature. This element of conformational flexibility associated with changes in ring pucker is likely to be a constant feature of polynucleotide chains when they interact with other molecules,including proteins, as well as when they interact with other polynucleotide chains.

ACKNOWLEDGEMENTS

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THREE LEVELS OF CHROMATIN STRUCTURE

W. Altenburger, W. Hörz and H. G. Zachau

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Federal Republic of Germany

Abstract - Experiments of our group on the three levels of chromatin structure are described. Special emphasis is given to the digestion of mouse liver chromatin by DNAase II. The conditions of DNAase II cleavage at either 200 bp or 100 bp intervals are examined. Histone H1 is required for cleavage in the 100 bp mode. The implications of the findings for chromatin structure and especially elements of superstructure are discussed.

Recent studies of chromatin structure have concentrated on the chromatin subunit, the nucleosome (1-3). It provides a first order condensation of the DNA double helix around a globular histone bead consisting of two copies each of the histones H2A, H2B, H3, and H4. It is clear that within the nucleus the nucleosome chain also called nucleo filament must be further compacted. In electron microscopic studies a thick fiber of 200-300 Å diameter can be visualized in addition to the nucleo filament which is 100 Å in diameter. Different models have been proposed to account for the thick fiber (4-6) but there is general agreement that histone H1 plays a central role at this level of superstructure. The organization of the thick fibers in relation to the nuclear matrix of the interphase nucleus (7) or within the metaphase chromosome structure (8) provides a further level of superstructure.

In the lecture experiments from our laboratory were discussed pertaining to each of these different levels of chromatin organization. Nucleosomes have been reconstituted from DNA and the histone constituents and examined by biochemical and electron microscopic methods (9,10). It turned out that by the criteria available a faithful reconstitution of the nucleosome cores was feasible that,however,the proper spacing of these cores along the DNA was not achieved neither in the absence nor in the presence of histone H1. It might be that for the assembly of properly spaced nucleosomes in addition to H1 a protein factor is required as indicated by the studies of Laskey et al. (11).

The spacing of nucleosomes in chromatin isolated from the nucleus was the subject of another series of experiments resting predominantly on the use of different nucleases (12). The data led to the delineation of DNA segments well protected within the nucleosome cores versus more accessible segments connecting the nucleosome cores, the linker DNA. In agreement with other reports (1-3) out of the 200 bp per nucleosome about 140-150 bp of DNA were found to be strongly protected.

Spleen acid DNAase has proved to be a novel probe into elements of superstructure associated with the transition from the nucleo filament to the thick fiber (13). Its use will be exemplified in more detail below.

Restriction nucleases have been introduced for the preparation of chromatin fractions (14), and their value could be documented for a number of different purposes. Experiments on the digestion of mouse liver nuclei with EcoRII revealed that mouse satellite DNA is organized in nucleosomes (15). Treatment of rat liver nuclei with EcoRI preferentially released chromatin fractions with highly repetitive DNA which could be purified subsequently to near homogeneity (16). Detailed analyses of the digestion products obtained with several restriction nucleases have led to the formulation of a "domain" model seeking to explain the organization of the nucleo filaments and/or fibers within the interphase nucleus (17).

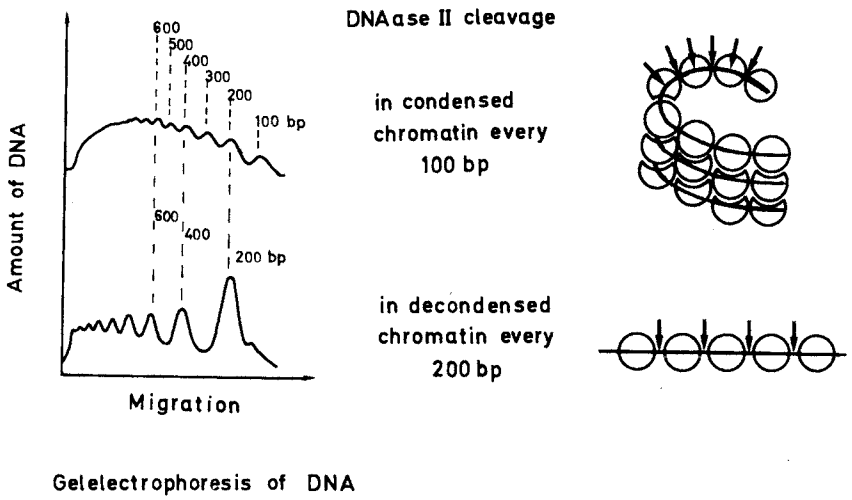

Fig. 1. Schematic representation of the DNAase II cleavage modes. See text for details.

This manuscript focuses on experiments directed towards the second level of chromatin structure. We have reported some unexpected findings resulting from our use of DNAase II to dissect chromatin (13,18,19). In contrast to other nucleases DNAase II cleaves native chromatin at a site within the nucleosome in addition to that in the linker region between nucleosomes thus giving rise to DNA digestion patterns with a 100 rather than the standard 200 bp periodicity (Fig. 1). What makes this result the more remarkable is that conditions which are known to change chromatin conformation affect the mode of DNAase II cleavage. Chromatin fully extended at low ionic strength and present as thin nucleo filaments has lost its nuclease susceptibility at the intranucleosomal site and is cleaved by DNAase II predominantly between nucleosomes with a 200 bp periodicity (Fig. 1). The expansion of the chromatin structure is reversible, the addition of appropriate salt concentrations results in contraction of the chromatin gel and restores the original cleavage mode of DNAase II.

One way of transferring nuclear material into the extended conformation is to wash nuclei repeatedly in NaCl and EDTA and suspend the resulting chromatin in dilute buffer. The DNAase II digestion patterns of this material in the presence or absence of divalent cations are shown in Fig. 2A, illustrating the switch from the 200 to the 100 bp cleavage mode of DNAase II.

The reversible contraction and expansion of chromatin is linked to the presence of histone H1 (20). It was important to determine therefore if H1 was also required for the divalent cation dependent change in the DNAase II cleavage pattern. To this end we first solubilized chromatin by digesting nuclei with low concentrations of micrococcal nuclease and subsequent lysis at low ionic strength (21,17). The soluble fraction was depleted of H1 by the tRNA procedure of Ilyin et al. (22). tRNA and short oligonucleosome chains were removed by Sepharose 2B chromatography. The high molecular weight chromatin which was shown to be H1 free by SDS gel electrophoresis was digested with DNAase II in the presence of 1 mM $CaCl_2$ or 1 mM EDTA. It is seen that under both conditions non-distinguishable 200 bp cleavage patterns are produced (Fig. 2B, lanes b) and c)). Note that the background of DNA material between the bands is lower than in the experiments with H1 containing chromatin cleaved in the 200 bp mode by DNAase II (Fig. 2A, lane a)). This reflects an even higher accessibility of the linker DNA relative to the DNA protected by the nucleosome cores. The change from the 100 bp to the 200 bp cleavage pattern on removing histone H1 was also observed with calf thymus chromatin (9).

DNAase II is known to cleave DNA in a haplotomic as well as diplotomic fashion, thus introducing single strand and double strand cuts into DNA. In order to examine the relative extent of single strand nicks in the DNAase II

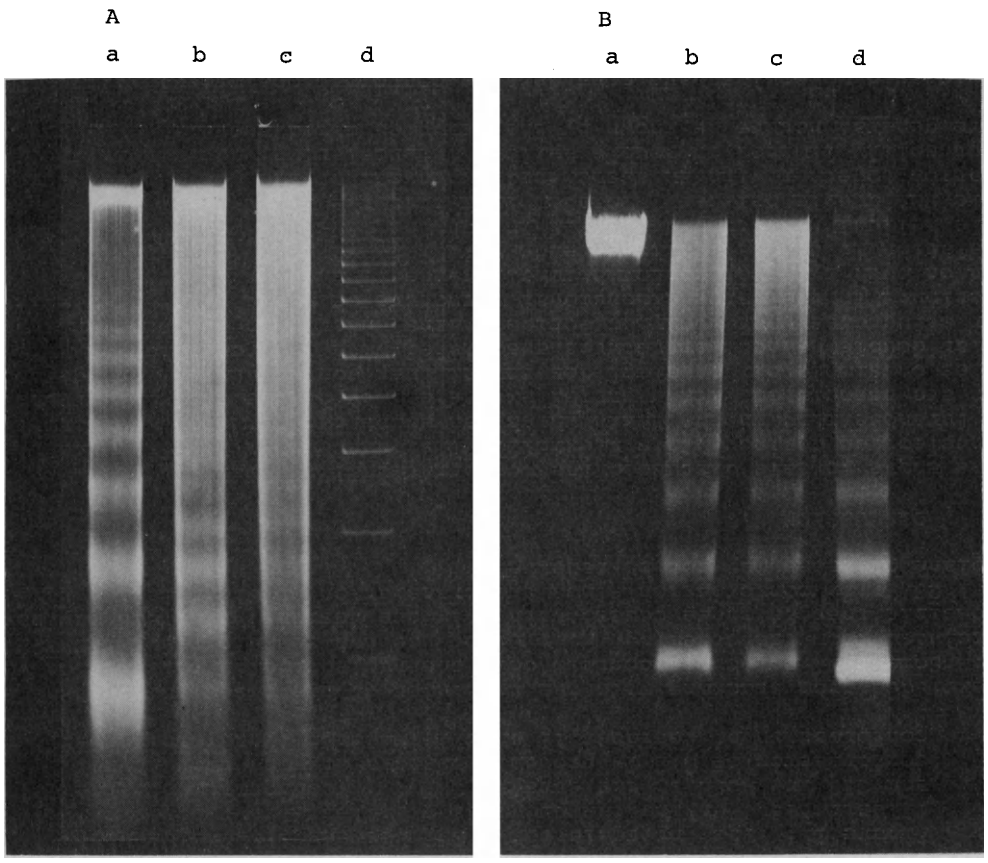

Fig. 2. DNAase II cleavage of chromatin at 100 bp intervals is dependent on the presence of histone H1. A. Chromatin was prepared by extracting mouse liver nuclei three times with 80 mM NaCl, 20 mM EDTA, pH 6.3. The resulting chromatin was washed twice in 10 mM Tris-HCl, pH 7.0, and digested in the same buffer with DNAase II (500 U/ml) in the presence of 1 mM phenylmethyl sulfonyl fluoride for 40 min at 37°. Additions were (a) none, (b) 1 mM $CaCl_2$, (c) 150 mM NaCl. DNA was extracted and analyzed on a 2 % agarose gel as described before (13). Lane d contains an EcoRII digest of mouse satellite DNA as a molecular weight marker (26). B. Mouse liver nuclei were digested with 15 U/ml micrococcal nuclease for 8 min at 37°, as described before (14). Chromatin was solubilized in 0.2 mM EDTA, 0.5 mM Tris-HCl, pH 8.0, and H1 removed by the tRNA procedure according to Ilyin et al. (11) employing the 40 mM NaCl, 3 mM EDTA, 5 mM Tris-HCl, pH 8.0, buffer. The H1-tRNA complex and small nucleosome chains were removed by chromatography on a Sepharose 2B column. The material passing through in the void was dialyzed against 10 mM Tris-HCl, pH 7.0, and digested in the same buffer for 20 min at 37°. Additions were (a) none, (b) 1 mM $CaCl_2$, DNAase II (300 U/ml), (c) 1 mM EDTA, DNAase II (100 U/ml), (d) 1 mM $CaCl_2$, micrococcal nuclease (1 U/ml). DNA was extracted and analyzed as in A.

degradation products of nuclei the DNA was denatured and analyzed by polyacrylamide gel electrophoresis (Fig. 3). This experiment is described in a thesis (23) but has not been otherwise published. As reported first for DNAase I digestion (24), the DNA shows a strong 10 nucleotide periodicity. The most intensely staining fragment is 90 nucleotides long as determined relative to a DNAase I digest where the 80 nucleotide fragment is strongest. These bands are ascribed to intranucleosomal nicking of the DNA with accessible regions present every 10 bp. It is remarkable that bands falling into the 10 nucleotide periodicity are also found at sizes around 200

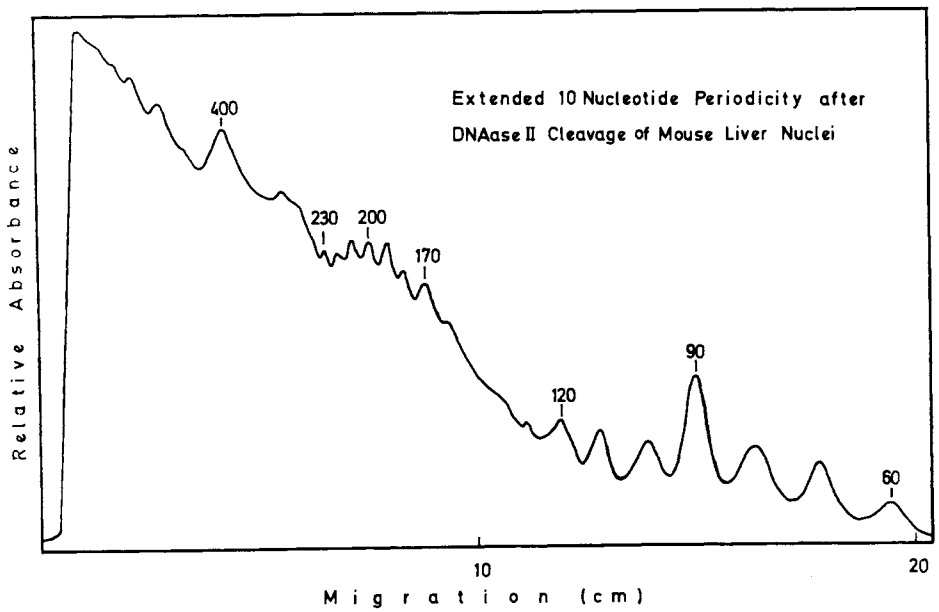

Fig. 3. Analysis of DNA from DNAase II digested nuclei under denaturing conditions. Mouse liver nuclei were digested with DNAase II (500 U/ml) for 1 h and the DNA extracted as described (14). 20 µg DNA were incubated with 10 M urea at 80° for 5 min and analyzed on a 6 % polyacrylamide gel containing 6 M urea according to Simpson et al. (27). The gel was stained with ethidium bromide, photographed, and the negative scanned as described (14).

nucleotides. This shows directly that at least some of the nucleosomes must be spaced accurately at integral multiples of 10 bp, a conclusion previously reached by Lohr et al. on the basis of DNAase I digestion patterns (25). At larger sizes broad bands are observed reflecting the 100 bp periodicity. It is apparent, however, that the 300, 500 etc. nucleotide fragments are underrepresented relative to the 200, 400 etc. nucleotide fragments. This would indicate that fragments which are generated by intranucleosomal splitting are nicked to a larger extent than fragments which are the result of only internucleosomal scission.

Concluding Remarks

The experiments described above clearly demonstrate the usefulness of DNAase II for structural investigations of chromatin. The results complement and extend those obtained with other nucleases since DNAase II appears to recognize certain features of chromatin which other nucleases are not sensitive to. A particular value of DNAase II rests in its property of being active in the presence as well as absence of divalent cations. It is therefore a powerful tool to investigate the degree of chromatin condensation which is strongly dependent on the ionic environment. Studies combining biochemical and electron microscopic techniques are in progress to better define the role of various parameters, such as histone H1, in the higher order structure of chromatin. Moreover it is our aim to contribute to the understanding of the DNAase II cleavage mechanism.

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BIOSYNTHESIS AND PROPERTIES OF NORMAL AND MUTANT *E. COLI* RNA POLYMERASE

R. B. Khesin

Institute of Molecular Genetics, USSR Academy of Sciences, Moscow 123212, USSR

ABSTRACT - Data are presented as to the effect of various changes in E.coli RNA polymerase on its interaction with template DNA and the biosynthesis of its subunits. The extent of immunological similarity between RNA polymerase subunits in different bacteria has been determined. The properties of E.coli and phage T7 enzymes are compared. The results are discussed in terms of the role of RNA polymerase changes in transcription regulation.

INTRODUCTION

The regulation of metabolism in bacteria, their adaptation and morphogenesis are primarily based on genome transcription regulation. The mechanisms which determine gene activity are highly diverse (Ref.1). RNA synthesis depends on chemical and structural changes in DNA. Special regulatory proteins act in two ways:

1. The activator- and repressor-proteins recognize specific DNA sequences, get bound to them and either assist RNA polymerase in initiating transcription or prevent it from doing so. The ρ-factor of termination may prematurely stop RNA synthesis.
2. Other transcription regulators interact with RNA polymerase instead of DNA. These are supposed to alter the ability of RNA polymerase to recognize various groups of promoters and use them for transcription initiation. Presumably different factors cause different specific rearrangements in RNA polymerase molecules so that they acquire the affinity to some promoters and lose the affinity to others.

This functionally determined structural diversity, the ability to assume various forms, must make RNA polymerase quite distinct from the majority of enzymes characterised by a high specificity for co-enzymes and substrates and from those enzymes, whose conformational rearrangements simply determine their activity level instead of changing their specificity. Therefore, we think it really important to study the various aspects of RNA polymerase: its structure and properties, biosynthesis and evolution in terms of its functions for transcription regulation. This paper presents some of such data, primarily the results obtained at our laboratory.

THE EFFECT OF MUTATIONAL CHANGES IN RNA POLYMERASE ON ITS INTERACTION WITH DNA

RNA polymerase comprizes a number of subunits forming the "minimal" (core) enzyme, and an instably attached polypeptide called the σ-factor (Refs. 1 & 2). Only when the latter is attached to the core enzyme does RNA polymerase specifically recognize the promoters. The natural question is: can changes in the core enzyme alter the specificity of RNA polymerase or is it the exclusive privilege of changes in the σ-factor?

We obtained a number of mutations altering the β and β' polypeptides of the core enzyme (Refs. 3 & 4). A.I.Gragerov and O.A.Larionov have demonstrated that certain mutations in either of these subunits may sharply alter the interaction of RNA polymerase with DNA, namely the rate of binding to DNA and the rate of complex dissociation, as well as the minimal temperature at which the enzyme unwinds the DNA double helix in promoters. G.M.Gorlenko's study of protein spectra in living mutant cells suggests that these changes in RNA polymerase also alter transcription specificity: for instance, in a temperature-sensitive rpoC1 (tsX) mutant the synthesis

of some proteins stops and that of others starts at non-permissive temperature. This may be due to an alteration of RNA polymerase's ability to recognize various promoters or react to various regulatory proteins. The first hypothesis is supported by the fact that normal and mutant enzymes interact in different ways with various template DNA's (e.g. the DNA of phages T2 and λ and oligo $(T)_{12}$).

The differences between normal and mutant RNA polymerases may be differently expressed depending on the tertiary structure, i.e. the supercoiling of template DNA. According to O.A.Larionov and A.I.Gragerov, one of the cold-sensitive (CS) mutant RNA polymerases (rpo B265) shows no difference from the normal one with regard to promoter opening on relaxed circular DNA, but is highly different on supercoiled ColE1 DNA.

Such expression of differences among enzymes in living cells was detected by S.M.Mirkin: the wild and mutant (rpoC3 and rpoB265) strains differ in resistance to coumermycin A, which, according to the well-known data (Ref.5) inhibits DNA-gyrase, which introduces the negative supercoiling into chromosome DNA. Supercoiling seems to affect different promoters in different ways, for V.N.Afanasiev found a change in the spectrum of newly-formed proteins upon coumermycin action on bacteria. This is not only due to the fact that supercoiling facilitates the "opening" of promoters (Refs. 6 & 7). The probability of the formation of cruciform structures by inverted repeats (palindromes) largely represented in promoters, was calculated by M.D.Frank-Kamenetskii et al. and proved to be negligible (in the order of 10^{-15}) in relaxed DNA, but many orders of magnitude higher in supercoiled DNA.

Thus, amino acid substitutions in various subunits of the core enzyme may alter the specificity of RNA polymerase's interaction with promoters even without decreasing its specific activity.

THE EFFECT OF RNA POLYMERASE CHANGES DURING BACTERIOPHAGE DEVELOPMENT ON ITS INTERACTION WITH DNA

Recently the first data were reported to the effect that mutational changes in the σ - factor may also alter the specificity of RNA polymerase for promoters (Ref.8). However it has long been supposed that σ -factor substitutions or the attachment of other polypeptides to RNA polymerase may alter its specificity and be a mechanism of transcription regulation during bacteriophage development. It has now been proved (Refs. 9 & 10).

The RNA polymerase purified from even T-phage-infected bacteria substantially differs from the normal enzyme in the minimal temperature at which it may unwind the DNA double helix at promoters (Refs. 11 & 12). Using different phage mutations which exclude the synthesis of new phage-encoded polypeptides, and recombining core enzymes and σ -like factors, I.Z.Zaitsev et al. have revealed the special role of a gene alc. The ability of RNA polymerase to open promoters is most altered by mutation in this gene, which is also known to adapt RNA polymerase to oxymethylcytosine-containing phage DNA and to inactivate it with respect to cytosine-containing DNA.

Infection-induced changes in RNA polymerase, the attachment of new polypeptides, alter its specificity. It begins synthesizing new RNA's on phage T2 DNA *in vitro*. These new RNA's, including "late" RNA's, are not formed by the enzyme of non-infected cells. At least, according to Yu.N.Zograff, the spectrum of proteins synthesized *in vitro* on the RNA which in turn has been formed *in vitro* by infected cell enzyme on T2 DNA, is quite different from the normal RNA polymerase patterns.

Thus, the attachment of new polypeptides to RNA polymerase alters the specificity of its interaction with various promoters and is used by bacteriophages as a mechanism of transcription regulation during their development. Transcription is switched on, sequencially in a similar way, at the various stages of morphogenesis in bacteria: during sporulation in bacilli (Refs. 9 & 10).

THE SYNTHESIS OF RNA POLYMERASE SUBUNITS

The regulation of RNA polymerase synthesis itself may be one of the mechanisms for controlling the level of transcription in the cell. Many mutational changes in RNA polymerase which decrease its activity or hamper its formation, have a stimulating effect on the synthesis of its subunits (Refs 11 & 13). For example, one of the temperature-sensitive mutations - rpoC1 (tsX) brings about a 2- to 6-fold increase in the enzyme subunit synthesis under non-permissive conditions (Ref. 14). When the activity of RNA

polymerase in living cells is partly inhibited by rifampicin, which gets bound to the enzyme, the synthesis of its subunits also increases. Finally, the combined effect of the mutation and rifampicin may increase the subunit synthesis rate 20-fold (Ref. 15). These facts suggest a compensatory increase in the synthesis of RNA polymerase components whenever it is damaged. A question arises: is its synthesis regulated at gene transcription or at the post-transcriptional level? To answer this we had to determine the rate of synthesis of the mRNA which encodes the enzyme's subunits.

A transducing λ -phage was obtained with the β-subunit gene (Ref. 16). A fragment of this gene was introduced by O.N.Danilevskaya into a vector plasmid. The presence of the β-subunit gene fragment in the plasmid was confirmed both electrophoretically and genetically. The same was shown by a heteroduplex analysis carried out by V.B.Fedoseeva. I.A.Bass and S.L.Mekhedov have shown by the hybridyzation of labelled RNA with plasmid DNA that the absolute amount of mRNA encoding the RNA polymerase subunit increases 1.5 - fold under the effect of rifampicin, while its relative amount (among all labelled RNA) increases nearly 3- fold. This stimulation of transcription of RNA polymerase genes may at least partly account for the doubling of the enzyme's subunit synthesis rate. The synthesis of RNA polymerase subunits may be further stimulated at the post-transcriptional level, for instance, due to a slower degradation of the corresponding mRNA's, or during translation.

Moreover, recently Zh.M.Gorlenko found that the introduction of small rifampicin concentrations into a cell-free system of coupled transcription and translation considerably accelerates the in vitro synthesis of the β-polypeptide directed by its gene, contained in the added DNA of the said transducing phage λ. This shows that rifampicin directly affects the properties of RNA polymerase during the transcription of its genes instead of acting through some other changes in intracellular processes.

Thus, whenever RNA polymerase is damaged, say when its activity is partly inhibited by an antibiotic, the transcription of its subunit genes is stimulated. The natural assumption is that the transcription of RNA polymerase genes is subject to autogenous regulation, i.e. RNA polymerase itself does not only transcribe these genes but also regulates the frequency of their transcription (Refs. 11 & 13). This is most easily accounted for by changes in the specificity of RNA polymerase under various effects.

One might inter alia expect that rifampicin, while partly inhibiting the enzyme's activity, would uniformly decrease the synthesis of all proteins in the cell. However, according to V.M.Aphanasiev, the antibiotic causes a sharp change in the spectrum of newly-formed proteins: the synthesis of some proteins is repressed while that of others, not only RNA polymerase components, sharply increases, as in the case of certain mutations in the enzyme's subunits.

One rather easily detects changes in the physical properties of RNA polymerase molecules caused by these mutations which stimulate its synthesis. The enzyme molecule is assembled out of isolated subunits in several stages, both in vivo and in vitro (Ref. 17):

$$\alpha + \alpha \longrightarrow \alpha_2 \xrightarrow{+\beta} \alpha_2\beta \xrightarrow{+\beta'} \alpha_2\beta\beta' \longrightarrow E$$

~4.5S ~6-9S ~10-11S ~14S

Once assembled, the molecules must "mature" so as to become active; the activation process is temperature-dependent and is stimulated by DNA and the σ - factor (Refs. 17 & 18). Premature and mature molecules substantially differ in the sedimentation rate : mature molecules have a sedimentation constant of about 14S, compared to 10-11S before maturation.

E.S.Kalyaeva and I.S.Sever have found that the above-mentioned temperature-sensitive mutation rpoC1 delays the enzyme maturation and a considerable part of its newly-formed molecules remains in the 10-11S peak (this accords with the data in Ref. 17). The maturation of molecules seems to consist in acquiring a tighter packing, a more compact structure. We do not yet know, whether the proportion of different molecular forms plays a role in the regulation of transcription of RNA polymerase genes.

Experiments on the core enzyme's self-assembly and its recombination with the σ - factor have shown that mutations often affect both the association of subunits and the enzyme's interaction with the regulatory polypeptide (Ref. 11). On the other hand, the excess subunits accumulated in a cell carrying mutations which stimulate their synthesis, quickly degrade (Ref. 19). E.S.Kalyaeva and I.S.Sever, however, have managed to obtain an alien suppressor mutation which stops the degradation of excess RNA polymerase

subunits thus normalizing the mutant phenotype: it is not impossible that a higher concentration even of abnormal subunits may stimulate their assembly.
It has recently been reported by other authors that the β and β' subunit genes are co-transcribed along with two ribosomal protein genes - rplJ (L10) and rplL (L7/L12) in the order: promoter - ribosomal genes - RNA polymerase genes (Ref.20). However such factors as rifampicin do not accelerate the synthesis of ribosomal proteins (Ref.21) though they do stimulate the formation of RNA polymerase mRNA and polypeptides, as stated above. On the other hand, a stringent or relaxed control at amino acid starvation carried out through the formation of guanosine tetraphosphate (ppGpp) is detected with regard to ribosomal protein synthesis, but not the synthesis of RNA polymerase subunits (Refs. 22 & 23). Hence the synthesis of RNA polymerase subunits must have a somewhat different mechanism of regulation at transcription stage: the regulation seems to be carried out at the region between the ribosomal and polymerase genes, but the mechanism is still obscure. At all events it does not seem to involve the attachment of RNA polymerase itself or any regulators which are present in the cell in small amounts: V.R.Yarulin and O.N.Danilevskaya have found that RNA polymerase binds the "2.6" restrict including the region between rplJL and rpoBC no more actively than another fragment without a promoter, and an increase in the number of "2.6" restrict copies in the cell obtained with the use of hybrid plasmids has no effect on the synthesis of RNA polymerase β and β' subunits.
Thus the properties of RNA polymerase molecules determine the activity of its own genes, the rate of subunit synthesis, the self-assembly and the reaction to regulatory factors.

THE EVOLUTIONARY VARIABILITY OF RNA POLYMERASE SUBUNITS

A protein is supposed to be the more conservative evolutionwise the more its functioning brings it in touch with other components: proteins, nucleic acids, co-enzymes etc. RNA polymerase is composed of subunits which are naturally in touch with each other. Whole molecules must specifically interact with template DNA, NTP and the regulatory factors. Therefore one might expect the components of RNA polymerase to be highly conservative and the enzymes of different bacteria to be highly similar. To investigate this point we studied the α-polypeptide in co-operation with Yu.A.Ovchinnikov's laboratory, since it is that laboratory which established its primary structure (329 amino acids) (Ref.24)
Indeed, the peptide maps of the α-subunit of RNA polymerase in E.coli and other enteric bacteria are either identical (S.marcescens) or show differences in only 10% of the spots (P.mirabilis) (Ref.25). As regards other proteins (e.g. anthranilic acid isomerase), 50% of spots show differences even in a much more closely related pair: E.coli - S.thyphimurium.
To further investigate the RNA polymerases of different bacteria, A.N.Lebedev and V.G.Nikiforov used the immunological method, i.e. the competition of different proteins for the binding with antibodies immobilised on cellulose. The antibodies were obtained through the immunization of rabbits by preparations of individual subunits of E.coli RNA polymerase. By comparing the binding of an isolated polypeptide and the same polypeptide within the core enzyme, one could distinguish the external and internal antigenic determinants.
The surface antigenic determinants of the α-subunit showed no differences at all within Enterobacteriaceae. It is only when E.coli was compared with a non-enteric bacteria like pseudomonades (P.putida) that differences were revealed. The external determinants of these bacteria were found to differ much less, i.e. to be more conservative than the internal determinants of the α-polypeptide enclosed in the whole enzyme.
This emphasizes the role of the surface structure of RNA polymerase in the α-subunit for its functioning, possibly for interaction with the regulatory factors and/or NTP, since the location of these on the α-subunit has recently been demonstrated by affinity labelling (Ref.26).
However the other subunits of RNA polymerase are not nearly so conservative. For instance, in P.mirabilis, which shows practically no difference from E.coli with regard to the α-subunit determinants, there is only a 40% and 20% homology for the external determinants of β and β' subunits, respectively, while in P.putida and A.vineladii the homology for and is less than 10%, compared to 30-80% for the α-polypeptide.
Suprisingly enough, even when the RNA polymerases of remote bacteria show no detectable amount of common determinants, their subunits are capable of

recombination with the formation of active molecules (Refs. 27 & 28). Possibly to ensure a complementary attachment of large surfaces at subunit association or the binding with long DNA stretches in promoters, the polypeptides must possess a very general structural similarity,but no local identity of the small regions that can create antigenic determinants. By contrast, the binding of small molecules like NTP or the specific interactions with regulatory factors and the transfer of their effect require the strictly specific structures, which form conservative antigenic determinants on the α - polypeptide.

Indeed, the nucleotide sequences in different promoters are known to practically lack any resemblance but be recognized by the same RNA polymerase (Ref.1). Similarily, different protein structures may recognize identical long DNA sequences.

However, as stated above, local changes, mutations, at different points of the enzyme molecule and the attachment of low-molecular weight ligands alter the ability of RNA polymerase to recognize different promoters. Most probably, these changes have no direct effect on the DNA recognition site, but affect other properties of RNA polymerase, especially the internal mobility of its polypeptides required for enzyme assembly (its maturation), the adaptation to different promoter structures and the organization of response to the regulatory factors.

The evolutionary, that is comparative, approach to RNA polymerase gives additional data as to the role of its subunits and the mechanism of its functioning. For instance, the simple RNA polymerases of some phages and eukaryotic mitochondria are monomeric (Ref.29) in contrast with the complex multimeric enzymes in bacteria and eukaryotic nuclei (Ref.30). Nevertheless both perform the same functions including the specific recognition of promoters. Furthermore, in bacteria one enzyme may assume different states which, inter alia, enable it to distinguish ribosomal genes from protein structural genes (Ref.31), and the differentiated eukaryotic RNA polymerases are at least partly composed of different subunits, so that one enzyme recognizes the genes of large ribosomal RNA, another those of 5S rRNA and tRNA, yet another the rest of the genes on which mRNA is synthesised (Ref.30).

Curious differences are observed between RNA polymerases of different species in accordance with their functions. For instance, phage T7 RNA polymerase is more specific with regard to RNA initiation on DNA than the E.coli enzyme, which is probably due to the smaller diversity of promoters on the viral chromosome compared to the bacterial one (Ref.32). The phage enzyme may use shorter templates for homopolymer (poly(A)) sinthesis than bacterial RNA polymerase. Cf: the stimulation of poly(A) synthesis on oligo $(dT)_{12}$ by the E.coli enzyme increases with an increase in oligo(A)-primer (according to the primer activity ApApApA > ApApA > ApA). Meanwhile T7 RNA polymerase is equally stimulated by ApA, ApApA and ApApApA but the attachment of C to the 5' - end, forming CpApA or CpApApA, somewhat decreases the primer activity of ApA and ApApA for the viral enzyme. This shows that after all it reacts with no less than four, instead of two, 3'-end nucleotides of the RNA product and elongates the latter less if it erroneously contains a nucleotide which is not complementary to the template. Here again bacterial RNA polymerase is different from the viral one.

Many of the differences between oligomeric and monomeric enzymes may be due to the fact that the former have to operate on complex templates containing a great many genes and, consequently, different promoters; this in turn requires interaction with various regulators. It seems plausible therefore that the complicated structure of RNA polymerases in bacteria and eukaryotic nuclei is designed for transcription regulation through changes in its specificity caused by structural rearrangements.

In conclusion one can say that there seems to be no other enzyme like RNA polymerase, in the sense that it raises an extremely wide range of problems from purely physicochemical (molecular) ones to biological problems concerning the regulation of the cell's vital activity, development and adaptation to the environment.

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ISOLATION, CLONING AND STUDIES ON THE STRUCTURAL ORGANIZATION OF EUKARYOTIC GENES

G. P. Georgiev

Institute of Molecular Biology, USSR Academy of Sciences, Moscow B-334, USSR

Abstract - The technology of the isolation of the DNA fragments containing structural genes and flanking sequences is discussed. Many of efficiently expressed structural genes of D.melanogaster are represented by a high number of identical copies scattered throughout the whole genome and surrounded by different DNA sequences. They are often located close one to another. Their location is very unstable but usually they are present in the sites of intercalary heterochromatin. Using cloning, two types of sequences from mouse genome hybridizing to a half and to a quarter of the total dsRNA-B were isolated. These very abundant sequences present in the great majority of pre-mRNAs and may have an important role in regulation of transcription or processing.

INTRODUCTION

Studies on the structure and function of the eukaryotic genome have been limited for a long time by the large size and heterogeneity of the latter. All information was obtained by analysing the total DNA or the whole nuclear pre-mRNA. Still, these studies along with some genetic experiments led to the establishment of several general fundamental properties of the eukaryotic genome organization.

It has been found that the eukaryotic genome contains different classes of sequences , highly repetitive simple sequences (satellites), indermediate repeats, and unique sequences (Ref. 1). Most of the structural genes or sequences responsible for transcription of mRNAs are unique occurring one or a few times in haploid genome. Several genes (ribosomal RNA and tRNA genes, histone genes) are represented by multiple copies usually clustered in a certain region of a chromosome (Ref. 2 & 3).

The primary product of transcription is a precursor of mRNA (pre-mRNA) which is several times longer than the mature mRNA (Refs. 4-6). In the course of mRNA maturation, a significant part of pre-mRNA is destroyed (Ref. 7). Thus, the majority of transcribed DNA is not involved in coding for proteins.

Genetic studies have shown that each band in D.melanogaster contains only one genetic unit on the average (Ref. 8). On the other hand, the average size of DNA in the band is very large, of about 35 kb. It indicates the existence of a large amount of excessive DNA. Thus, apart from structural genes, the eukaryotic genome contains a number of non-transcribed and transcribed but not translated DNA sequences whose functional role is a subject for many speculations (Refs. 9-11).

Recently, the introduction of genetic engineering techniques made it possible to isolate and study individual genes instead of the total DNA; as a result, the information flow was very much accelerated. Two discoveries have been made with the aid of this techniques. The first one is the discovery of interventing sequences in many structural genes which are responsible for the main part of transcribed but not translated DNA (Refs. 12-17). The second one is the discovery of a large group of multiple structural genes with varying location in different parts of the genome (Refs. 18 & 19).

In the present paper, I shall describe the latter in more detail. Also the general strategy in the isolation and cloning of genes will be discussed, as well as some new data on the isolation of some particular sequences present in the eukaryotic genomes.

ISOLATION, CLONING, AND SELECTION OF EUKARYOTIC GENES

There are two main strategies. The first and probably the most practicable one is to prepare random fragments of DNA, to obtain with them a great number of clones, and then to find among them those containing the sequences of interest. This approach has been successfully realized in constructing gene libraries (Ref. 20). Long (~20 kb) fragments of eukaryotic DNA were ligated with synthetic restriction sites and inserted into ~ DNA. The recombinants were then covered with a phage capside, the procedure increasing many times the infectivity. Clones were obtained at a very high multiplicity and then the studied genes were selected by two-step colony hybridization of heavily labeled cDNA to 10^6 clones. Once the gene libraries are established, further isolation of clones is very fast.
Another strategy is based on the enrichment of total DNA with the investigated DNA sequence before cloning. One of the techniques successfully used (Ref.13) involved fractionation of the restriction DNA fragments on a RPC-5 column and by gel electrophoresis followed by selection of fractions hybridizing to the corresponding cDNAs. After such purification, about 1/1000 of all the clones obtained contain the investigated sequences.
Another way for selection is the use of the R-loop technique (Ref.14).
An alternative method of preselection has been worked out in our laboratory (Refs. 21 & 22). This "gap method" consists of the following main steps:
(1) High molecular weight DNA (~100 kb on the average) is prepared.
(2) A number of single-stranded nicks (one nick per 1-10 kb) are produced using pancreatic DNAase.
(3) Nicks are converted into short gaps (100-500 bases) with the aid of exonuclease III.
(4) DNA is restricted by a certain endonuclease (Hind III, EcoRI, Bam, etc) completely or to fragments of a desired size (underrestriction).
(5) The restricted DNA is hybridized to mRNA and the hybrids are fished out by oligo(dT) cellulose, or poly(U)-Sepharose chromatography. The hybrids are eluted with the aid of RNAase TI and deproteinized.
(6) The selected DNA (purified ~100-1000 times in a certain structural gene) is ligated with the restricted pBR-322 (Boyer) DNA.
(7) The ligated material is again hybridized to mRNA, and the hybrids eluted from poly(U)-Sepharose are used for transformation. The second step gives additional ~100 times purification.
This typical procedure may be changed. For example, it is possible to prepare first the recombinant DNA containing randomly sheared eukaryotic DNA fragments and plasmid DNA, and thereafter to produce gaps by DNAase I endonuclease treatment and purify the gene containing DNA by hybridization to mRNA.
Using the above procedure, we were able to isolate several clones containing mouse structural genes efficiently expressed in Ehrlich carcinoma cells.
The method has been also applied for the isolation of the albumin gene from the rat genome. In the first experiments, restriction endonucleases used for the rat and pBR-322 DNA cutting were EcoRI and Hind III taken in mixture. Four out of eighty clones obtained from 240 μg rat DNA contained sequences hybridizing to albumin mRNA as followed from the colony hybridization experiments. This indicates a very high (~100,000X) degree of purification. Unfortunately, the inserted rat DNA fragment was rather small (1.5 kb), and hybridized to only about 3% of the length of the total albumin mRNA. All four clones isolated hybridized to the same fragment of mRNA. Thus, although the choice of restriction endonucleases in this experiment was not optimal, the result obtained indicated the applicability of the method for isolation of specific DNA sequences. On the other hand, it is necessary in every case to make a correct choice of restriction endonucleases in order to obtain DNA fragments of the optimal length.
To date many groups of workers using different strategies have obtained a number of clones containing eukaryotic genes with flanking sequences, and this led to a number of discoveries. Some of them are discussed below.

MULTIPLE STRUCTURAL GENES OF DROSOPHILA MELANOGASTER AND THEIR PROPERTIES

Ilyin and Tchurikov (Refs. 22 & 23) cloned the EcoRI fragments of the D. melanogaster genome and selected clones whose DNA bound the highest amounts of mRNA prepared from the culture cells of D.melanogaster. Three such clones, Dm 225, Dm 234, and Dm 118, have been isolated. Their pro-

perties are presented in Table 1 (Refs. 18 & 24). All of them hybridize to a significant proportion of mRNA, from 0.3 to 1.1%.

TABLE 1. The properties of the studied EcoRI fragments

Cloned fragments	Size of EcoRI fragment, kb	% of mRNA bound	S value of mRNA	Number of copies per genome
Dm 225	2.9	0.8	20S	200-250
Dm 234	1.6	0.3	30S	250
Dm 118	1.0 +3.0	1.1	15S	50-100

As the average content of individual mRNA is lower than 0.1%, one may conclude that transcription of the selected genes far exceeds the average level.

Thus, all the three genes are actively transcribed. Upon hybridization, each of them combines with mRNA of a definite size. Dm 225 DNA hybridizes to 20S mRNA, Dm 234 - to 30-35S mRNA, and Dm 118 DNA - to 15S mRNA. These S values correspond to RNAs of about 2.3 kb, 4-5 kb, and 2 kb, respectively. The size of cloned DNA fragments is 2.9 kb (Dm 225), ~1.6 kb (Dm 234), and ~4 kb (Dm 118). Thus, only Dm 225 and Dm 118 may contain the whole structural gene or a significant part of it while the Dm 234 fragment corresponds to less than a half of the gene.

Data indicating active translation of gene 225 were also obtained. mRNA was prepared from polysomes of different size and hybridized to Dm 225 DNA (Ref. 24). The peak of RNA binding was located in polysomes containing about 20-25 ribosomes per mRNA which correlated well with the size of mRNA. In other words, the ribosomes are attached to the whole length of mRNA, and it seems very probable that mRNA is in the process of translation. For two other mRNAs, such analysis has not yet been done but it seems quite possible that they are also translated since the corresponding mRNAs have been prepared from polysomes.

In the next series of experiments, the DNAs from clones were hybridized to the total DNA of D.melanogaster labeled by nick translation. Under the saturation conditions, Dm 225 DNA binds about 0.5% of the total Drosophila DNA. It is a very high figure. If Dm 225 sequence is unique, it should bind only ~ 0.002% of DNA, and such a binding was indeed observed in the cases when DNA fragments containing unique sequences have been studied. Therefore, one may conclude that Dm 225 DNA is represented in the genome by~ 250 copies. In the same way, we detected the number of copies for gene 234 to be equal to 250, and for gene 118 about 50. Thus, all the three genes under investigation are multiple genes.

It is important that the structural gene itself is repeated. It was first proved in experiments where DNA-DNA hybridization was performed in the presence of unlabeled mRNA taken as a competitor. In the case of Dm 225 DNA, the 75% competition takes place. Thus,about 75% of the repetitive sequences presented in Dm 225 DNA can be accounted to a structural gene. Even a higher competition has been obtained in the case of Dm 234 DNA which probably as a whole corresponds to the structural gene. The situation is more complex with Dm 118 DNA. It consists of two EcoRI subfragments. Both are repeated about 50 times per genome but only one of them corresponds to the structural gene.

A more detailed analysis of the properties of Dm 225 DNA was performed thereafter. The DNA excised with the aid of EcoRI endonuclease was further cleaved to five fragments by HaeIII endonuclease. These subfragments were separated electrophoretically and their arrangement in Dm 225 DNA was detected (Fig. 1). In hybridization experiments, mRNA combines with all subfragments. Its 3'-end is mapped in the fragment B. Thus, almost all Dm 225 DNA, except a part of the fragment B, corresponds to the structural gene coding mRNA and probably the whole gene is included in Dm 225 DNA. This result again confirms the repetitiveness of the whole structural gene 225.

The next question solved with gene Dm 225 was whether all copies of this gene in the genome were identical or not. To answer this question, the total DNA of D.melanogaster was restricted by EcoRI endonuclease, the same enzyme which had been used for original excising Dm 225 DNA. Thereafter, the DNA was fractionated according to its size in polyacrylamide gel, transferred onto a nitrocellulose filter, and hybridized with ^{32}P-labeled Dm 225 DNA. The distribution of the bound label along the filter was analysed by autoradiography. The distribution of the total DNA EcoRI

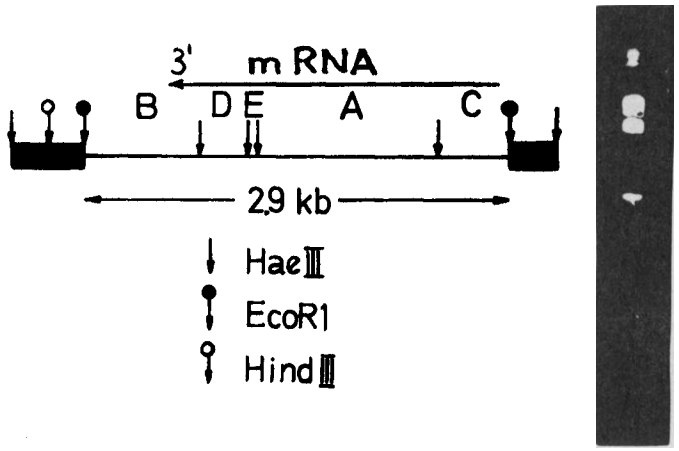

Fig. 1. The restriction and transcription mapping of Dm 225 DNA fragment. On the right, the electrophoregram is presented demonstrating the separation of HaeIII fragments of Dm 225 DNA.

restricts was very heterogeneous along the filter. However, the labeled material was found only in one band. This band contained DNA of 2.9 kb in length. Thus, all copies of DNA in the genome containing Dm 225 sequences in the EcoRI restricts had exactly the same size. If the same experiment was performed but using two endonucleases, EcoRI and HaeIII, hybridization again took place only with fragments equal in size to those prepared from Dm 225 DNA by the EcoRI+HaeIII treatment. In other words, all 250 copies of Dm 225 gene in the genome of D.melanogaster were identical. The same result was obtained in the work with two different lines of cultivated cells (Fig. 2).

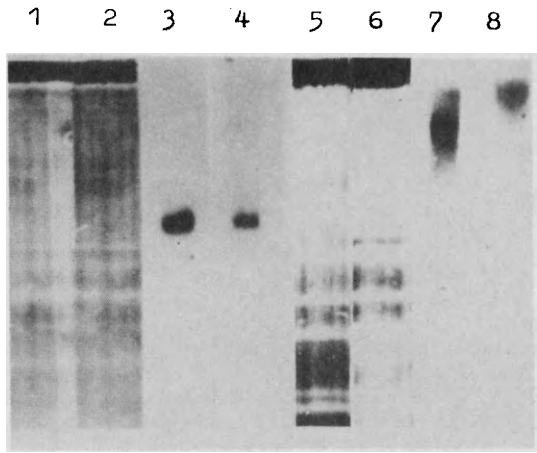

Fig. 2. The hybridization of /^{32}P/ Dm 225 DNA to restricted DNA of D.melanogaster. 1,2 - electrophoretic fractionation of D.melanogaster DNA treated by EcoRI endonuclease; 3,4 - autoradiograms of the same samples after hybridization to Dm 225 DNA; 5,6 - the same as 1 but after HindIII endonuclease treatment; 7,8 - the same as 3 but after HindIII endonuclease treatment. 1,3,5,7 - DNA was prepared from cell line 67J25G; 2,4,6,8 - from cell line 67J25D.

The experiment was also conducted with Dm 234 and Dm 118 sequences. EcoRI restricts of the total DNA were used for blotting and hybridization. Dm 234 was found to bind with only one band equal in size to Dm 234 (~1.6 kb). In the case of Dm 118, EcoRI endonuclease cut the sequence in two fragments, 1 and 3 kb in length. Only one of them, namely, fragment B (1 kb) hybridized to mRNA and at the same time it hybridized to only one band of restricted DNA. Thus, in all cases, the multiple structural genes themselves are very homogeneous.

The next question was whether sequences surrounding all copies of Dm 225 gene in the genome were different. To answer it, the total DNA of D.melanogaster was restricted with HindIII enzyme which did not cut the Dm 225 fragment. Thus, this enzyme excised the investigated gene with its flanking sequences. After blotting to a nitrocellulose filter, hybridization with Dm 225 /^{32}P/ DNA was performed. The labeled DNA was now hybridized with heterogeneous populations of sequences. One may conclude that different copies of Dm 225 gene in the genome have different flanking sequences. Moreover, the distribution of the label in DNA from two different cell sublines of D.melanogaster is different. Thus, sequences surrounding Dm 225 DNA in the two sublines are not the same. It is necessary to note that these two sublines originated from the same parental line but were grown separately for several hundred generations, one of the lines being adapted to grow in a serum-free medium. Thus, one may suggest that gene 225 is localized in a number of different sites of the genome and that the sites can vary in different cells (Fig. 2).

For further analysis, new clones containing the Dm 225 sequence were prepared (Ref. 25). In these experiments, HindIII fragments of the D.melanogaster DNA were used. Seven different clones were obtained, and their DNAs were restricted either by HindIII or by EcoRI endonucleases, or by their mixture (Fig. 3). All seven clones contained 2.9 kb Dm 225 fragment, limited by two EcoRI sites. Five of them contained also 1.4 kb EcoRI-HindIII fragment adjacent to the 5'-end of Dm 225 mRNA. This 1.4 kb fragment was homologous to another polysomal mRNA. Fragments adjacent to the 3'-end of Dm 225 mRNA were very heterogeneous. In all cases, their size was different. Thus, the conclusion about the heterogeneity of flanking sequences for Dm 225 gene was confirmed directly.

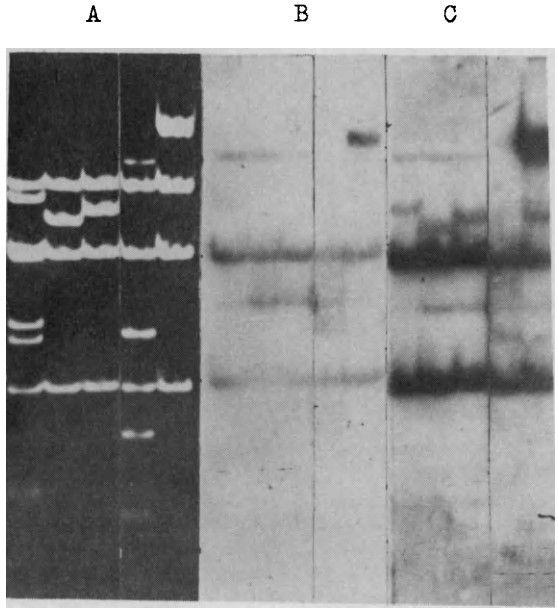

Fig. 3. The properties of DNA prepared from five clones containing pBR-322 plasmid DNA and HindIII fragments of D.melanogaster DNA hybridizing to Dm 225 DNA. A - electrophoregram of the material obtained after restriction by a mixture of HindIII and EcoRI endonucleases; B - hybridization of filters prepared from slabs (Fig. A) to /^{32}P/ mRNA; C - the same as B but /^{32}P/ pre-mRNA.

Then hybridization of the fragmented DNA of clones with mRNA and nuclear pre-mRNA was studied (Fig. 3). 2.9 and 1.4 kb bands bound high amounts of mRNA. It is interesting that one of the clones contained a third band (~7 kb) which bound even higher amounts of mRNA. This band was analysed in more detail and was found to hybridize with about 2% of the total mRNA, thus representing the expressed structural gene. Moreover, according to experiments on hybridization with DNA this gene like others is also multiple.

One may conclude that different multiple structural genes are often localized very closely one to another in the genome. In other words, there are certain regions in the genome where these genes are preferentially located.

The above mentioned conclusions were confirmed in experiments on the *in situ* hybridization of labeled DNA or labeled cRNA transcribed from cloned DNA to polytene chromosomes of *Drosophila* salivary glands (Refs. 18 & 25). It was found that the DNAs of each of the three genes under study hybridized to a large number of sites in the chromosomes. All cells obtained from the same animal had the same distribution of hybridization sites. For example, Dm 225 DNA hybridized to 40 sites on polytene chromosomes obtained from heterozygous (gtw^a x gt^{13z}) animals (Fig. 4A. Such hetero-

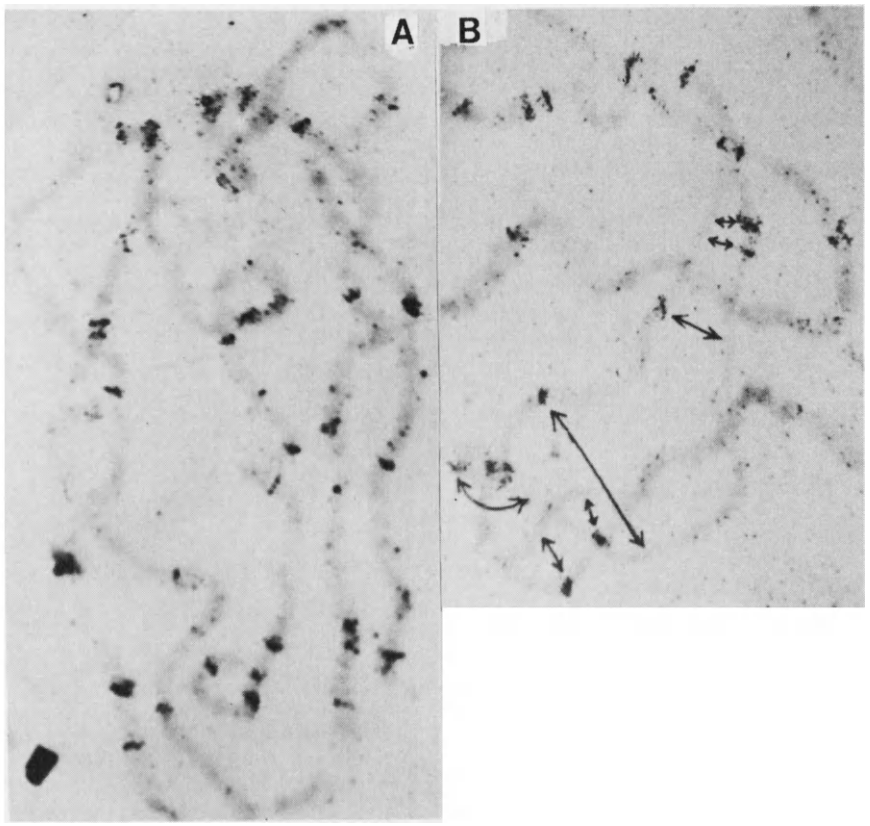

Fig. 4.A. *In situ* hybridization of *D.melanogaster* polytene chromosomes with Dm 225 cDNA. The latter was transcribed with the aid of E.coli RNA polymerase from λgt-Dm 225 DNA. For hybridization the chromosomes of ♀ gtw^a/♂ gt^{13z} were used. B. The differences in the localization of Dm 225 hybridization sites in the different strains of *D.melanogaster* as seen in the unpaired regions of chromosomes of a gtw^a/gt^{13z} hybrid. The non-conjugated zones are localized between regions 98 and 92 and between regions 88 and 86.

zygous animals were used in order to obtain a high extent of polyteny and therefore thick chromosomes. In these chromosomes, one can see sometimes the region of asynapsis, i.e. regions where the homologous chromosomes are not paired (Fig. 4B). In these regions, one can follow the distribution of hybridization sites between the parental chromosomes. We found that this distribution was quite different. Therefore, detailed investigation of hybridization with chromosomes from animals of different Drosophila stocks was performed. First of all, the gtw^a and gt^{13z} animals were compared. Animals of any of these stocks had 20-25 hybridization sites on their chromosomes but only six of them were common. The localization of all others was different. When individuals from the same stock were compared these differences were less prominent but still 20-30% of hybridization sites were different.

In agreement with some independent biochemical data, genes Dm 225 and Dm 234 have very similar distribution in the genome. The score of chromosomes from 15 animals gave about 70 sites where these two genes may occur. The localization of gene 118 was even less stable. About 150 sites were found where gene 118 can be located.

The study of the sites where the genes, in particular Dm 225 and 234, are located has demonstrated their non-random distribution in chromosomes, i.e. a remarkable coincidence of these sites with the location of the so-called intercalary heterochromatin in D.melanogaster. The intercalary heterochromatin is characterized by the following properties: (1) a high frequency of ectopic pairing or pairing between non-homologous sites in these regions of polytene chromosomes; (2) late replication; (3) occurrence of underreplication resulting in the presence of the so-called "weak spots" (for reference see Ref. 18). The total number of such sites in the chromosomes is close to one hundred. Their functions remain obscure.

We found that about 90% of hybridization sites for genes Dm 225 and 234 coincided with the sites of the intercalary heterochromatin known from literature. On the other hand, about a half of the latter is covered by hybridization sites for the two mentioned genes. The same is true of gene 118. One may conclude that multiple structural genes scattered throughout chromosomes are localized in the regions of the intercalary heterochromatin; apparently, these genes are easily transposed from one site of the intercalary heterochromatin to another. Often the studied genes are localized not only in the two sites of the intercalary heterochromatin but also in the fibril formed in the course of ectopic pairing between the sites, as followed from the in situ hybridization. Thus, the genes themselves may be involved in the process of ectopic pairing.

It is interesting that clustered multiple genes with stable localization such as the histone or rRNA genes are also localized in the intercalary heterochromatin. Moreover, recent observations have indicated the possibility of their translocation although it is much less prominent than in the case of the three mentioned genes (Refs. 27 & 28).

A general scheme can be drawn according to which eukaryotic chromosomes contain a number of regions designated as intercalary heterochromatin where different multiple genes are concentrated. Although the nature of these genes remains obscure, one may suggest that they are involved in the synthesis of abundant proteins used for the own needs of the cells. Therefore, the corresponding genes should be actively expressed. These genes may be easily translocated but only among the mentioned regions (nests for multiple genes) which are organized in such a way as to make such translocations possible. It is not unlikely that the negative control of gene expression in these sites is less prominent than in other sites of the genome. Differences in the number and distribution of multiple genes in the genome may determine many individual differences between animals.

It should be pointed out in conclusion that the described type of genome organization is not an exception but a significant proportion of the eukaryotic genome is organized in this manner. Except the above four genes, two others with similar properties have been described in the Hogness laboratory (Ref. 19). The instability in location of these genes was also observed recently. All in all, these genes can constitute 2-3% of the whole genome. Such genes were also detected recently in yeasts (R.Davis, personal communication).

STUDIES ON CERTAIN DNA SEQUENCES IN MOUSE GENOME

As I have mentioned above, one of the main recent achievements in the molecular biology of eukaryotic gene is the discovery of split genes and splicing. Splicing seems to be one of the most important parts of pre-mRNA processing. It should involve a number of very specific steps at which the sequence recognition takes place. For this and other reasons, it is still important to study certain DNA sequences present in many different transcriptional units. The use of cloning technology much facilitated these studies.

As an example, I will present recent data on the isolation of DNA sequences complementary to double-stranded regions in pre-mRNA. It was shown previously that at least the major part of pre-mRNA molecules contained double-stranded hairpin-like structures (Refs. 29-31). The double-stranded (ds) regions of pre-mRNA were subdivided into four different classes (Ref. 32). One of the most interesting classes comprises sequences designated as ds RNA-B. Their length is of about 200 base pairs and they are able to snap back after pre-mRNA melting. Using the renaturation kinetics analysis, we found ds RNA-B to be very homogeneous and to consist of a few kinds of sequences (Refs. 31 & 32). A similar conclusion was achieved later in sequencing studies (Ref. 33).

It was suggested that ds RNA-B might be involved in processing by serving as a signal for processing enzymes. This idea was supported by experiments demonstrating the existence of complementarity between rather short sequences in mature mRNA and dsRNA-B.

In recent cloning experiments developed in our laboratory (Ref. 34), we found that many randomly selected clones contained sequences binding the isolated and denatured ds RNA-B. In saturation conditions, each clone bound a very significant proportion of the total ds RNA-B. A group of clones, for example, combined with 30-35% of ds RNA-B. If the bound RNA was eluted from a filter and hybridization was repeated, 50-60% of the eluted RNA could be bound again. This is probably the maximal efficiency of the reaction. Thus, one can conclude that the original amount of ds RNA able to hybridize with cloned DNA constituted about 50% of the total ds RNA. The RNA purified by hybridization and elution from one of the clones hybridized equally well to the DNA of this clone and to DNA of any other clone of the same group (Table 2). Thus, the same kind of sequence is present in many different clones. This sequence was designated as a ds RNA-B1 (or B1-sequence). It corresponds to about a half of the total ds RNA-B.

TABLE 2. Hybridization of /^{3}H/ labeled ds RNA-B sequences preselected by binding to DNA of clones №35 and №14 with DNA prepared from different clones

№ of experiment	DNA on filter (clone №)	Hybridization, %		
		Total ds RNA	ds RNA complementary to DNA of clone №35	ds RNA complementary to DNA of clone №14
1	4	24	50	
	14	7	0	
	15	14	32	
	31	22	60	
	35	22	47	
	75	19	35	
	87	17	37	
2	3	30	50	48
	14	11	0	58
	35	21	70	0
	61	17	0	46
	63	10	0	35
	89	15	31	0

For each experiment, 27,000 cpm of total /^{3}H/ dsRNA or 500 cpm of preselected /^{3}H/ ds RNA was taken.

We isolated also clones containing another sequence, designated as B-2 which was able to combine with about a quarter of the total ds RNA-B. Clones containing the B1 and B2 sequences do not cross-hybridize. Thus, The B1 and B2 sequences are completely different. On the other hand, the melting profiles of hybrids between cloned DNA and ds RNA demonstrate the good quality of hybrids and the absence of a prominent mismatching. As ds RNA originates from many different pre-mRNAs, one may conclude that all sequences belonging to the same class in particular B1 are very similar if not identical.
The sequences B1 and B2 are widely distributed throughout the mouse genome. In the saturation conditions, the cloned DNAs hybridize to 0.6% and 0.4% of the total cellular DNA in the case of the sequences B1 and B2, respectively. The corresponding figures for hybridization of nuclear pre-mRNA are 1.5 and 0.7%. The number of these sequences is about 4 times higher than the number of palindromes in DNA or of ds RNA in pre-mRNA. It means that a significant part of them in the genome probably is not organized in palindromes. The role of these very specific sequences still remains unknown. We often see them in close neighbourhood with sequences complementary to mRNA, i.e., with structural genes. In any case, the cloning of these sequences allowed direct approaches in studies of the primary structure, their organization in the genome and function. This work is in progress now.

The data presented in the paper demonstrate several lines of recent progress in studies on the eukaryotic gene organization induced by application of genetic engineering technology. New data are accumulated so fast in this field now that one may expect the formulation of the main principles of the gene structure and function in higher organisms in the nearest future.

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PRIMARY ORGANIZATION AND FUNCTIONING OF THE NUCLEOSOME CORE PARTICLE OF CHROMATIN

A. D. Mirzabekov, V. V. Shick, A. V. Belyavsky, S. G. Bavykin and A. Rich*

Institute of Molecular Biology, Academy of Sciences of the USSR, Moscow 117984, USSR
**Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA*

Abstract - The sequence arrangement of histones along the DNA in the nucleosome core has been determined. It has been shown that histones are arranged within discrete DNA segments of about 5-7 nucleotides long separated by regularly spaced histone-free gaps of about 3-5 nucleotides long. A linearized model of the nucleosome core has been proposed. The histone-free gaps appear to be located on one side of the DNA molecule. The lateral binding of histones to DNA creates an asymmetric neutralization of DNA phosphate groups. Some features of the model are discussed in terms of nucleosome function. (1) Histones appear to leave the minor and part of the major groove of DNA exposed and accessible to recognition by some proteins. (2) Histones are unlikely to form a "cage" around DNA; the nucleosomal DNA may therefore be readily released from the histone octamer during replication and transcription. (3) The asymmetric neutralization of DNA by histones may be the driving force for nucleosome folding.

INTRODUCTION

The major part of the eucaryotic genome is organized in the form of a series of nucleosomes. The basic structural elements of nucleosomes in all cell types seems to be a core particle containing DNA of about 140 base pairs in length complexed with a histone octamer containing two copies each of histones H2A, H2B, H3 and H4. In addition, the nucleosome contains histone H1 and DNA of a variable length (10-100 base pairs) (for review see ref. 1). Recent studies of the core particles (Ref. 2) and their crystals (Ref. 3), suggest that the core is a flat disc having dimensions of 110 x 110 x 57 Å; about 1.75 turns of DNA in the B form are believed to be coiled on the outside of the core as a left handed superhelix with histones located within the core. Histones are bound to DNA mainly by ionic interactions between negatively charged phosphate groups of DNA and positively charged lysine and arginine residues of histones.

Here we present our data on the study of the primary organization of the nucleosome core, propose the model of its structure and discuss the mechanism of core particle function.

PRIMARY ORGANIZATION OF THE NUCLEOSOME CORE

The primary structure of proteins and nucleic acids describes the sequence arrangement of monomers along the polymer chain. Similarly, for specific complexes such as nucleosomes, ribosomes, complexes of RNA polymerase with DNA, etc., the primary organization would describe the sequence arrangement of protein molecules along the DNA chain. Investigation of the primary organization of nucleoproteins is still at its beginning. We have recently described a new method for sequencing proteins along DNA chain (Ref. 4). Fig.1 outlines this sequencing procedure and demonstrates that a protein molecule is bound to one or both of the DNA strands having a certain length at the unique site. The protein is crosslinked to DNA and this is accompanied by a simultaneous splitting of a DNA strand at the crosslinking point in such a way that only the 5'-terminal DNA fragment remains attached to the protein. Measuring the size of the crosslinked DNA fragments enables one to locate the position of one or several protein molecules on one or both of DNA strands. The procedure has been described (Refs 4,5) for crosslinking lysine ε-amino groups in proteins to partially depurinated DNA under mild

conditions followed by DNA cleavage at the point of crosslinking. By using this approach, the sequence arrangement of histones along DNA in the core (Fig. 2) has been determined (Ref. 6). A number of conclusions related to the internal structure of the core can be drawn from this sequence.

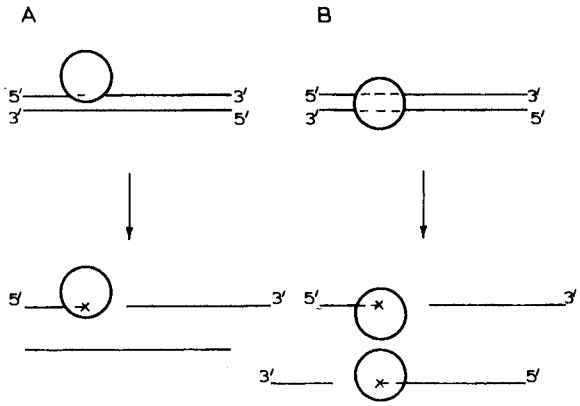

Fig. 1. Outline of the experiments locating the histone proteins on DNA. The protein molecule at a specific site on the DNA is crosslinked to it and the DNA strand is cleaved at the point of crosslinking. The size of the crosslinked single-stranded 5'-terminal DNA fragments determines the position of the protein. (A) Protein bound to one strand. (B) Protein bound to both DNA strands. ○ - protein, ——— - DNA, X - the point of crosslinking.

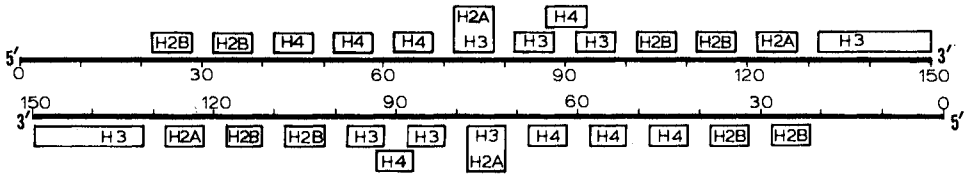

Fig. 2. The sequencial arrangement of histones on DNA of 150 base pairs long in the nucleosome core. The positions of histones are shown in boxes, and distances are indicated in nucleotides from the 5'-ends of DNA.

(1) Each histone interacts apparently through its NH_2- and COOH-terminal and central regions with several adjacent or dispersed discrete DNA segments of about 5-7 nucleotides in length.

(2) The discrete DNA segments covered with histones are separated by histone-free intervals or gaps of about 3-5 nucleotides in length. These histone-free gaps are located at a regular distance of 10 nucleotides from each other and are found at about 10 x n nucleotides from the 5'-ends of DNA where n is an integer.

(3) No histones appear to be bound to the first 20 nucleotides from the 5'-ends in both of the DNA strands.

(4) Each segment of the core DNA is bound to one histone except that the segment at 70-80 is attached to two histones. This indicates that histones are bound primarily to only one strand of the core DNA or are arranged in a similar, symmetrical manner on both DNA strands. There is not enough space on one DNA strand to bind all 8 histone molecules. In addition, a number of the data support the presence of symmetry in core particles (Refs 3,7). This strongly suggests that histones are bound similarly to both complementary DNA strands.

The data shown in Fig. 2 are very similar to those published earlier (Ref. 6) and have been obtained from cores containing DNA with 150 base pairs isolated from Drosophila melanogaster instead of mouse Ehrlich ascites

tumor cells used previously. Use of the Drosophila core provides higher resolution due to the better separation of Drosophila histones and enable us to locate histone H4 more precisely at position about 85-95 instead of 80-90 (Ref. 6).

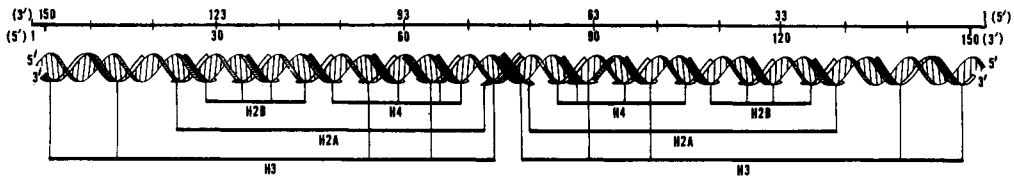

Fig. 3. A linearized model of the nucleosome core. For details see ref. 6. The figures indicate the distance of base pairs in nucleotides from the 5'-ends of both complementary DNA strands. The exact location of the histone-free gaps is taken from Sollner-Webb et al. (Ref. 7). Histones are shown as black (in front of DNA) or open (behind DNA) straps bound to sugar-phosphate backbone from the side of the major DNA groove (Refs 4,10).

Fig. 3 shows a linearized model of the core particle (Ref. 6) based on the data of Fig. 2. Histones are arranged along the DNA believed to be in the B form with 10 nucleotides per turn. The histones are shown bound to the sugar-phosphate backbone from the side of the major groove in agreement with data on partial protection by histones from methylation in the major groove by dimethyl sulfate (Refs 4, 10). The distribution of the histone regions between two molecules of each histone in the core shown in Fig. 3 has been substantiated elsewhere (Ref. 6). It is well known that different nucleases split nucleosomal DNA at regular 10 nucleotide intervals. Further, the cuts are at a distance of about 10 x n nucleotides from the 5'-ends (Refs 7-9). The position of sites accessible to nucleases in the core DNA coincides very well with the location of the histone-free gaps; this fact strongly suggests their identity. Sollner-Webb et al. (Ref. 7) have located with high precision the position of phosphodiester bonds in core DNA digested with staphylococcal nuclease, DNAase I, and DNAase II at distances of 10 x n, 10 x n+2, and 10 x n+3 nucleotides from the 5'-ends, respectively. These digestable bonds are concentrated within DNA regions 4 nucleotides in length and are symmetrically arranged on the complementary DNA strands. Histone-free gaps and histone-covered segments of 4 and 6 nucleotides in length respectively, are arranged along the core DNA in Fig. 3 according to the data of Sollner-Webb et al. (Ref. 7). The model suggests that some region of histone H4 located in the major groove can interact simultaneously with the adjacent segments 50-60 of one DNA strand and 85-95 of the complementary strand.

One striking feature of the core structure appears in the linearized model: there is a lateral alignment of histones on the DNA double helix. It is evident from the model that the histone-free gaps are found on the same side (the upper side in Fig. 3) of the helix on both DNA strands while histones cover the opposite, lower part of DNA. One should apparently place these uncovered accessible regions of DNA on the surface facing outward, i.e. towards a solution when the DNA with its bound histones is folded into the nucleosome (Ref. 6). One should notice that the position of the histone-free gaps is partly displaced from the position of the most exposed sites located on the top of the helix in Fig. 3.

FUNCTIONAL STATES OF NUCLEOSOME CORES

Transcription and replication of DNA are accompanied by interaction of some proteins with DNA, movement of polymerases along its chain, and by partial or complete unwinding of the DNA complementary strands. It is still a matter of debates concerning what happens to nucleosomes during these processes but some structural changes obviously take place.

The ultimate objective of the structural studies is to understand how the structure works. The data on the primary organization of the core presented above can provide some insight into nucleosome functioning.

(I) The DNA grooves are more or less free of histones

Recognition of specific nucleotide sequences in DNA by proteins occurs as a result of their interaction mainly with some functional groups of DNA bases exposed in the minor or major grooves of the DNA double helix. DNA appears to lie on the surface of the nucleosome and is accessible for interaction with a number of proteins, for example, nucleases (Refs 7-9). Methylation of chromatin and nucleosomes with dimethyl sulfate has demonstrated that the minor groove of DNA is fully exposed to this reagent while the major groove is shielded only by about 20% (Refs 4 & 10). These data suggest that the minor groove and partly the major groove of DNA in chromatin can be available to specific interaction with proteins and other ligands (Ref. 4).

(II) Histones are unlikely to form a "cage" around DNA

This is illustrated in Fig. 3 and Fig. 4. Histones interact with discrete DNA segments of less than 10 nucleotides long and therefore embrace less than a turn of the DNA helix. All of the histone-free gaps appear to be located on the same external side of the DNA in the core particles. As a result of such a lateral alignment, histones do not encircle the DNA continuously from all sides and do not form a "cage" around DNA. Rather they embrace it in the form of open helical clamps and leave one side of the helix uncovered. This core structure may enable DNA to be readily released from the histone octamer through the open part of the clamps without significant rearrangement of the octamer. This is consistent with experiments demonstrating easy dissociation and reassociation of the crosslinked octamer with DNA (Ref. 11) and could account for some features of DNA replication. During replication old histone octamers preserve their integrity, do not incorporate new histones and segregate conservatively through several rounds of replication (Ref. 12).

Fig. 4. Diagram of the binding of histones to DNA in the form of open helical clamps. Note that the histones do not encircle the DNA.

(III) The potential role of the asymmetric neutralization by histones of phosphate groups of DNA in bending of the core

Despite of normally rigid form, DNA is folded around the core into a left handed superhelix with a radius of about 50 Å (Ref. 3). Camerini-Otero and Felsenfeld (Ref. 13) estimated that the free energy of histone interactions in the nucleosome would be adequate to account for DNA folding. Mirzabekov and Rich (Ref. 14) have suggested that asymmetric neutralization by histones of DNA phosphates might play a significant role in its bending. An example of such a nucleic acid bending is seen in yeast phenylalanine transfer RNA (Ref. 15). Asymmetric neutralization by a spermine molecule of phosphates in the deep groove of the double helical RNA region located between the anticodon and dihydrouridine stems seems to cause a 25° bending of the helix formed by these stems.

The rigidity of the double stranded DNA is mainly maintained by the electrostatic repulsion between charged phosphate groups. Neutralization of phosphates with cations progressively increases DNA flexibility (Ref. 16). Upon more effective neutralization of DNA with polycations - spermine (Ref. 17), spermidine (Ref. 18), polylysine (Refs 19 & 20) or histones (Ref. 21), DNA collapses into folded toroidal structures. Moreover, upon concerted charge neutralization and progressive dehydration DNA collapses into beaded nucleosome like fibers (Ref. 22). Some experimental data and the theory of these processes have been discussed in detail by Manning (Ref. 23).

The asymmetric neutralization of the core DNA is the result of the lateral alignment of histones on the surface of the DNA double helix. Fig. 5

illustrates the scheme of bending that could be induced by asymmetric neutralization of DNA phosphates. In the diagram the histone-free gaps are tentatively arranged around the most exposed nucleotides located on the top of the helix. The lower part of the helix covered with histones acquires flexibility as a result of charge neutralization (Ref. 23). The electrostatic repulsion of unneutralized phosphate groups located on the other, upper side of the DNA in Figure 5 brings about the bending of the helix towards the opposite side. The manner, curvature and location of the bending would depends on many factors, in particular, on the arrangement of unshielded phosphate groups. Several models of DNA bending in the nucleosome have been proposed either by kinking DNA towards the minor groove (Ref. 24) or the major groove (Ref. 25) or by its continuous deformation (Refs 26 & 27). The repulsion between charged phosphates located on the top side of the DNA strands (Fig. 5) separated by the minor or the major groove would induce kinking towards the opposite major or minor groove, respectively. The repulsion between charges located in other DNA regions could also cause smooth bending.

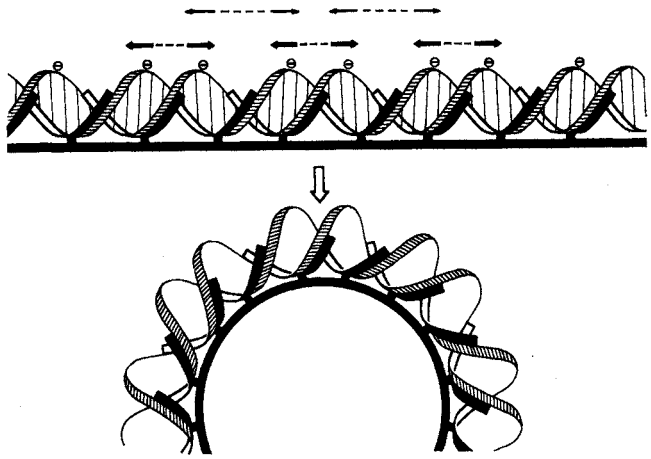

Fig. 5. Diagram of smooth bending of core DNA induced by asymmetric neutralization of DNA phosphates by histones. The bending is a result of the electrostatic repulsion between unneutralized phosphate groups uniformly located on the top of both complementary DNA strands across the minor groove (⟵) or the major groove (⟵). ⊖ - several negative charges of unshielded DNA phosphates.

If the core DNA is kinked the same amount towards both grooves, there would be about 24 kinks in the model in Fig. 3 each bent about 26° (360°x 1.75 ÷ 24 = 26°). This value would decrease if the bending is distributed among several adjacent base pairs but increase up to about 52° if kinking occured only towards one of the grooves.

In order to characterize the bending of DNA caused by its asymmetric neutralization one has to estimate the size and the location of open and shielded DNA regions. In the sequencing experiments, the size of the histone-free gaps and the segments covered with histones has been estimated approximately at 3-5 and 5-7 nucleotides, respectively. This value can be estimated in another way by considering the number of charged groups in the histone octamer and in the DNA 150 base pairs in length. The DNA has 300 negatively charged phosphates. The histone octamer of calf thymus contains a total of 224 positive charges (lysine, arginine and NH_2-terminal residues) and 82 negative charges (glutamic acid, aspartic acid, and COOH-terminal residues resulting in a net positive charge of +142. These calculations are based on the known primary structures of calf thymus histones (for review see ref. 28). The maximal size of the histone-free gaps can be estimated using these numbers. We first exclude the 20 phosphates at each of the 5'-ends that are uncovered with histones, and the remaining DNA contains 260 negative charges. If we assume that 142 charges are neutralized by the histone octamer, only 118 negative charges remain to be distributed among 22 histone-free gaps in the model. This estimation is based on a simple assumption that all

the positively charged groups in the histones which are not neutralized by the negatively charged carboxyl groups in the proteins participate in the shielding of the DNA phosphates. This assumption is justified at least for lysine residues of histones all of which are shielded in chromatin against acetylation with acetic anhydride (Ref. 29). This rough approximation suggests that on the average there may be as many as 5 negatively charged phosphate groups in each histone-free gap and all the other 5 phosphate groups are shielded in the adjacent DNA segments covered with histones. These estimations are in a good agreement with each other and with the results of nuclease digestion experiments of Sollner-Webb *et al.* (Ref. 7) that are used in our model.

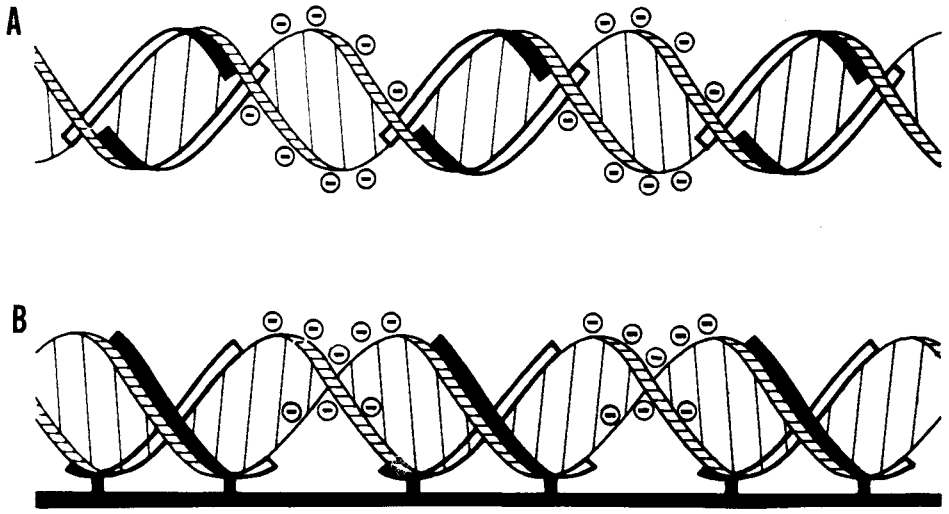

Fig. 6. Two views of the arrangement of unshielded DNA phosphates (⊖) in the linearized model of the core in Fig. 3 (Ref.7). (A) View from the side of DNA unshielded by histones; the body of the histone octamer of the core is behind the DNA. (B) View 90° away ; the histone octamer body is at the bottom shown as a black strip.

Fig. 6 shows the location of unshielded and neutralized phosphate groups (4 and 6 phosphates on each turn of one DNA strand, respectively) in a short segment of the linearized model of the core (Ref. 7). According to Coulomb's law, electrostatic repulsion between two charges is inversily proportional to the squared distance between them. The repulsion would therefore be most intensive between the nearest unneutralized phosphate groups of two DNA strands separated by the minor groove. The repulsion between unshielded phosphates separated by longer distances, for example, across the major groove, would be less significant. Thus, on these simple considerations the bending or even kinking towards the major groove (Ref. 25) could be the pretominant manner of folding of the core DNA although some continuous deformation or kinking towards the minor groove might also occur. It should be noted that the resultant force of repulsion between charges located across the minor groove appear to deviate from the axis of the double helix. It is tempting to speculate that this deviation might direct the formation of the left handed superhelix in the core DNA. This asymmetric electrostatic repulsion produced by the lateral neutralization of DNA phosphates by histones, perhaps together with specific histone-histone interactions, can be the main component of the delicate balance of forces involved in the folding of the core DNA. The disturbance of this balance leading to the nucleosome unfolding can be brought about *in vitro* by the action of urea (Refs 30 & 31), low (Ref. 32) and high (Ref. 33) ionic strengths.

Disturbance or modification of the asymmetric neutralization of DNA by histones might play an important role in folding-unfolding processes which may occur in nucleosomes during the DNA transcription and replication. For example, no nucleosomal beads could be detected by electron microscopy in actively transcribed ribosomal genes. The DNA of these genes is also slightly less extended that the normal protein-free DNA (Refs 34-36). The disappearance of nucleosomes is likely to be due to their unfolding since these ribosomal genes are still covered by basic proteins (Refs 34 & 37).

Changing from an asymmetric to a random on more non-asymmetric distribution of unshielded phosphate groups in the core DNA could be brought about in many ways. For example, shifting histones along the DNA strands towards histone-free gaps by a few nucleotides,neutralization of the basic residues in histones,by chemical modification (acetylation, phosphorylation), binding acidic non-histone chromosomal proteins, RNA polymerase or low molecular weight chromosomal RNA would all have the effect of disturbing the electrostatic balance in nucleosome. Some of these processes probably regulate chromatin activity. The same reasoning could be applied to the DNA region covered with histone H1. Finally, nucleosomal unfolding could also break the chromatin higher order superstructure.

It is also attractive to speculate that the proposed mechanism of the DNA bending caused by the asymmetric neutralization of DNA phosphates by proteins might be a major factor in increasing superhelicity of DNA catalyzed by DNA gyrase (Ref. 38).

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POLYALKYLATING RNA COMPLEMENTARY TO SELECTED DNA AS A TOOL FOR GENE-DIRECTED MUTAGENESIS

R. I. Salganik and G. L. Dianov

Institute of Cytology and Genetics of the USSR Academy of Sciences, Siberian Department, Novosibirsk 630090, USSR

Abstract - Modified polyribonucleotides carrying a number of alkylating groups were used for alkylation of complementary sites of DNA to induce gene-directed mutations. Polyfunctional alkylating mustard, N,N,N'-tri-(β -chloroethyl)-N'-(p--formylphenyl)-propylene diamine-1,3,moieties were attached to the T7 phage early transcripts through the aliphatic di-(β -chloroethyl) groups. The aromatic β -chloroethyl groups were activated by the reduction of the neighbouring formyl residues when it was necessary for intracomplex alkylation of DNA. It was demonstrated that modified transcripts alkylate only the complementary H-strand of T7 DNA and does not interact with L-strand. The transcripts modified up to 4% were hybridized with T7 DNA to form R-loops and then RNA molecules were covalently bound to the complementary DNA sites due to the activation of aliphatic alkylating groups. T7 DNA molecules alkylated by modified RNA's transcribed from 0.3 or 1.3 T7 phage genes were used for transfection of E.Coli. In the progeny of phages generated by the transfection only 0.3 or 1.3 gene mutants respectively were identified.

Complementary oligonucleotides carrying single alkylating groups at 3' or 5' ends, have been previously suggested as specific devices for the cleavage of the DNA chain in the vicinity of the end of the base-pairing oligonucleotide(1). However to solve some biological problems one has to modify long stretches of DNA. Problems of this kind are directed mutagenesis as well as complete inactivation of the genes selected. The basic assumption was that complementary polynucleotides,carrying a set of randomly attached alkylating groups, can be used as agents affecting long stretches of DNA that correspond to particular genes. Such polyalkylating polynucleotides were suggested to be capable of modifying the guanine and adenine residues strictly along the complementary sites of DNA under conditions of hybridization. To prepare RNA molecules carrying a set of alkylating groups, polyfunctional nitrogen mustard N,N,N'-tri-(β -chloroethyl)-N'-(p-formylphenyl)-propylene diamine-1,3 has been used(see Note a). The synthesis and properties of the compound will be described elsewhere(2). The highly reactive aliphatic di-(β -chloroethyl) group of the compound was utilized to attach it covalently to RNA molecules. The reactive capacity of the aromatic β --chloroethyl group is very low because of the inhibitory effect of the neighbouring formyl residue at the para position of the benzene ring. However, the reduction of the formyl residue can activate this group. Sodium boron hydride was employed as a reducing agent for this purpose. The advantage of such governed alkylating groups is that their activation can be carried out after hybridization of the polyalkylating RNA to the complementary DNA site. Modified in this way RNA becomes capable of alkylating solely the nucleotide composing the complementary site corresponding to the selected gene. Before applying this approach, we had to ascertain whether the modofied RNA remains capable of forming a complementary complex with relevant DNA(or RNA) In model experiments, the capacity of poly I, prealkylated with N,N-di-

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Note a. The abbreviation used is: PNM

-(β -chloroethyl)-4-amino benzaldehyde(3) to form a complementary complex with poly C was assayed. The formation of the complex was manifest in hyperchromism. As shown in Table 1, when not more than 10% of the inosine residues were modified, poly I was still able to form the complex with poly C.

TABLE 1. Effect of the extent of poly I modification on its capacity to form a complex with poly C and on the stability of the complex

Modification extent of poly I (%)	Hyperchromism during the formation of the complex with poly C	T_m, °C
0	31	67
1.1	28	63
3.9	28	58
8.8	28	57
9.9	22	54
10.4	19	45
11.2	0	-

The complex was formed in 0.1 M phosphate buffer, pH 7.0, at 25°C.

However, the modification produced a decrease in the T_m of the complex. When the modification rate exceeded 4%, the sensitivity of poly I to RNase T_1 rose sharply as a result of the development of one-stranded loops.

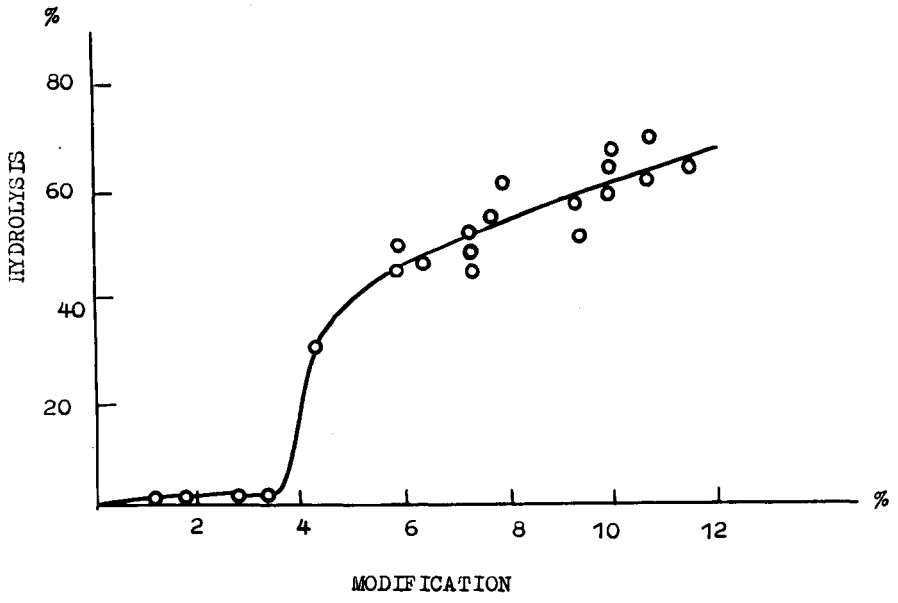

Fig. 1. Enzymic hydrolysis of the modified poly I in poly I : poly C complexes under the effect of RNAse T_1. Hydrolysis was carried out for 2 hrs at 23°C in 0.1 M potassium phosphate buffer, pH 7.0. Concentration of RNAse T_1 was 20 units/ml. Unhydrolyzed polynucleotides were precipitated with 0.5 N perchloric acid for 20 min at 0°C and cen-

trifuged at 5,000 r.p.m. for 10 min. The supernatant was hydrolyzed in 0.5 N perchloric acid for 20 min at 100°C. The optic density of the hydrolysate was measured at 248 nm.

In view of these data 4-5% modification of complementary RNA was considered sufficient for our experimental purposes(4). T7 phage was chosen as target, and the polyalkylating derivatives of the transcripts from the early region of phage T7 were applied for the directed gene modification. The T7 RNA molecules were transcribed from T7 DNA by E.coli RNA polymerase(5) or extracted from phage T7-infected E.coli(6,7). The transcripts were modified by PNM as follows. RNA was incubated with 0.8 mM of the reagent in a 50% methanol - $5 \cdot 10^{-3}$ M Tris-HCl buffer, pH 7.5, at 25°C for 10 minutes. Paper chromatography of alkaline hydrolysate of the modified RNA demonstrated that the reagent is covalently bound to 10-12% of guanine and to the same amounts of the adenine residues. No modified derivatives of cytosine and uracil were recovered. The total yield of modified base residues was 5-6% (8). According to Minkley(9), transcription occurs solely on the H-strand under conditions where E.coli RNA polymerase is used to transcribe T7 DNA. Previous experiments with the incubation of prepared transcripts with the H- and L-strands of T7 DNA have shown that, in spite of modification, the transcripts are selectively hybridized only to the H-strand like the intact ones are (8,10).

A point of major issue was to ascertain whether or not the polyalkylating RNA is able to provide the highly selective alkylation of the complementary DNA strands. Discrete T7 early RNA with tritium labelled guanine residues was generated by E.coli RNA polymerase transcription of T7 DNA. Being attached to RNA, the polyalkylating reagent alkylates only the guanine and adenine residues(9). The transcripts were treated with the polufunctional reagents, and, as a result, the ^{3}H-guanine moieties acquired potentially active alkylating groups. The modified transcripts were incubated with the H- or L-strands of T7 DNA under hybridization conditions and then the potentially active alkylating groups, carried by the transcripts, were activated by reducing the neighbouring formyl residues with sodium boron hydride. As a result of the alkylation reaction, the ^{3}H-transcripts were covalently bound to the corresponding DNA base moieties. Hybridization of the polyalkylating ^{3}H-transcript (1 μg/ml) with the H-strand of T7 DNA (10 μg/ml) was carried out for 12 hrs at 25°C in a 0.01 M Tris-HCl buffer, pH 7.5, containing 0.3 M sodium chloride and 40% formamide. The reaction mixture was diluted with 0.01 M Tris-HCl (pH 7.5) to achieve a 20% solution of formamide. The aromatic alkylating groups were activated with 0.01 M sodium boron hydride for 30 min at 25°C. For intracomplex alkylation of DNA, the reaction mixture was additionally incubated for 120 min at 40°C. Nucleic acids were precipitated with two volumes of ethanol. The precipitate was dissolved in small amounts of 90% formamide and incubated for 30 min at 37°C to denaturate the complementary interacting strands of DNA and RNA(11). Thereafter 50 volumes of 0.01 M Tris-HCl (pH 7.5), 100 μg of pancreatic RNAse and RNAse T_1 (30 units of activity) were added to hydrolyse the unbound RNA which was freed from the RNA-DNA hybrids. RNA enzymic hydrolysis was performed for 120 min at 25°C. Under these conditions, only the covalently bound parts of RNA remained attached to DNA, being resistant to enzymic hydrolysis. The hydrolysate products were separated from polynucleotides by gel filtration on Sephadex G-50 columns.

Incubation of polyalkylating RNA with the L-strand of T7 DNA, as well as subsequent treatment, were all performed identically. In the control experiments, the H- and L-strands were hybridized to the unmodified transcripts. It was found that the labelled guanine residues of the RNA, which carried alkylating groups, were covalently bound to the H-strand of T7 DNA, but not to its L-strand (Fig.2). From these experiments, it is obvious that the modified transcripts alkylate only the complementary H-strand. In spite of their high activity, the alkylating groups carried by RNA do not interact with the non-complementary L-strand, under conditions optimal for hybridization. The data indicate that the complementary interaction of polyalkylating RNA with the relevant DNA ensures very high specificity of alkylation. To modify the discrete early gene of T7 phage, corresponding individual RNA transcripts were obtained. These ^{32}P labelled T7 early RNA's were separated by electrophoresis in 2.25% polyacrylamide - 0.5% agarose gels. By electroelution and subsequent phenol extraction, the individual RNA, corresponding to the gene 1.3, and the RNA's, corresponding to the genes 0.3 and 1.1, were isolated. It is well established that 1.3 gene specifies T7 ligase while 0.3 gene codes the protein responsible for overcoming host restriction. The function of 1.1 gene is unknown(12,13).

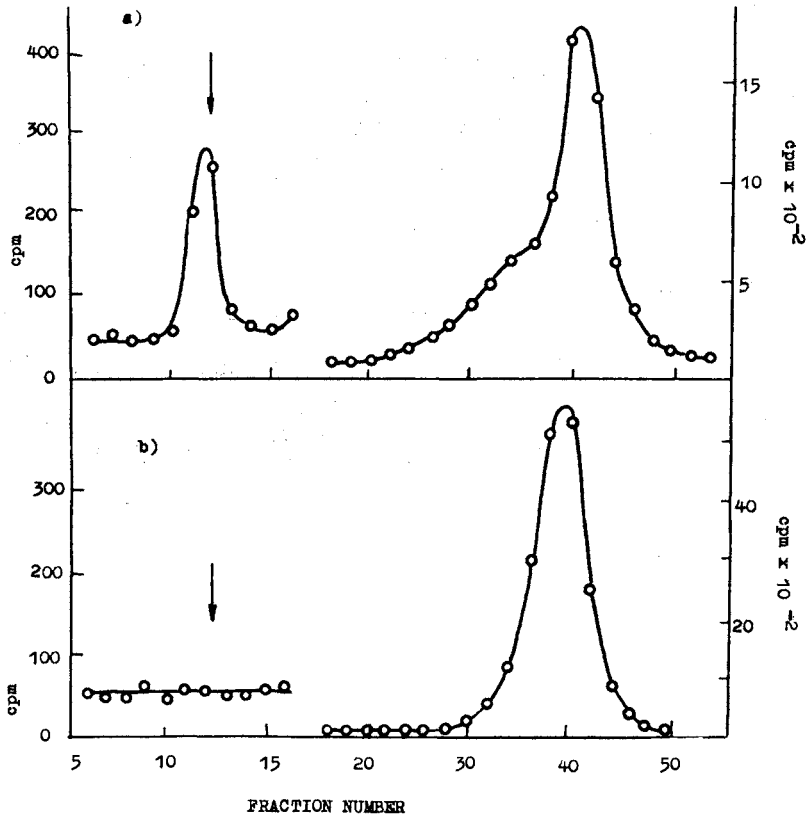

Fig. 2. Sephadex G-50 elution profiles of RNAse treated. H-strands (a) and L-strands of T7 DNA (b) after reaction with ^{3}H-GTP labelled polyalkylating transcript of T7 early genes under hybridization conditions. The left part of the figure is magnified. The arrow indicates the position of DNA elution.

The isolated RNA's were modified by treatment with the PNM as described above. As a result, PNM was attached to 3-4% of the RNA base moieties. Modified individual T7 RNA's, carrying alkylating groups, were employed for obtaining T7 DNA with the corresponding R-loops(14). After activation of these alkylating groups by the reduction procedure, the RNA molecules in the R--loops are bound covalently to DNA and, unlike unmodified RNA, cannot be removed by denaturation. By means of electron microscopy, the R-loops formed by the covalently bound T7 early RNA's were located at one end of the T7 DNA, where the R-loops formed by the unmodified T7 early RNA's are located (14). As suggested, the modified T7 early RNA's alkylate correctly the complementary DNA sites.

The T7 DNA with covalently bound RNA's of the selected genes was used for the transfection of E.coli C 1757, a strain bearing an amber suppressor and lacking restriction activities. The inability of T7 mutants to overcome host restriction systems and ligase deficiency were tested in several B and C strains of E.coli. When tranfection was effected with T7 DNA having an R--loop formed by the polyalkylating 1.3 RNA, plaques were identified containing only 1.3 gene mutants, which were unable to plate in the ligase deficient E.coli BL 2 strain. 25 plaques were identified in the progeny of phages, generated by transfection with T7 DNA having an R-loop formed by the polyalkylating 0.3 and 1.1 RNA's; of these, 3 contained 0.3 gene mutants(15). The data reported here offer evidence to show the possibility of addressed mutagenesis by means of polyalkylating polynucleotides complementary to the selected genes. The modified polynucleotides may be applied in vivo at the periods of DNA replication when one-stranded regions assesible to hybridization and highly sensitive to alkylation appear(16).

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EFFECTIVE SOLID PHASE METHOD FOR THE SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES

V. K. Potapov, S. I. Turkin, V. P. Veiko and Z. A. Shabarova

Chemistry Department, A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Bldg.A, Moscow 117234, USSR

Abstract - A new type of polymeric support is suggested for solid phase synthesis of oligonucleotides prepared by modification of polysterene grafted on the surface a polytetrafluoroethelene support. The adequacy of the support is exemplified with the synthesis of a number of 6-15 nucleotide long chains. To increase the yield of the target product in terms of the diester scheme, an approach was developed based on the step-wise blocking of the internucleotide phosphate by anilide group introduced via triphenyl-phosphine-carbon tetrachloride complex. The synthesis has been carried out in a semiautomatic device with a column type reactor; addition of the nucleotide takes on an average 12 hours. For the triester solid phase method, an effective scheme of block synthesis of oligonucleotides without condensing agents and with 3'-5'-end chain elongation has been elaborated.

INTRODUCTION

Synthetic oligodeoxyribonucleotides of a required structure can be used for solving some important problems of molecular biology (1,2). Hence the necessity of rapid methods for the synthesis of these compounds. The adequacy of any such method is judged by the rate of synthesis that should be high, by simplicity and reproducibility. The task is facilitated by the fact that the chains to be synthesized should not contain more than 12-15 nucleotides as they can be elongated into polymers by enzymatic methods.

The triester methods that is being developed in some laboratories (2-5) is the most advantageous; so is the procedure based on the use of polymeric supports (6-9) and it is the solid phase method that is most satisfying, as the high rate here is combined with total automation. The key problems involved in this method are the following:

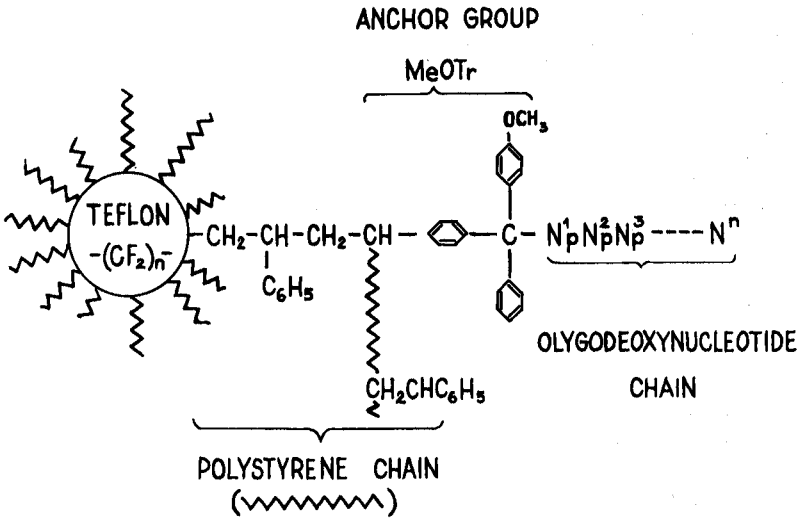
1. A support with an optimal macromolecular structure should be selected and methods of its preparation elaborated.

2. A method for the synthesis of the internucleotide bond should be chosen.

RESULTS AND DISCUSSION

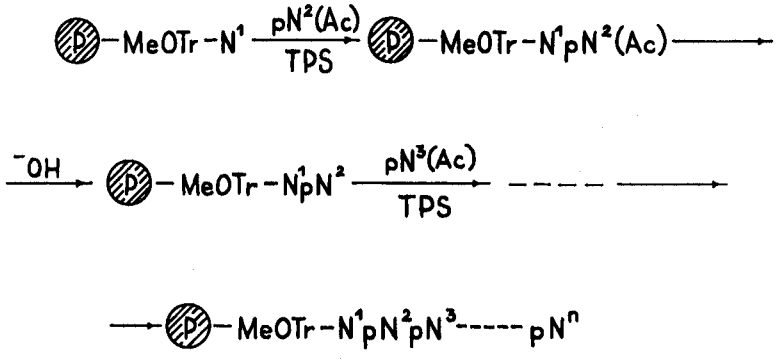
The results that we have obtained in a systematic study of polymeric supports of different structure (9,10) have allowed a new type of support (11) to be suggested for the synthesis of oligonucleotides. This carrier combines the best properties of the polymeric supports studied previously. The support has been obtained by chemical modification of polystyrene grafted on the surface of polytetrafluoroethylene by irradiation. The amount of grafted polystyrene can be varied from 10 to 50% of the weight of teflon. The resulting polymer, depending on the degree of grafting, can be used as a support for synthesis of biopolymers with the amount of ancher groups of 0.1-0.2 mmol/g, or as a solid phase reagent (for example, polystyrenesulphochloride) of a capacity of 2.5 mmol/g.

As the reaction centres are localized on the surface of the granule, the role of the diffusion decreases, and the high conformational mobility of polystyrene chains that are not cross-linked by divinylbenzene, can decrease the steric hindrances both in modification of the polymer and in the synthe-



sis of oligonucleotides. The support possesses a low sorptional activity and swells very moderately due to the teflon being extremely inert to all reagents and solvents. The major difference between a grafted support and any cross-linked polymers is that the former allows an extensive (30-50%) modification when introduced into anchor groups. Owing to the great depth of the modification, as early as at the first steps of the synthesis the nature of the grafted phase changes. For example, the greater is number of internucleotide phosphate groups in an oligonucleotide synthesized by the diester method, the lower is the hydrophobicity of polystyrene. Thus, a grafted carrier, owing to its being highly loaded, lacks the major drawback of styrene polymeres, i.e. the incongruence between the hydrophobicity of the supports and the polar nature of oligonucleotides synthesized on them.

Studying the characteristics of the supports, we synthesized on them a number of oligonucleotides of the deoxy series with a chain length of 6-15 nucleotides. To compare our results with those for polar supports (6,7), the oligonucleotides were synthesized by the standard diester scheme (1), with the nucleotide component being preactivated as in (12) at every step. The anchor group was the well-explored methoxytrityl group which can be readily introduced to the polymer via a two-step modification (11). A two-fold excess of triisopropylbenzenesulphonylchloride over that of the mononucleotide was used for condensation in all cases. The excess of the mononucleotide grew as did the oligonucleotide chain from 5-fold at the first step to 10 to 12-fold at the last, the reaction at the internucleotide phosphate group being taken into consideration.



N=dT, danC, dbzA, dibG

TPS=2,4,6-(i-C_3H_7)$_3$ $C_6H_2SO_2Cl$

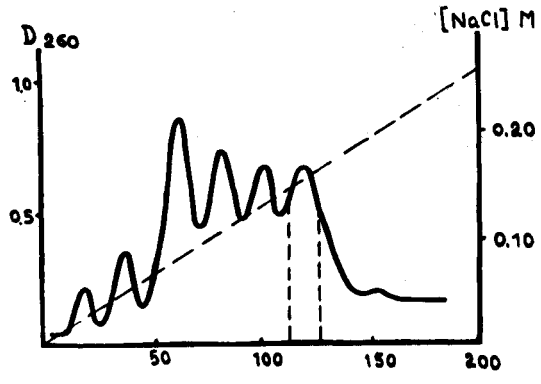
The full synthesis of the nucleotide chain consists of seven successive standard operations and lasts 8 hours. The synthesis was performed in a semiautomatic synthesizer with a column type reactor. During synthesis the activated mononucleotide is pumped through the cycle, and at the washing and deblocking stages, the products are discharged. To control the quality of the washing of the polymer from the reagents, a conductometric flow-cell was used. At the intermediate steps and at the end of the synthesis, the oligonucleotide on the polymer is totally deblocked by treatment with concentrated ammonia (50°, 12 hours). The mixture of oligonucleotides is removed from the support by treatment with 1% trifluoroacetic acid at -30°C and fractionated by ion exchange chromatography on DEAE cellulose as described by Tomlinson and Tener, pH 7.5. The results are shown in the Table. The first six oligonucleotides are fragments of encephaline gene, the rest are primers for reverse transcription for oncornavirus RNA.

Table

The yields of oligodeoxyribonucleotides

Nos	Oligonucleotide ($A_{260} \cdot 10^{-3}$)	Initial nucleoside on polymer (μmoles)	Isolated oligonucleotide		Yield (per cent)
			A_{260}	μmoles	
I	CATGTA (67)	17	70	1.04	6.1
II	TGGGGGG (81)	12	15	0.18	1.6
III	CCCATACA (83)	11	30	0.36	3.3
IV	AGGAACCC (92)	8	25	0.27	3.5
V	AGCTTCAA (90)	11	20	0.22	2.0
VI	TTCCTTTGA (86)	12	60	0.70	5.8
VII	TTTTTGTGG (89)	34	160	1.60	4.7
VIII	TATAATG (83)	7	100	1.20	17.0
IX	TATAATGCAT (116)	5	35	0.30	6.0
X	$(T)_{15}$ (132)	4.8	130	1.00	20.00

As seen in the Table, the best results are obtained in the syntheses of polypyrimidine sequences (VI, VII) and worst in the synthesis of oligoguanilate (II). The fractionation profiles of the total products of synthesis for oligonucleotides II and VII are shown in Fig. 1 and Fig. 2.



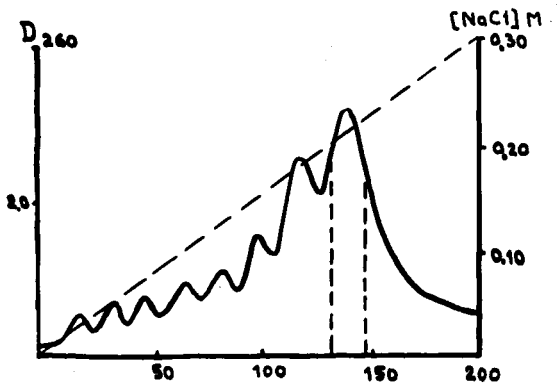


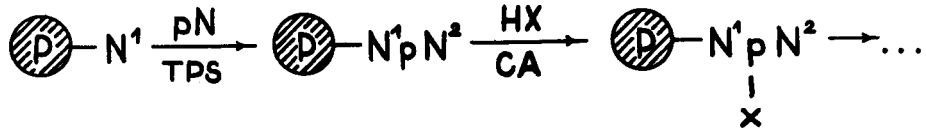
Fig. 1. Fig. 2.
Column chromatography on DEAE-cellulose (DE-32), column 0.9x20 in linear gradient of 0.1-0.3 M NaCl, 7 M urea, 0.01M Tris-HCl buffer, pH 7.5. Fractions of 3 ml were collected every 8 min.
Fig. 1. Synthesis of $d(TG_6)$. Fig. 2. Synthesis of $d(T_5GTG_2)$

The zones corresponding to the target product were isolated and rechromatographed by the same method at pH 3.5. The homogeneity of all oligonucleotides was proved by polyacrylamide gel electrophoresis after introduction of a ^{32}P-labelled phosphorous acid residue to the 5'-end of the chain by means of T4 phage polynucleotide kinase. The base sequence was determined by the modified method of Maxam-Gilbert (13). Determination of the composition of the reaction mixture after every step of synthesis, which was carried out for all the synthesized oligonucleotides, has clearly shown that excess of the condensing agent is a negative factor. Cleavage of the internucleotide bonds, which occurs at every step, leads to increase of the relative amount of low molecular weight components, the process becoming especially pronounced at the final steps.

Breakage of an oligonucleotide chain can bring about activation of the internucleotide phosphorous atom, and the only way to avoid it is to block the internucleotide phosphorus residue, i.e. to use the triester synthesis. There are two possibilities here:

a) blocking of the diester phosphate group after each chain elongation or

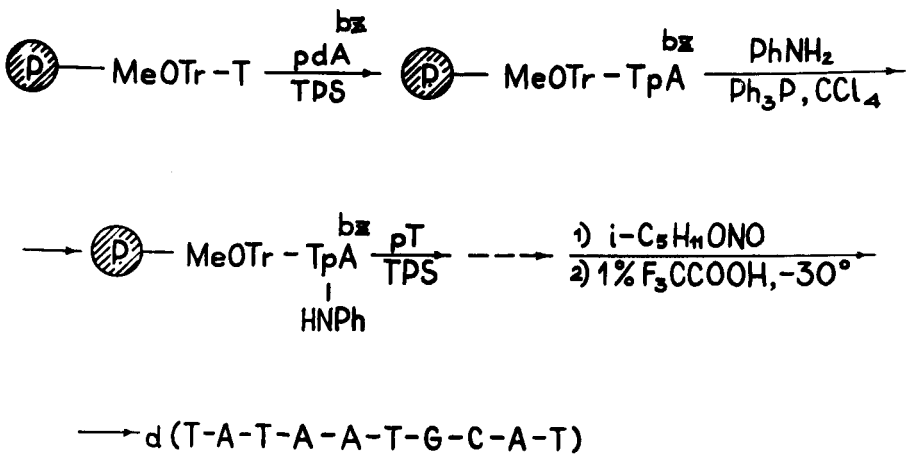
b) performing the whole synthesis by the triester method, when the internucleotide linkage is formed with the participation of the activated nucleotide diester.



where X is a protecting group, Y is a phosphate activating group, C.A. is a condensing agent.

Realization of the first scheme will require a protecting agent that would be resistant to alkali used to remove the acetyl residue from the 3'-end of an oligonucleotide; a fast, mild an effective method for introduction of such group to the oligonucleotide and its removal at the completion of synthesis should be elaborated.

It turned out than an anylide group that is readily introduced to an oligonucleotide with the help of triphenylphosphine-carbon tetrachloride as a condensing agent (14) and easily removed by treatment with isoamylnitrite (15), meets very well the above requirements.



Synthesis of oligonucleotides VIII-X performed in accordance with this scheme showed that the amount of low molecular weight components in the final mixture (Fig. 3) of oligonucleotides is significantly lower compared to that obtained by the diester synthesis. The yield increases more than twofold

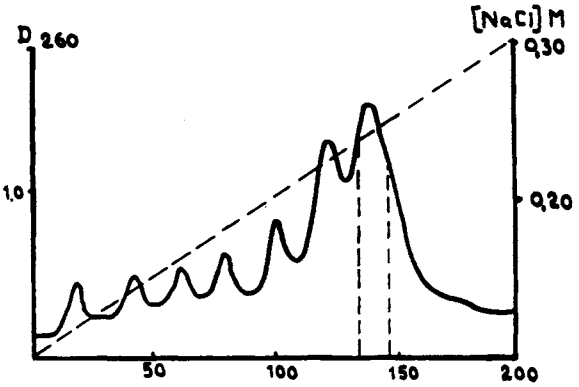
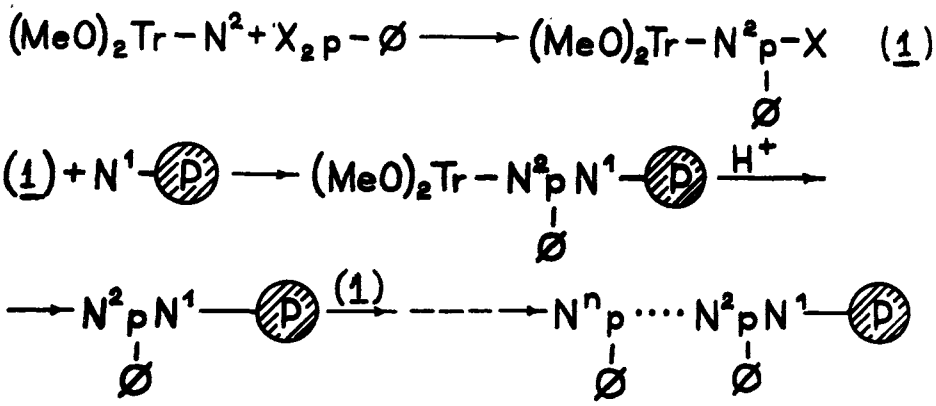


Fig. 3. Column chromatography on DEAE-cellulose (DE-32), column 0.2x20 in linear gradient of 0.1-0.3 M NaCl, 7 M urea, 0.01 M Tris-HCl buffer, pH 7.5. Fractions of 3 ml were collected every 8 min. Synthesis of d(TATAATGCAT)

This approach is a good combination of the advantages of the diester method (accessibility of the initial protected mononucleotides and the reproducibility of all operations) and those of the triester method (absence of reactions involving the internucleotide phosphate group).

We believe that the use of polymeric supports in the triester method that is specially designed for the solid phase version is very promising. Our detailed study of the various aspects of the problem which included our previous results about the synthesis of oligonucleotides without condensing agents (9,16) and the new data (2-5,8) has allowed us to choose a scheme of triester synthesis that is optimal for the solid phase version.



X is 1.2.4 - triasol-I-yl, and N-deoxynucleoside with protected isocyclic amino group, Ø is a p-chlorphenyl.

The synthesis proceeds via a reverse scheme, i.e. the first nucleoside is attached to the polymeric support via the 3'-hydroxy group. Activated nucleotide (1) is obtained in solution by treatment with 5'-O-dimethoxytrityl-nucleoside with a stoichiometric amount of a phosphorylating complex (PC), i.e. p-chlorphenyldichlorphosphate-1.2.4-triazol-N-benzylimidazole (1:4:16) in acetonitryl solution. The formation of the internucleotide bond occurs after the interaction of activated nucleotide (1) with a more reactive 5'-hydroxy group of the nucleoside on the polymer. The removal of dimethoxytrityl protective group is performed by mild acidic treatment with simultaneous quantitative control (and yield determination) by spectrophotometric measurements in the visible region.

The study and improvement of all the steps of the scheme is exemplified by the synthesis of octanucleotide d(TGCACATG) that is self-complementary at all the four terminal bases and capable of forming a DNA-like duplex:

```
5'... T-G-C-A-C-A-T-G T-G-C-A-C-A-T-G ... 3'
       | | | | | | | | | | | | | |
3'. . . G-T-A-C-A-C-G-T G-T-A-C-A-C-G-T ... 5'
```

The oligonucleotides of such structure are handy objects for elaborating optional conditions for every step, as, being internally symmetrical, they contain only two types of dinucleoside-phosphate blocks, i.e. d(TpG) and d(CpA). The scheme of their synthesis is given below

$$(MeO)_2TrN^1 \xrightarrow[-30^\circ]{P.C.} (MeO)_2TrN^1p(\varnothing)-X$$

$$\xrightarrow{N^2} (MeO)_2TrN^1p(\varnothing)N^2 \quad (\underline{2}) \text{ or } (\underline{3})$$

$$\xrightarrow{H^+} N^1pN^2 \quad (\underline{2a}) \text{ or } (\underline{3a})$$

($\underline{2}$) N^1=T, N^2=BzG

($\underline{3}$) N^1=BzC, N^2=BzA

The yield at the first step is almost quantitative, at the second step is 85% (the pure substance isolated by column chromatography on silica gel). Part of protected blocks (2) and (3) was deblocked and used as a nucleoside component in the assembly of an octanucleotide via a (2+2)+(2+2) scheme:

$$(\underline{2}) \xrightarrow[2.(\underline{3a})]{1.P.C.} (MeO)_2TrTpGpCpA \quad (\underline{4})$$

$$(\underline{3}) \xrightarrow[2.(\underline{2a})]{1.P.C.} (MeO)_2TrCpApTpG \xrightarrow{H^+} CpApTpG \quad (\underline{5})$$

$$(\underline{4}) \xrightarrow[2.(\underline{5})]{1.P.C.} (MeO)_2TrTpGpCpApCpApTpG$$

Our study has demonstrated that whatever the size of a block, the phosphorylation step terminates within 10-15 min and addition of a nucleoside block within 2 hours.

Thus, realization of this scheme with the use of polymeric supports allows the time of synthesis to be reduced 2 to 3-fold compared to the diester scheme.

There is no doubt that with a machine-aided solid-phase method oligonucleotides will become available and this will mean further progress in the nucleic acids chemistry, molecular biology and genetic engineering.

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E. COLI RNA-POLYMERASE INTERACTIONS WITH THE SUBSTRATES AND TEMPLATE. THE PRIMARY STRUCTURES OF THE PHAGE 434 PROMOTERS

E. D. Sverdlov and K. G. Skryabin*

Shemyakin Institute of Bioorganic Chemistry of the USSR Academy of Sciences, Moscow 117988, GSP-1, USSR
**Institute of Molecular Biology of the USSR Academy of Sciences, Moscow 117988, GSP-1, USSR*

Abstract - The restriction endonuclease Eco RI splits the phage λ imm 434 DNA at 6 sites. The smallest of the formed fragments Eco RI-G contains the operator O_R and promoters P_R and P_{RM}, gene cro and N-terminal part of the gene CI of the phage 434. λ DNA portion of the fragment contains the sequences of P_o promoter, the gene CII and of the gene O N-terminal part. The complete primary structure of the Eco RI-G fragment was determined. It consists of 1287 base pairs of which 361 belong to the immunity region of the phage 434. The amino-acid sequences of the phage 434 protein cro and N-terminal part of the CI repressor were deduced from the nucleotide sequence. The proposed sequences of the phage λ CII protein and of the N-terminal part of O protein was also obtained. The sequence of the phage 434 promoter-operator region is quite different from that of the phage λ .

INTRODUCTION

Studies on the mechanism of E.coli RNA polymerase activity could probably be much more informative, if the templates containing a single or at least, a few promoters with known primary structure were used. Recently (1) we have detected that the phage λ imm 434 DNA was splitted by restriction endonuclease Eco RI at 6 sites (Fig. 1).

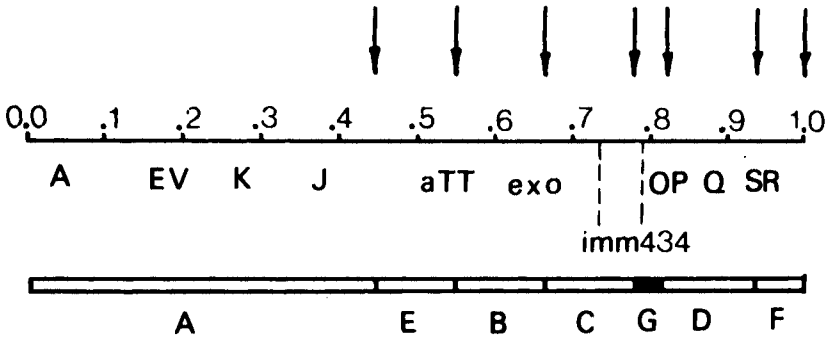


Fig. 1. Map of the Eco RI cleavage sites in λ imm 434 DNA. The positions on the map are related to the left terminus of the DNA taken as 0. The endpoints of the imm 434 region are indicated by the dotted lines. The Eco RI cleavage sites are represented by arrows. The localizations of the genes are shown below the map. The fragment Eco RI-G with the structure determined is given black.

The smallest of the formed fragments -Eco RI-G- was shown to direct the synthesis of the short oop-RNA (2) and, therefore, contains P_o promoter. From the comparison of its position on the physical map of λ imm 434 (1)

with the genetic map of the corresponding region of the phage λ DNA (3,4) it may be concluded that Eco RI-G fragment includes the phage 434 gene cro and gene CII of the phage λ.

We used this fragment to study the interactions of RNA polymerase with the substrates during the initiation of the RNA synthesis (5). The analysis of the obtained results permitted supposition that the additional promoter is present in the fragment. To get a deeper insight into the processes of the transcription of the fragment we have established its primary structure.

The structure determination.

The cleavage sites locations in the Eco RI-G fragment of the imm 434 DNA for different restriction enzymes and the schematic representation of the sequence determination are shown in Fig. 2.

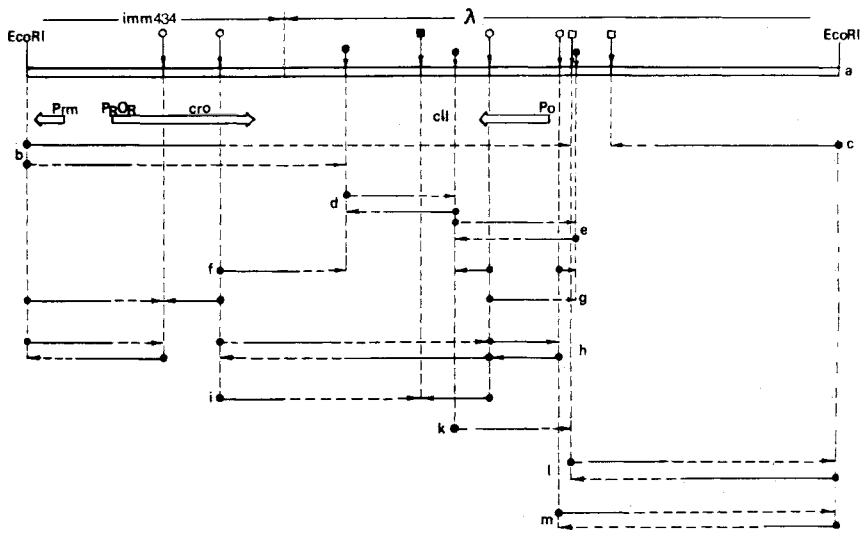
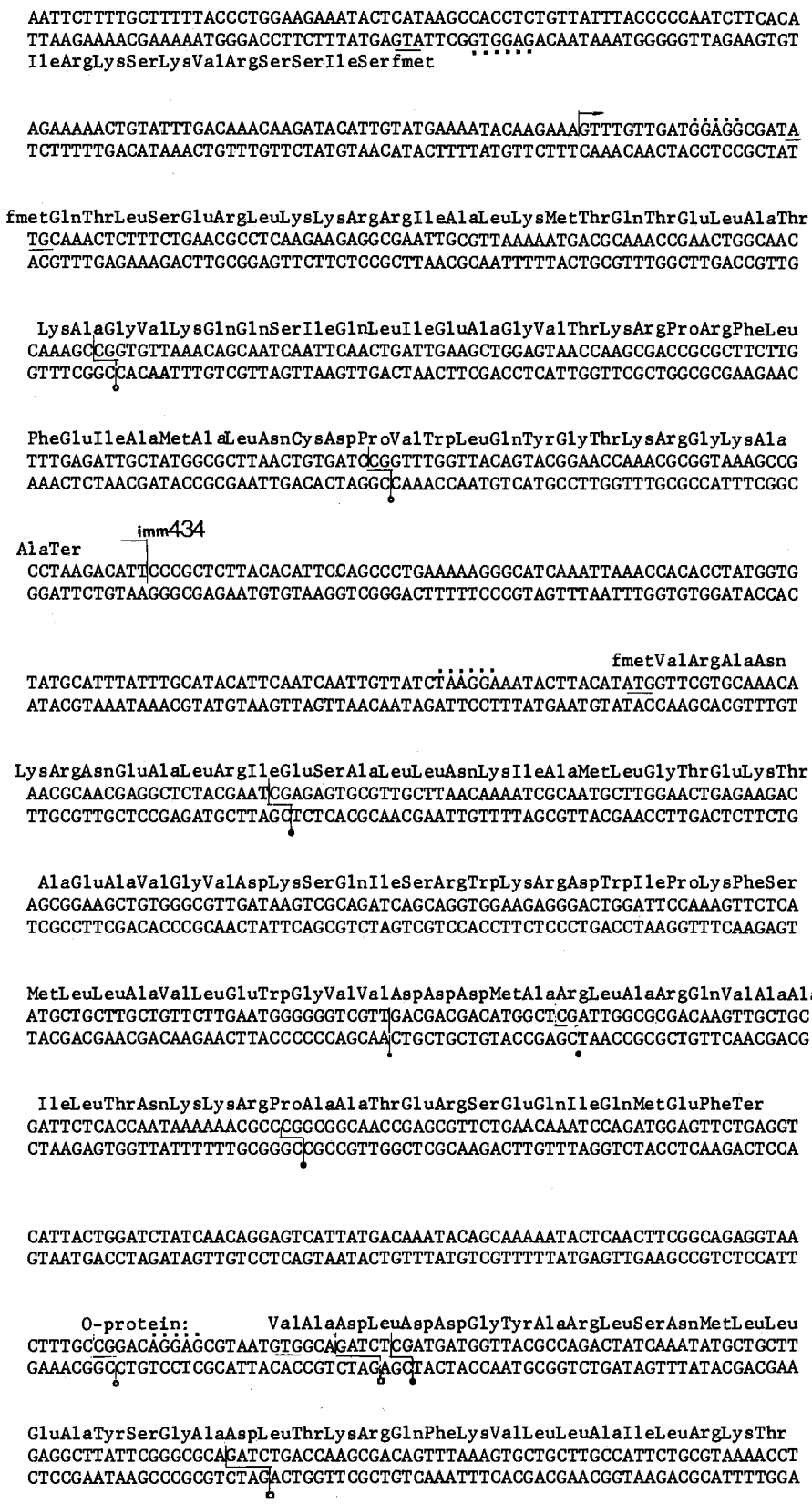


Fig. 2. Cleavage sites locations of different restriction enzymes in the Eco RI-G fragment of λ imm 434 DNA (a) and the schematic representation of the sequence determination (b). The whole length of the fragment-1287 base pairs (b.p.). The vertical arrows indicate the cleavage sites of HpaII - ♀, TaqI ♀, HindII - ▼ and of BglII - - ♀. The open arrows indicate the left-ward and right-ward RNA transcripts from the promoters P_R, P_{rm} and P_o. The positions of the operator O_R and genes cro, CII and O are shown below the map. The horizontal arrows (b - l) represent fragments and their strands selected for sequence analysis. The arrows directed from left to right correspond to the l-strand. The r-strand is represented by the opposite directed arrows. The solid parts of the arrows indicate the fragment region with the sequences established. ^{32}P Labelled 5'-ends are marked by full circles. In the cases when fragments for the sequence determination were obtained by additional cleavage with the second restriction endonuclease, each of them is represented by a single arrow directed to the site of the second splitting. If the labelled at a single terminus fragments were obtained by strand separation they are represented by the parallel opposite directed arrows.

The sequences of the digestion products were determined by Maxam-Gilbert technique (6), using the partial apurinization with formic acid to locate the purines positions as it has been described by us (7) and other authors (8). To separate the radioactively labelled termini the fragments were cleaved by treatment with a second restriction endonuclease or their strands were separated as shown in Fig. 2.

The obtained sequence is presented in Fig. 3.



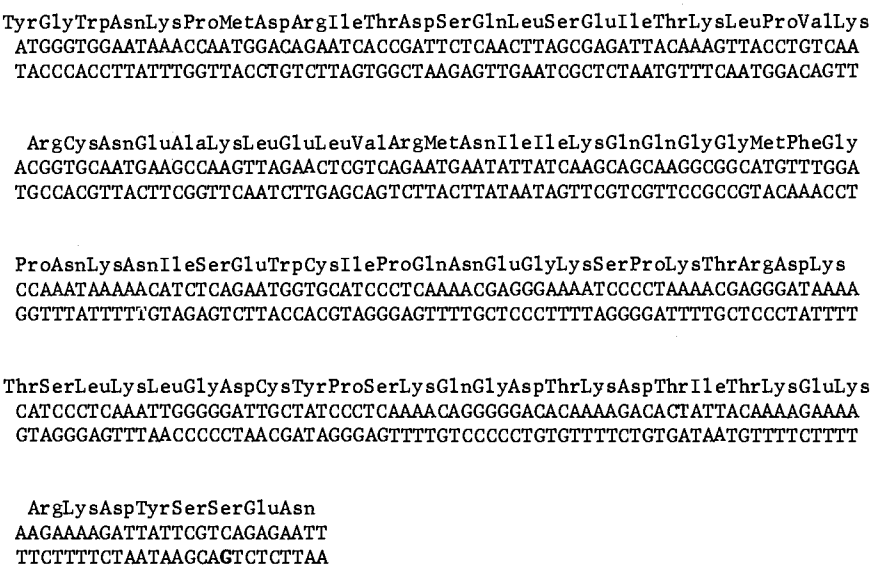


Fig. 3. The complete sequence of the Eco RI-G fragment of λimm 434 DNA. The 1 strain is on the top. The right boundary of the phage 434 immunity region and the cleavage sites of different restriction endonucleases are indicated. The stars mark the nucleotides complementary to the 3'-end of the ribosomal 16S RNA. The initiating codons are underlined. The terminating codons are shown by symbol Ter. Peptides, corresponding to the nucleotide sequences are arranged near to the strand with the sequence adequate to m-RNA.

Eco RI-G fragment of the λimm 434 DNA consists of 1287 base pairs. The part which belongs to the phage 434 immunity region is the left part of the fragment, as it may be concluded from direction of the oop-RNA gene (2). The position of the end-point of the phage 434 immunity region has been derived from the comparison of the fragment structure with the sequence of the corresponding phage λ region (9).

The phage 434 sequence consists of 361 b.p. and the rest of the fragment contains the phage λ DNA sequences.

The structure of the imm 434 region.

Basing on electron microscopy data (the results will be published elsewhere) it may be concluded that the RNA polymerase forms the stable complex within the phage 434 immunity region of the Eco RI-G fragment. Moreover, the fragment directs the synthesis of two relatively short proteins in cell-free coupled transcription-translation system. There are two combinations of the initiating codons ATG with the stretches of several nucleotides complementary to the 3'-end of ribosomal 16S RNA. The combinations might provide the sites of the translation initiation (10-12). One of them is arranged in the 1-strand and corresponds to the reading frame which could be read for 71 aminoacids residues. The termination of the protein is signalled by an ochra TAA codon, which is situated near to the end-point of the immunity region. The position of the coding sequence is analogous to that of the cro gene in the phage λ DNA (9). Therefore it appears reasonable to assume that the sequence represents the gene cro of the phage 434. The corresponding protein has 5 aminoacid residues more than the cro protein of the phage (13). This result is in agreement with the recently reported molecular weights of the proteins (14). Another potential initiating combination is arranged in r-strand of the fragment (position 33 - 46, Fig. 3). It perhaps, provides the sites of initiation of the phage 434 CI repressor translation. The fragment contains

information for N-terminal amino acid residues of the protein.

It is of interest to compare the sequences of the regions which are arranged between the sites of the translation initiation in the phage 434 DNA and in DNA of the well-known phage λ (15). The comparison (Fig. 4) indicates that though the translation initiating sites are arranged analogously in both cases, the sequences of the intervals, which obviously contain promoters P_R and P_{rm} and operator O_R (4) are quite different.

Fig. 4. Comparison of the right promoter-operator regions of the phages λ (16, top) and 434. The initiating codons are underlined. The stars indicate the nucleotides complementary to the 3'-end of the ribosomal 16S RNA. The sequences which are simmetric about 2-fold rotational axes, are boxed.

In their investigations on the structure of the right promoter-operator region of the phage λ DNA (15-16) Ptashne and his associates managed to recognize three symmetrical G-C rich regions with A-T rich spacers between them. The corresponding region of 434 DNA does not follow this principle. The region is A-T rich sequence and A-T quantity increases from the left to the right. Though symmetrical sequences exist in this case, they are not arranged similarly to that of λ , and their structures are different from the structures of the phage λ symmetrical sequences. Besides, we have not been able to recognize the Pribnow boxes (17) in the 434 promoter sequence. It should be noted that the amino acid sequences of the cro gene and of the N-terminal part of the CI gene of the phage 434 are strongly different from those of the λ phage (13,16).

The phage 434 is the nearest relative to the phage λ . For this reason it could be suggested that the laws determining the specificity of the interaction between regulatory DNA sequences and corresponding proteins should be the same in both cases. If it is true, the primary structure of DNA does not reflect these laws directly.

The λ DNA part of the fragment.

The Eco RI-G fragment contains 926 base pairs which are located to the right of the imm 434 boundary and, therefore, belong to the λ DNA. According to genetic and physical maps of the DNA space arranged near the immunity region (3) this sequence should include the genes CII and oop-RNA, y- and ori-regions and, perhaps, N-terminal part of the gene O of the phage λ . In fact, the sequence of the oop-RNA gene is arranged in the position 714-789 from the left end of the fragment. A preceeding sequence obviously regards to the P_o promoter (2). The primary structure of the oop-RNA region is in agreement with the sequence determined by Schwartz et al. (9). In the position 458 - 474 there is the combination of the oligonucleotide TAAGGA complementary to the 3-end of the ribosomal 16S RNA and initiating codon ATG followed by purine nucleotide (12). It may well be that this stretch is the starting point of the translation of the phage CII gene. The initiating codon is followed by 121 sence codons and then terminator TAG. After this work has been completed, the report of Schwartz et al. (9) about the establishment of the CII and N-terminal part O-gene sequences was published. The primary structure of this part of the region is in agreement with sequence determined by other authors (9, 19). The sequence of Y-region which is of great importance for the transcription regulation was also determined in our work. This region is positioned within interval between imm 434 boundary

and the start of the CII gene. Our sequence is in agreement with the sequence published (9, 18) when this work was practically completed.

Acknowledgement - This report is the summary of the work which has been carried out at two Institutes of the USSR Academy of Sciences. The work was conducted by professors Yu.A. Ovchinnikov and A.A. Baev, and, besides the authors of the report, the participants are S.O.Guryev, A.S.Krayev, G.S. Monastyrskaya and V.M. Zacharyev.

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THE USE OF ULTRAVIOLET-INDUCED POLYNUCLEOTIDE-PROTEIN CROSSLINKS IN STUDIES OF NUCLEOPROTEIN STRUCTURE

E. I. Budowsky, M. F. Turchinsky, N. E. Broude, I. V. Boni, I. V. Zlatkin, K. S. Kussova, N. I. Medvedeva, G. G. Abdurashidova*, Ch. A. Aslanov* and T. A. Salikhov*

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow 117988, GSP-1, USSR

**Institute of Bioorganic Chemistry, Uzbek SSR Academy of Sciences, Tashkent, USSR*

Abstract - The utilization of ultraviolet-induced polynucleotide-protein crosslinking has been discussed as a possible approach in structural and functional studies of nucleoproteins. With respect to the E.coli translation system this approach has yielded the following results: i) Identification in A- and P-sites of proteins contacting with aminoacyl-tRNA and N-acetylaminoacyl-tRNA respectively. ii) The observation of considerable changes in 16 S RNA contacts with ribosomal proteins resulting from interaction of initiation factor 3 with the 30 S subunit. iii) Considerable difference in interaction of ribosomal protein S1 with the 30 S subunit than with oligo- and polynucleotides including the isolated 3'-terminal fragment of 16 S RNA.

INTRODUCTION

Bioorganic chemistry attempts to describe in chemical terms the structure of different cellular components and systems and to shed light on the mechanisms of their function. Nucleic acids in particular, accomplish their function only in nucleoprotein complexes; the formation of such complexes is due to cooperative non-covalent interactions between parts of macromolecules involved. Elucidation of nucleoprotein structure therefore requires information about the structure and three dimensional arrangement of interacting species and their parts.

Any transition of a nucleoprotein from one functional state to another is due to and/or accompanied by changes in the spectrum of non-covalent interactions between the components of the complex. Therefore in order to understand the mechanism of functioning of a nucleoprotein one has to possess a technique for characterization of these interactions in different functional states of the complex.

Several approaches have been used for studies of different structural states of complex multicomponent nucleoproteins, ribosomes among others. Different methods of measuring the association of individual proteins with RNA or incomplete RNP (1) were very convenient for the elucidation of ribosomal self-assembly. Three-dimensional arrangement of proteins in a ribosome can be investigated by immunoelectron microscopy (2,3). Use of bifunctional crosslinking agents and photoaffinity agents (4-6) was most useful for the elucidation of distances between the components of the ribosome. It should be pointed out that these approaches permitted to determine only the distances between certain groups in a macromolecules. Each component of the ribosome (RNA's and proteins) interacts as a rule with several counterparts simultaneously. Therefore there exists an important problem to define the direct contact (interaction) between ribosomal components.

It is apparent that in each case both interacting partners must be identified on the basis of the interaction. However, since the quarternary structure of nucleoproteins is stabilized by cooperative weak non-covalent interactions the identification of interacting components using incomplete complexes is impossible as a rule. Interactions in complexes containing only an incomplete set of components (incomplete complexes) may differ in

significant respects from interactions in native nucleoproteins. This problem therefore can be unequivocally solved only by forming the covalent linkage between macromolecules interacting with each other inside the native nucleoprotein; this approach provides also an opportunity for isolation and analysis of these fragments. For this purpose one has to activate nucleoside or amino acid residues within the nucleoprotein. The activation should not result in any degradation of the polynucleotides or polypeptides or in any damage to higher structure of the components or of the whole complex; The activation however must provide an opportunity for the formation of the covalent linkage resulting from the direct reaction of the activated residue with a partner brought into close proximity.

This problem has no general solution at present. However, marked differences in the spectral characteristics of nucleic acids and proteins permit to accomplish the selective excitation of nucleic bases in nucleoproteins. This transition to the excited singlet state or to the triplet state upon UV-irradiation transforms nucleic bases to strong electrophilic, nucleophylic or radical reacting species (for review see Ref. 7).

It has been shown that UV irradiation induces the formation of the covalent crosslinks between amino acid residues and nucleic bases (for review see Refs. 8,9). The low specificity of formation of UV-induced crosslinks between nucleic acids and amino acid residues is most useful in studies of nucleoprotein structure since it allows to "freeze" contacts between most different although not between all interacting parts of polynucleotides and proteins.

Photochemical reactions only weakly depend on pH, ionic strength and temperatures; this allows crosslink induction under the conditions optimal for nucleoprotein complexes. The effectiveness and specific pathway of a photochemical reaction, however, depends significantly on the wavelength of the incident light, on the presence or absence of quenching agents or sensitizers; this allows a degree of control of the specificity in crosslink induction.

Identification of proteins interacting with tRNA in the A- and P-site of E.coli ribosomes.

Many authors have shown that UV irradiation of nucleoproteins leads to the formation of polynucleotide-protein crosslinks (10-15). The reliable information concerning the contacts between polynucleotides and proteins within the native nucleoprotein can however be derived only from studies of crosslinks induced within the intact (from the standpoint of higher structures) nucleoprotein. Since ultraviolet irradiation results in a modification of both polynucleotides and proteins (16) possibly leading to changes in the structure of the complex. one has to begin from the determination of the ratio between the yield of crosslinks and the damage to nucleoprotein.

It has been shown earlier that considerable number of polynucleotide-protein crosslinks may be formed in ribosomes when about 50 quanta (λ =254 nm) are absorbed per nucleotide (15). At such doses the functional activity of 70 S ribosomes (measured by the formation of a complex $Phe\text{-}tRNA^{Phe}\cdot poly(U)\cdot 70\ S$) is retained for about 70%; sedimentation profiles of 30 S and 50 S subunits as well as of 70 S ribosomes remain unchanged and no scissions of the polyribonucleotide chains of 16 S and 23 S RNA species is observed. The covalent crosslinking of ribosomal proteins with RNA and of tRNA with the ribosome is described by single-hit kinetics. At last, at doses below 50 quanta per nucleotide ribosomes completely retain the ability to transfer N-acetylphenylalanine residue from the $N\text{-}AcPhe\text{-}tRNA^{Phe}\cdot poly(U)\cdot 70\ S$ complex to puromycin.

All these data point out that at doses below 50 quanta per nucleotide, polynucleotide-protein crosslinks are formed in the native nucleoproteins. The data presented below were obtained with doses around 30 quanta per nucleotide.

Before the step of translocation the aminoacyl-tRNA and peptidyl-tRNA are known to occupy the A-site of the ribosome, after the translocation the peptidyl-tRNA is displaced to the P-site (17). The spectrum of proteins located closely to tRNA, bound in the A- or in the P-site, as well as of proteins modification or removal of which affects the binding of tRNA to

the ribosome was determined by several authors (17-19). It has not yet been established, however, which proteins directly interact with tRNA bound to the A- and P-site of the ribosome. In order to identify these proteins we employed the technique of ultraviolet induction of polynucleotide-protein crosslinks within the complexes (complexes I and II; Phe-tRNA^{Phe}·poly(U)·70 S and N-AcPhe-tRNA^{Phe}·poly(U)·70 S; tRNA in the A- and P-site respectively (17). These complexes are retained by nitrocellulose filters and the radioactivity retained when [^{32}P]-tRNA is used is proportional to tRNA content (Fig. 1).

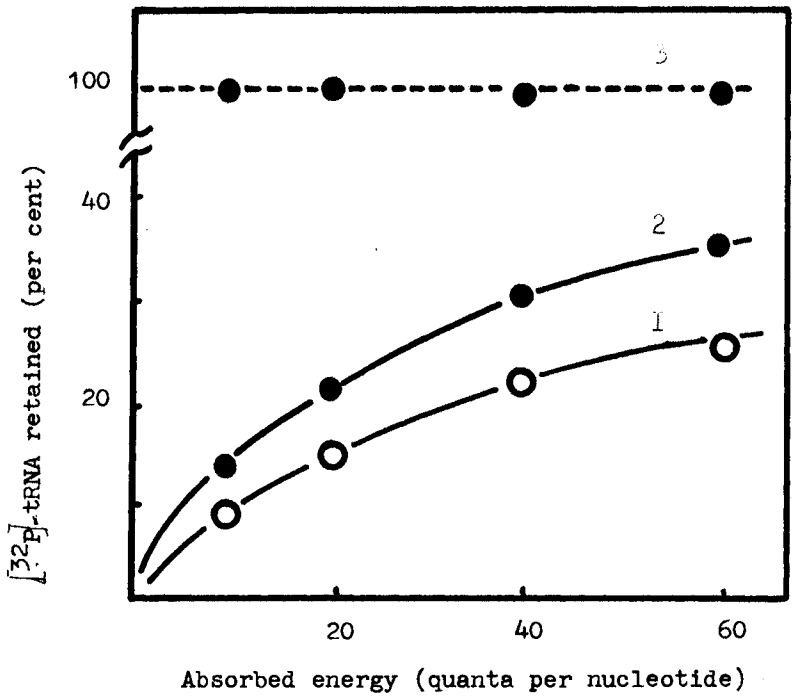

Fig. 1. The effect of ultraviolet irradiation (λ = 254) of complexes Phe-tRNA^{Phe}·poly(U)·70 S (filled circles) and N-AcPhe-tRNA^{Phe}·poly(U)·70 S (open circles) on the retention of [^{32}P]-tRNA by nitrocellulose filters. Continous lines : EDTA added to complexes before filtration to a final concentration of 15 mM; dashed line : filtration in the initial buffer containing 10 mM Mg^{+2}.

In an excess of EDTA (over Mg^{+2}) the complexes dissociate and free Phe-tRNA^{Phe} and N-AcPhe-tRNA^{Phe} is no longer retained by Millipore filters (Fig. 1). Ultraviolet irradiation of complexes leads to an exponential increase of the amount of [^{32}P]-tRNA bound by filters in the presence of EDTA (up to a dose of about 30 quanta per nucleotide) (Fig. 1). This is explained by the covalent crosslinking of tRNA with ribosomal proteins. Since only one crosslink with a protein is sufficient to retain tRNA on the filter, curves 1 and 2 reflect the formation of the first crosslink. Taking into account that 6-8 proteins are crosslinked to tRNA in complexes I and II with roughly similar efficiency (Table 1) one can estimate the minimum quantum yield of the crosslink formation. On the basis of the data of Fig. 1 the quantum yield for the formation of different crosslinks between tRNA and proteins in complexes I and II exceeds 10^{-3} crosslinks per quantum per nucleotide; in other words the yield is comparable to that of the photoreactions of pyrimidine components of polynucleotides (7)

TABLE 1. Proteins of E.coli 70 S ribosomes covalently crosslinked with tRNA after ultraviolet irradiation (λ = 254 nm, 20–30 quanta per nucleotide) of complexes Phe-tRNA^{Phe}·poly(U)·70 S and N-AcPhe-tRNA^{Phe}·poly(U)·70 S (tRNA localized in the A- and P-site respectively).

Relative activity is shown as a percentage of the total radioactivity present in two-dimensional gels containing proteins with cross-linked fragments of [^{32}P]-tRNA. The average total radioactivity present in the whole gel in one experiment was equal to 20 000 - 40 000 counts/min; background - 200 counts/min/square cm of the gel.

Ribosomal proteins	Complex I Phe-tRNA^{Phe}·poly(U)·70 S	Complex II N-AcPhe-tRNA^{Phe}·poly(U)·70 S
S5	19,5	-
S7	2,8	2,2
S9	16,2	18,4*
S10	14,9	-
S11	-	*
L2	12,8	20,1
L4	-	18,7
L6	18,8	-
L7/L12	-	15,6
L16	15,0	-
L25/S17	-	3,6
L27	-	21,4

* Radioactivity was approximately equally distributed between proteins S9 and S11.

After irradiation, complexes I and II were subjected to exhausting treatment by the mixture of RNAses A and T_1 and proteins containing covalently attached fragments of [^{32}P]-tRNA were tentatively identified from their position on a two-dimensional electrophoregram (20). Additional identification was carried out for several proteins: the material from the first gel was separated in the system of Traut (21) iodinated with [^{125}I], digested with trypsin and peptide maps obtained in this way were compared with those of ribosomal protein markers (22).

The results obtained indicate (Table 1) that both in the A- and P-site tRNA has contacts with proteins of both ribosomal subunits; the spectra of proteins bound to tRNA in the A- and P-site are significantly different and only protein S9 and L2 are common for both sites. This is the first identification of ribosomal proteins directly interacting with tRNA in the pre- and post-translocation state.

Changes of the polynucleotide-protein contacts in 30 S E.coli ribosomal subunits upon binding of the initiation factor IF 3.

Nucleoproteins represent cooperative system and removal or addition of different components (even of minor ones) might lead to very important changes in the quartenary structure of a nucleoprotein. This statement is illustrated by the following specific example. The formation of the complex between 30 S ribosomal subunit and IF 3 initiation factor represents a necessary prerequisite for the translation of natural mRNA (23,24). The attachment of IF 3 affects physical characteristics of the 30 S subunit (25,26)

and reactivity of ribosomal proteins towards N-ethyl maleimide and iodination (27,28). Since the ability to react with the latter agents decreases for some proteins and increases for others one may assume that the action of IF 3 was probably not due to a generalized shielding effect but rather to some action of a more general nature directed on higher levels of the subunit structure and leading to a change in the interaction between RNA and proteins. Indeed the attachement of the IF 3 to 30 S ribosomal subunit has fundamentally changed the interaction between 16 S RNA and ribosomal proteins.

TABLE 2. Proteins crosslinked with 16 S RNA after ultraviolet irradiation (λ = 254 nm) of 30 S ribosomal subunit from E.coli and of the complex between 30 S subunit and IF 3 initiation factor.

Proteins crosslinked		S2	S3	S4	S5	S6	S7	S8	S10	S9+ S11	S12	S15+S16+ +S17	S18+S19+ +S20+S21	IF3
Relative radioactivity %	30S	-	-	2	-	-	68	-	-	3	-	11	17	-
	30S IF3	3	3	8	8	2	30	5	4	11	3	8	11	7

Solutions of 30 S subunits containing 16 S [^{32}P]-RNA and 30 S·IF 3 complex were irradiated (at 40 quanta per nucleotide) at 0° with stirring with unfiltered light of a low pressure mercury lamp (λ = 254 nm); nucleoproteins were then treated with a mixture of EDTA and urea added to final concentrations of 40 mM and 2 M respectively and RNA was hydrolyzed by a mixture of RNAse A and T_1. The hydrolysates were fractionated by the two-dimensional electrophoresis in a polyacrylamide gel (see Ref. 20). After the separation the gel was subjected to autoradiography and radioactivity of region containing proteins with crosslinked RNA fragments were counted. Radioactivity in each zone was expressed as percentage of the total radioactivity of the gel (usually 2000 - 4000 counts/min per gel).

The quantum yield of IF 3-16 S RNA crosslink during the irradiation of IF3·30 S complex is around 5×10^{-3} crosslinks per quantum per nucleotide. Similar quantum yield is observed for the crosslinking of 16 S RNA and about a half of proteins of the 30 S subunit with the exception of S7 which is crosslinked much more effectively. In the free 30 S subunit, however S7 is predominantly crosslinked to RNA, considerably lower labeling (fragments of [^{32}P]-16 S RNA crosslinked to proteins) is found in the regions of proteins S15-S17 and S19-S21 and still less (by one order of magnitude) in the region of S4 and S9/11. Thus the binding of the initiation factor IF3 (molecular weight 21 000) to 30 S ribosomal subunit or the "preparation" of the 30 S subunit to binding of natural mRNA does indeed result in a fundamental alteration of polynucleotide-protein contacts in the ribosome.

S1 protein has no contacts with 16 S RNA in the 30 S ribosomal subunit of E.coli.

Ribosomal protein S1 is also indispensable for the initiation of translation of natural messengers (29-31). This protein possess strong polynucleotide-binding activity (32-34) and particularly forms a complex with the isolated 3'-terminal fragment of 16 S RNA having 49 nucleotides in length (35). Assuming that similar interaction takes place in 30 S subunit Dahlberg and Dahlberg came to the conclusion that this interaction lead to despiralization of 3'-terminal fragment, necessary, according to their view, for specific complementary pairing with a preinitiation region of natural mRNA (35). The presence of contacts with 16 S RNA in 30 S

ribosomal subunit was demonstrated for several split proteins using the technique of ultraviolet-induced polynucleotide-protein corsslinks formed with the single-hit kinetics and a quantum yield of $3x10^{-4}$ - 10^{-3} crosslinks/quantum/nucleotide (15). Although the sensitivity of the technique permits to detect crosslinks with the quantum yield about 10^{-5}, ultraviolet irradiation of 30 S ribosomal subunit containing [^{3}H] or [^{125}I] labelled S1 does not lead to any crosslinking of S1 protein with 16 S rRNA. In parallel experiments when the complexes of S1 protein either with isolated 3'-terminal fragment of 16 S RNA, obtained by treatment of ribosome with cloacine DF13 (35) or with poly(U) are irradiated crosslinks were formed with the quantum yields 10^{-2} and $5x10^{-3}$ crosslink/quantum/nucleotide respectively. Thus the protein S1 apparently has no contacts with 16 S rRNA within the ribosome or at least its contacts are quite different from those with isolated 3'-terminus. These data are confirmed by the recently published results suggesting that the absence of 3'-terminal fragment in 16 S rRNA of the 30 S subunit does not affect the binding of S1 (36). These results undermine the hypothesis of Dahlbergs and specifically demonstrate that the data obtained with incomplete artificial complexes may not be valid for the discussion of structure and function of complete nucleoproteins. It should be pointed out that the other nucleotide binding site of the S1 (37) does not participate in the interaction of this protein with the 30 S subunit since the addition of oligodeoxynucleotides does not affect the binding of S1 with 30 S ribosomal subunit. Hence, the binding of S1 with 30 S subunit is determined, most probably, by the protein-protein interactions.

CONCLUSION

Data presented in this communication indicate that the technique of ultraviolet-induced polynucleotide-protein crosslinking is very fruitful in studies of the structure and function of nucleoproteins consisting of many components, of the translation system in particular. One should not, however, forget that ultraviolet irradiation leads to a damage of both polynucleotides and proteins and therefore results obtained at higher doses may reflect not contacts in native nucleoproteins but rather changes of these contacts in the course of a UV-induced degradation of complexes (see Refs. 38-40).

The formation of ultraviolet-induced polynucleotide-protein crosslinks permits the isolation and analysis of fragments of macromolecules interacting in the initial native complexes down to minimal fragments consisting of nucleic acid bases bound to amino acid residues. Studies of structure of minimal fragments and of mechanisms of their formation opens new ways for the elucidation of specific interactions between proteins and nucleic acids.

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STRUCTURAL STUDIES OF THE ELONGATION FACTOR G FROM *ESCHERICHIA COLI*

Yu. B. Alakhov

Institute of Protein Research, Academy of Sciences of the USSR, 142292 Poustchino, Moscow Region, USSR

Abstract - The trypsin and thermolysine effects on EF-G maintaining its native structure and the trypsin effect on EF-G modified by 1,2-cyclohexanedione have been studied. It has been shown that the EF-G polypeptide chain has two regions accessible for the proteolytic attack, one of which is situated in the N-terminal part of the polypeptide chain and the other - in the C-terminal part. Limited proteolysis of native EF-G results in cleavage of the polypeptide chain in the mentioned sites and formation of two large fragments. The high stability of the large fragments formed to further proteolysis is due to their maintaining of the compact structure which, according to the CD and scanning calorimetry data, is the same in the whole protein and its fragments. The fragments of limited proteolysis exist in the whole protein molecule as independent structural units - domains.

INTRODUCTION

It is known that the protein-synthesizing system contains, besides the ribosome, mRNA and tRNA, the protein translation factors and, in particular, EF-G, the elongation factor of protein biosynthesis. It catalyzes the GTP-dependent translocation of the peptidyl-tRNA·mRNA complex from the A-site to the P-site on the ribosome leading to the release of the deacylated tRNA from the P-site and from the ribosome. It is also known that EF-G catalyzes the ribosome-dependent GTPase reaction in the absence of mRNA and aminoacyl-tRNA (Refs. 1-3). This activity of the factor is called uncoupled GTPase activity as it is not connected directly with translocation (Ref. 4). It has been shown that the uncoupled GTPase reaction passes through an intermediate stage of the formation of a ternary complex which includes the ribosome, EF-G and GTP (Refs. 4-8). The formation of a binary complex between EF-G and GTP precedes the formation of the ternary complex (Refs. 9 & 10). EF-G is a protein consisting of one polypeptide chain with the MW of about 81,000 (Refs. 11 & 12). The polypeptide chain consists of about 740 amino acid residues. Alanine is the N-terminal and lysine is the C-terminal amino acid. (Refs. 12 & 13).

To study in detail the physico-chemical principles of the EF-G functioning we have undertaken an investigation of its primary structure. At the first stage we used limited proteolysis of the factor into large fragments. The method of limited protein proteolysis under native conditions is widely used for investigation of structural and functional properties of a number of proteins. In particular, it has been shown that at limited trypsinolysis of native EF-G several fragments are formed which are relatively stable to further trypsin action (Ref. 14).

The study of the fragments of limited EF-G trypsinolysis permitted to align them in the polypeptide chain (Ref. 13). The study of the trypsin and thermolysine effects on the native EF-G and of trypsin on EF-G modified with 1,2-cyclohexanedione (Ref. 15) permitted to localize the regions of the polypeptide accessible to the proteolytic attack.

LIMITED PROTEOLYSIS OF EF-G

Incubation of EF-G with trypsin results in the formation of five fragments T_3-T_7 rather stable to further trypsin action. Fragment T_5 is the most stable to further trypsinolysis and fragment T_3 changes into fragment T_4 by splitting off the N-terminal part consisting of 69 amino acid residues (fragment T_7). Fragment T_6 which is the N-terminal part of EF-G with the molecular weight of about 6,000 decomposes rather quickly. Localization of tryptic fragments in the polypeptide chain of EF-G is represented in Fig. 1.

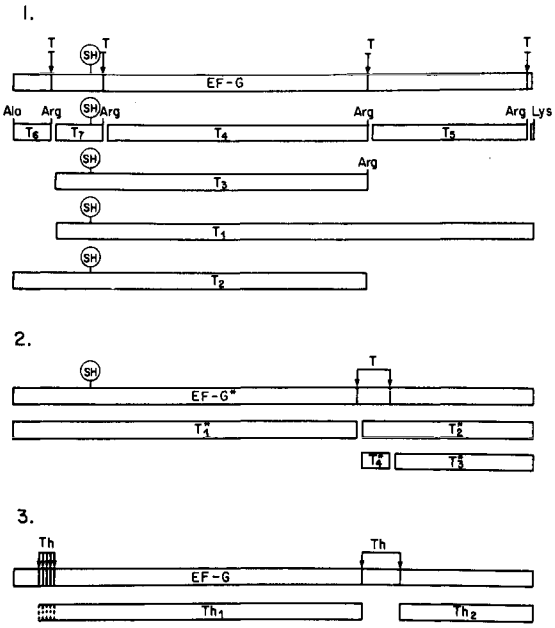


Fig. 1. Alignment of fragments of limited proteolytic hydrolysis along the EF-G polypeptide chain. Tryptic digest (1.); tryptic digest of EF-G modified by cyclohexanedione (2.); thermolytic digest (3.).

Limited hydrolysis of EF-G by thermolysine results in the formation of two large fragments (Th_1 and Th_2) with the molecular weights of 45,000 and 25,000 (Fig. 1). The fragment with MW of 25,000 represents the C-terminal part of the EF-G polypeptide chain which is shorter than the analogous tryptic fragment at the N-terminus. The fragment with MW 45,000 is analogous to the tryptic fragment T_3 but the C-terminus is shortened by 25-35 amino acid residues. The N-terminal part of this fragment is heterogeneous which is caused by several disruptions of the polypeptide chain.

The study of the N-terminal sequences permitted to localize the fragments in the polypeptide chain of the G-factor and to identify the sites of the proteolytic attack of the EF-G polypeptide chain.

As has been mentioned above, the fragment with MW 25,000 (Th_2) represents the C-terminal part of the EF-G polypeptide chain shortened at the N-terminus by seven amino acid residues in comparison with the analogous tryptic fragment T_5. The amino acid sequence of the tryptic fragment T_5 is as follows:

Th Th Th
Glu↓Phe-Asn↓Val-Glu-Ala-Asn↓Val-Gly-Lys-Pro-Gln-Val-Ala-Tyr-Arg-Glu-Thr-...

Th ↓ are the sites of the thermolysine attack.

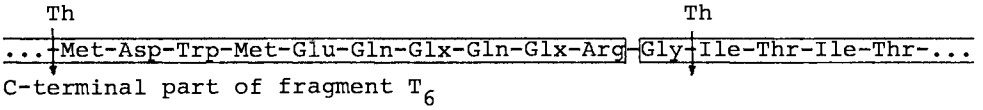
The amino acid sequence of the thermolytic fragment Th_2 is as follows:

Val-Gly-Lys-Pro-Gln-Val-Ala-Tyr-Arg-Glu-Thr-Ile-Arg-...

Thus, the site of the thermolysine attack of the EF-G polypeptide chain is in direct proximity to that of the trypsin action.

The fragment with MW of 45,000 is analogous to the tryptic fragment with MW of 49,000 but it is shortened at the C-terminus of the molecule by 25-35 residues, i.e. the region of the EF-G polypeptide chain containing the C-terminal part

of tryptic fragments T_3 and T_4 and the N-terminal part of the tryptic fragment T_5 is accessible to thermolysine action. This region includes about 40 amino acid residues. It should be noted that thermolysine has a much broader specificity than trypsin and therefore the probability of multiple disruptions of the protein polypeptide chain is naturally higher. Nevertheless, the region of the polypeptide chain attacked by thermolysine is rather small. This is apparently due to maintaining of the compact structure by the fragments formed. On the other hand, the N-terminal part of the fragment with MW of 45,000 is heterogeneous which is caused by several disruptions of the polypeptide chain in the region containing the C-terminal part of the tryptic fragment T_6 and the N-terminal part of the tryptic fragment T_7:



C-terminal part of fragment T_6

Th ↓ are the main disruptions of the EF-G polypeptide chain by thermolysine in its N-terminal part.

Most of disruptions (about 60%) occur at isoleucine situated near the N-terminal glycine in the tryptic fragment T_7 and the other disruptions occur in the C-terminal region of the polypeptide chain contained in the N-terminal fragment T_6.

Thus the study of thermolytic fragments permitted to localize two exposed regions in the EF-G polypeptide chain (Fig. 1), one of which, about 10 amino acid residues long, is situated in the N-terminal part of the molecule and the other, about 40 amino acid residues long, is situated at a distance of one third of the polypeptide chain length from the C-terminus of the molecule.

We have shown earlier (Ref. 13) that all tryptic fragments of EF-G (T_3-T_7) have arginine as the C-terminal amino acid, i.e. cleavage of the protein polypeptide chain occurs only at arginine residues. Therefore we studied the trypsin effect on EF-G modified by 1,2-cyclohexanedione at arginine residues. It turned out that at trypsin treatment the modified EF-G splits into four fragments T_1^*-T_4^* (Fig. 1). Fragment T_1^* is the N-terminal part of EF-G since its N-terminal amino acid sequence coincides with that of EF-G. Therefore fragment T_2^* is the C-terminal. Fragment T_3^* has the same C-terminal sequence as EF-G and fragment T_2^* and differs from the latter by the lack of 25-35 amino acid residues at the N-terminus. Since fragments T_1^* and T_2^* completely cover the polypeptide chain of EF-G (Fig. 1) it was interesting to check whether the N-terminal fragment and its mixture with the C-terminal fragment possess functional activity. The ability of the G-factor to form a complex with the ribosome and [^{14}C]GDP in the presence of fusidic acid was used as a functional test (Refs. 4 & 16). It appeared that neither fragment T_1^* nor the mixture of fragments T_1^*+T_2^* display the ability to form the complex. These fragments, apparently because of a weak interaction, cannot form a hybrid molecule possessing functional activity.

The study of the fragments resulting from tryptic hydrolysis of EF-G modified by cyclohexanedione permitted also to confirm the presence of an exposed region of the polypeptide chain containing 25-35 residues at a distance of one third from the C-terminus of the molecule.

Thus proteolysis of the native EF-G results in cleavage of its polypeptide chain at two points with the formation of two large fragments. At tryptic hydrolysis the N-terminal region consisting of 69 amino acid residues and containing the GTP-binding center (Refs. 17 & 18) detaches from one of these fragments. The high stability to further proteolysis of the fragments formed is conditioned by their compact structure. They probably exist in the whole protein molecule as independent structural units - domains. To test this assumption and to determine the stability of the G-factor and its fragments in solution we studied them by circular dichroism and scanning microcalorimetry.

THE STUDY OF EF-G AND ITS TRYPTIC FRAGMENTS BY CIRCULAR DICHROISM AND SCANNING MICROCALORIMETRY

The method of fragment separation which we used for the structural characterization (Ref. 13) was unsuitable for isolation and the study of the molecule state in solution as isolation proceeded in denaturing conditions and it was difficult to evaluate nativity after renaturation from the 6 M urea solution. Therefore in this case isolation was done under conditions preserving protein molecules from denaturation. To prevent unspecific aggregation, separation was achieved by gel filtration on Sephadex G-150 at high ionic strength (0.35 M

KCl) and raised temperature (35°C). Under these conditions we succeeded to obtain a high yield of the C-terminal fragment T_5 and of the mixture of fragments T_3 and T_6. It turned out that the mixture of fragments T_3 and T_6 is a stable complex which is not decomposed by prolonged dialysis or by gel filtration. The complex decomposes only in the presence of 6 M urea. The stability of this complex is evidently due to the fact that fragment T_6 is the N-terminal region of the G-factor and of fragment T_2, i.e. adjoins directly the beginning of the polypeptide chain of fragment T_3 and therefore specifically interacts with fragment T_3 forming a hybrid molecule similar to fragment T_2 by its properties. All further studies were done on this hybrid molecule (designated further as T_2^*) and fragment T_5.

Figure 2 (A) represents CD spectra of fragments T_2^* and T_5. A comparison of the spectra shows that the curves of these fragments considerably differ in shape.

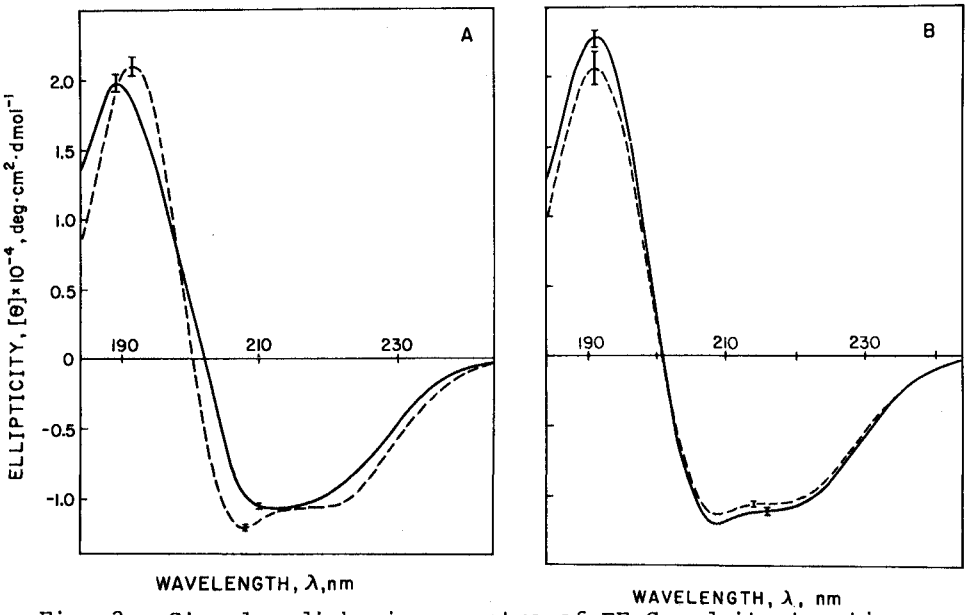


Fig. 2. Circular dichroism spectra of EF-G and its tryptic fragments. (A), circular dichroism of fragment T_5 (———) and T_2^* (- - -). Vertical lines show the shift from the mean value at different concentrations and cuvette thickness. (B), circular dichroism of EF-G (———) and that calculated from curves (———) and (- - -) in (A) according to the equation:

$$0.321[\theta]^{26\cdot10^3} + 0.679[\theta]^{55\cdot10^3}.$$

This points to the difference in their secondary structures which also follows from Table 1. Figure 2 (B) represents a CD spectrum experimentally obtained for EF-G and that calculated from the curves for separate fragments. It follows that there is practically a complete additivity of optical properties of the fragments taken separately and within the G-factor.

TABLE 1. Secondary structure of EF-G and of its proteolytic fragments

	Content of the secondary structure (%)	
	α	β
EF-G	33 ± 1	27 ± 1
T_5	29 ± 1	30 ± 1
T_2^*	34 ± 1	22 ± 1

The error of ±1% indicated in the Table corresponds to the error in measuring the values $[\theta]_{210}$, $[\theta]_{219}$ and $[\theta]_{225}$ equal to ±2%.

Thus, in terms of the polypeptide chain packing, these fragments are independent structural units (domains) in the EF-G molecule. Figure 3 (A) (curve c) represents the melting curve of the G-factor in 20 mM potassium phosphate

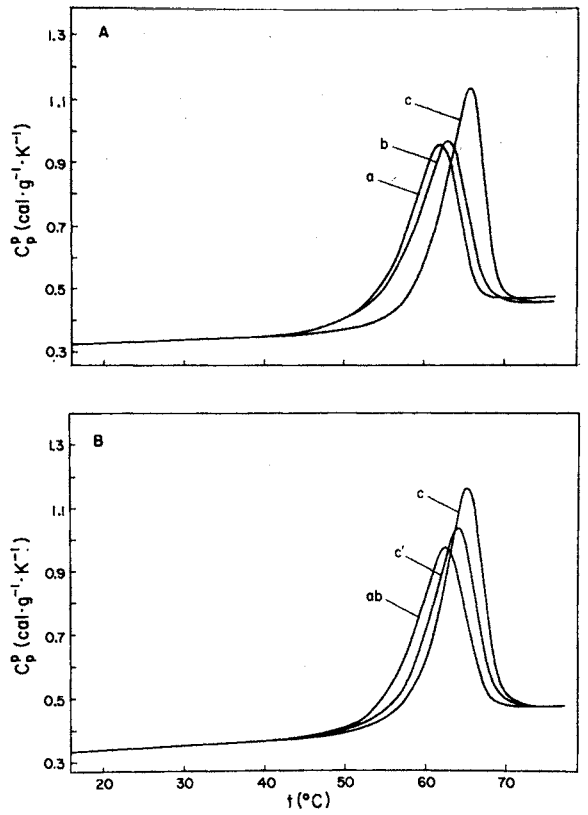


Fig. 3. Temperature dependence of partial specific heat capacity of EF-G and its fragments. (A), EF-G (curve c); T_5 (curve a) and T_2^* (curve b) in 20 mM potassium phosphate, pH 7.5. (B), EF-G (curve c) in 20 mM potassium phosphate, pH 7.5; EF-G (curve c') in 20 mM potassium phosphate, 350 mM KCl, pH 7.5, and the algebraic sum of specific capacities of fragments T_5 and T_2^* (curve ab) in 20 mM potassium phosphate, pH 7.5.

(pH 7.5). Under these conditions the G-factor is a relatively stable molecule having the melting temperature of 65.5°C. Its melting is characterized by a rather symmetric peak of heat absorption. At the first glance, it seems that this symmetry indicates high cooperativity of the transition and the absence of intermediate states. However, a comparison of the calorimetric enthalpy with the effective one shows a considerable difference between these values; in this case, calculated per mole of the G-factor, they differ by a factor of three (see Table 2). Therefore we have to conclude that there are several

TABLE 2. Thermodynamic parameters of melting of the factor EF-G and of its fragments T_5 and T_2^* at pH 7.5, 20 mM potassium phosphate

Object	Q_g, J·g^{-1}		T_m, °C	
	–	350 mM KCl	–	350 mM KCl
EF-G	19.2 ± 1.0	18.4 ± 1.0	65.5 ± 0.2	64 ± 0.2
T_5	19.6 ± 1.0	19.6 ± 1.0	61.8 ± 0.2	–
T_2^*	19.6 ± 1.0	19.6 ± 1.0	62.9 ± 0.2	–

Object	ΔH_{cal}, kJ·mol^{-1}		$\Delta H^{v.H}$, kJ·mol^{-1}	
	–	350 mM KCl	–	350 mM KCl
EF-G	1560 ± 80	1490 ± 80	598 ± 30	540 ± 30
T_5	510 ± 80	–	444 ± 40	–
T_2^*	1085 ± 80	–	420 ± 40	–

cooperative units within the G-factor or that denaturation proceeds in several stages, i.e. it can be assumed that the G-factor has a domain organization. What are, from this point of view, the parameters of fragments T_2^* and T_5 (curves b and a in Fig. 3 (A))? It is seen in Table 2 that for T_5 both enthalpy values are similar, i.e. this fragment melts as an ordinary globular protein with a compact structure and represents a domain within the G-factor. On the other hand, the effective and calorimetric enthalpies do not coincide for fragment T_2^* which can be explained by its domain organization. However, it is not yet clear whether the boundaries of the constituent domains coincide with the sites of the trypsin attack.

Figure 3 (B) represents the melting curves of the G-factor in solution with a low ionic strength (20 mM potassium phosphate) (curve c) of the G-factor in solution with a high ionic strength (20 mM potassium phosphate, 350 mM KCl) (curve c') and the curve ab which shows the change of heat absorption and is calculated as a sum of heat absorption changes of fragments T_2^* and T_5. It is seen that the rise of the ionic strength decreases the stability of the molecule and the curve of the heat absorption change shifts towards the algebraic sum of two fragments.

It follows from the CD data and calorimetric measurements that tryptic fragments T_2^* and T_5 are globular compact structures and exist as independent structural units within the whole EF-G molecule. Their interaction within the native protein seems to be small taking into account non-cooperativity of the whole factor. Nevertheless this interaction exists as both fragments somewhat stabilize each other within the factor and their melting becomes more cooperative (cf. $\Delta H^{V \cdot H}$ of the fragments and the factor in Table 2).

This is also seen in Fig. 3 (B) representing the melting curve for the G-factor (curve c) and the curve ab which is an algebraic sum of specific heat capacities of the fragments. The nature of the forces determining this interaction is unclear but it can be assumed that an important role is played by salt bonds and electrostatic interactions. This conclusion is based on the following: an increase of the ionic strength not only destabilizes the factor, but also decreases cooperativity and consequently $\Delta H^{V \cdot H}$ of its melting (Fig. 3 (B), curve c'). As a result, the melting curve approaches, by its shape and position, the curve characteristic for the mechanic mixture of non-interacting fragments. It is difficult to evaluate the energy of the domain interaction in the G-factor without the data on the interaction constant. It is possible to say only the following: (a) the enthalpy of interaction is negligeably small as specific melting heats of the factor and fragments coincide within the error of experiment. Therefore the main role is played by entropic forces. This does not contradict the importance of electrostatic interactions. (b) The free Gibbs energy is not large either, since the disruption of the covalent bond between the fragments leads to their dissociation even at a low ionic strength (data of electrophoresis in polyacrylamide gel under non-denaturing conditions). The disruption of the bond does not necessarily lead to conformational changes making the interaction unfavourable. A sharp decrease of the effective domain concentration in the given volume at the small constant of mutual affinity is sufficient for dissociation.

To determine quantitatively thermodynamic parameters of the structure stabilization in the G-factor domains and their interaction within the native molecule it is necessary to carry out further investigations. Nevertheless it should be noted that the revealed weak connection between the domains and its sensitivity to the ionic strength may be important from the functional point of view.

Thus the study of the fragments of limited proteolysis permitted to obtain data on the general composition of the EF-G molecule which consists of at least two independent structural units - domains. At the ends of the larger domain there are regions of the polypeptide chain accessible to the proteolytic attack. One of the regions in the N-terminal part of the molecule adjoins directly the GTP-binding center of EF-G and the second region is situated at the boundary between the domains. Cleavage of the protein molecule into a strictly determined number of large fragments is a convenient approach to the determination of the primary structure of a large molecule such as that of the G-factor.

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THE CYCLING OF Ca^{2+} ACROSS THE INNER MITOCHONDRIAL MEMBRANE

E. Carafoli, M. Crompton, P. Caroni, K. Schwerzmann and I. Roos

Laboratory of Biochemistry, Swiss Federal Institute of Technology (ETH), Universitätstrasse 16, 8092 Zürich, Switzerland

Abstract - Mitochondria contain 2 separate pathways for Ca^{2+} transport. One is used for the uptake, and it consists of an electrogenic uniporter driven by the membrane potential generated by respiration, and inhibited by ruthenium red. The other route is used for the release of Ca^{2+}, and has so far been identified and characterized in mitochondria from heart, brain, endocrine tissues, exocrine tissues, and skeletal muscles. It consists of a ruthenium red-insensitive system, which specifically exchanges Ca^{2+} for Na^{+}. In other mitochondrial types (liver, kidney, lung), the Na^{+}-activated release route is absent. The available evidence, however, suggests separate pathways for Ca^{2+} uptake and release also in these mitochondria. The simultaneous operation of the uptake and release routes results in the continuous cycling of Ca^{2+} across the inner mitochondrial membrane.

INTRODUCTION

The inner mitochondrial membrane is the barrier which separates the intramitochondrial solutes from the extramitochondrial environment. It contains a series of specific carriers which catalyze the transport of a series of essential anions, among them respiratory substrates, adenine nucleotides, and inorganic phosphate, and of at least one essential cation, Ca^{2+}. The mechanism and the regulation of the transport of Ca^{2+} have been the subject of intensive investigation since the discovery of the process by Vasington and Murphy in 1962 (Ref. 1). Recent experiments have led to the development of new concepts, which place it in a position of particular significance from a perspective of metabolic cell regulation. In the present article, the field will be reviewed, with special attention to the development of these recent new concepts, and to their possible consequences in the domain of metabolic regulation.

WHY IS Ca^{2+} SO IMPORTANT IN BIOLOGY?

The largest portion of the Ca^{2+} of the body (more than 99%) is found in the bone. The small amounts of Ca^{2+} present in the circulating fluids and in the soft tissues, however, play a role of fundamental importance: Ca^{2+} is a biological messenger capable of modulating the generation of the primary "signals" at the level of the plasma membrane, and of carrying the signals to their respective intracellular targets (for a recent review, see Carafoli and Crompton, Ref. 2).The "targets" of the messenger function of Ca^{2+} represent a very long, and continually expanding, list of biochemical events which occur in the soluble phase of the cell and in the various, membrane-enclosed, intracellular compartments. One of their common properties is to be regulated by Ca^{2+} in the μM concentration range, a fact which in turn requires that the intracellular activity of Ca^{2+} is allowed to oscillate only around this very low level. This is achieved by a series of pumping systems, which eject Ca^{2+} from the cell, or sequester it into intracellular compartments.
A discussion of the reasons for the evolutionary choice of Ca^{2+}, among the other cations abundantly present in biological systems, as a biological messenger, would lead to very interesting considerations of molecular evolution, (Carafoli and Crompton, Ref. 2), which would, however, be beyond the scope of this presentation. Here it may suffice to say, that Ca^{2+} is a very "flexible" ligand, uniquely capable of interacting optimally with the irregular complexing cavities normally offered by biological systems (Refs. 3 and 4). This versatility of Ca^{2+} may have well been the key factor in dictating its evolutionary choice as a biological messenger: the development of specific and efficient membrane transport systems has probably been a comparatively easy task in the case of Ca^{2+}, permitting its maintenance in the intracellular milieu at the very low levels of activity demanded by the messenger function. Diffe-

rent ways of transporting Ca^{2+} across membranes have been developed, and are now known: electrophoretic transport systems, in which Ca^{2+} crosses the membrane in response to a membrane potential, membrane ATPases, which are specifically stimulated by Ca^{2+}, and specific Na^{+}/Ca^{2+} exchange carriers.
Specific Ca^{2+} transporting systems have so far been described in the plasma membrane, and in 2 intracellular membranous systems, the mitochondria and the endo-(resp. sarco-)plasmic reticulum. Across the plasma membrane, Ca^{2+} diffuses continually from the extracellular spaces down its concentration gradient, and across the plasma membrane it is continually ejected by energy-requiring (ATPases, or other) processes. In fact, the long term maintenance of Ca^{2+} within cells at levels which are much lower than outside must depend on its ejection across the plasma membrane. The plasma membrane, however, (Table I) represents but a minor fraction of the total Ca^{2+} transport surface of average cells. It may thus well be that intracellular membranes play a predominant role in the rapid regulation of cell Ca^{2+}, a fact which is frequently demanded by metabolism.

TABLE I. Total area of Ca^{2+}-transporting membranes in some representative cells

	Area (m^2 / gm tissue)		
Cell	Plasma membrane	Mitochondria	Endo-or sarcoplasmic reticulum
Liver	0.55 (11.4%)	2.65 (54.8%)	1.63 (33.7%)
Heart	0.10 (0.8%)	10.60 (87.0%)	1.48 (12.1%)

Modified from Ref. 2

The figures reported in Table I further indicate that the largest Ca^{2+} regulating system of average cells is represented by the inner mitochondrial membrane. Whether the mitochondrial system is the most important also in the rapid and fine regulation of Ca^{2+} at the low concentrations required by some biological processes cannot be said at the moment. Essential, comparative information on the kinetic parameters (e.g. K_m, V_{max}) of the transport process in mitochondria and, e.g., the endo-(sarco-)plasmic reticulum is indeed still lacking.

THE ENERGY-LINKED UPTAKE OF Ca^{2+} BY MITOCHONDRIA

The main properties of the energy-linked uptake system are summarized in Table II. Of particular interest is the specific inhibition of the process by very low concentration of ruthenium red, an observation which has been instrumental in the development of the concept of a separate pathway for the release of Ca^{2+} (see below). Also noteworthy is the total capacity of mitochondria for Ca^{2+} storage. In the presence of the simultaneous penetration of inorganic phosphate, and thus presumably in conditions similar to those prevailing in situ, up to 3 μmoles of Ca^{2+} can be accumulated per mg of mitochondrial protein. This corresponds, in a tissue like heart, which contains approximately 100 mg of mitochondrial protein per g of tissue, (Ref. 5) to a total of about 300 μmoles per g of tissue. In the past, it has generally been assumed that the uptake of massive amounts of Ca^{2+} by mitochondria would invariably lead to irreversible damage to the organelle. It has become evident more recently, however, that mitochondria containing up to 20 or 30 times their normal Ca^{2+} content may be structurally intact and functionally competent (Refs. 6-8, see also Ref. 9 for a review), although some of their biochemical parameters, e.g. the translocation of adenine nucleotides, may be modified (Refs. 8 and 10). These findings are of particular interest, since they indicate that the mitochondrial Ca^{2+} "sink" without evident functional damage, may accumulate very large amounts of Ca^{2+} in vivo, as may be normally required in some tissues, or occasionally required, due to pathological stresses, in others.
Some of the kinetic parameters of the Ca^{2+} uptake system are also summarized in Table II. The K_m value of about 10 μM is close to the range, where intracellular metabolic regulation by Ca^{2+} occurs (in addition, it may be rendered artificially higher by the isolation procedure). The V_{max} of the process of Ca^{2+} uptake can, under optimal conditions, be as high as 10 nmoles of Ca^{2+} per mg of mitochondrial protein per sec. (corresponding, in a tissue like heart, to 1μmole of Ca^{2+} per g of tissue per sec.). It must be stressed, however, that in the conditions prevailing in vivo, the rate of Ca^{2+} uptake may be slowed down by substances naturally present in the cytosol. Among them, a special role may be played by Mg^{2+}, which has been shown (Ref. 11) to transform the rate versus concentration curve for Ca^{2+} uptake from hyperbolic to sigmoidal, at least in heart mitochondria. The ionized concentration of Mg^{2+} in the cytosol of tissues

like heart or liver is not known, but it is reasonable to assume that the rate of Ca^{2+} uptake, at least at extremely low Ca^{2+} concentrations, may be limited by Mg^{2+}.

TABLE II. General properties of the mitochondrial Ca^{2+} uptake process

Requirements	an energy source (respiration, ATP splitting)
Inhibited by	inhibitors of energy conservation
Inhibited by	lanthanides, ruthenium red, also by Mg^{2+}
Maximal levels attainable	
a = without phosphate	100 - 150 nmoles of Ca^{2+} per mg of protein
b = with phosphate	up to 3 μmoles of Ca^{2+} per mg of protein
V_{max} of uptake	3 - 10 nmoles of Ca^{2+} per mg of protein per sec.
K_m	about 10 μM
Mechanism of uptake	electrogenic, charge uncompensated

One last point mentioned in Table II which must be discussed is the electrogenicity of the Ca^{2+} uptake process. Recent research (Refs. 12-14) has shown that the process is driven by the membrane potential developed by respiration across the inner mitochondrial membrane (Ref. 15), and occurs with a charge transfer of 2. Earlier (Ref. 16) as well as more recent (Ref. 17) suggestions that the process may be partially charge-compensated, i.e., occur with a charge transfer of less than 2, have not been supported by experimental evidence (Ref. 18).

THE PROBLEM OF THE DISEQUILIBRIUM OF THE Ca^{2+} UPTAKE PROCESS

If the process of Ca^{2+} uptake were to reach equilibrium with a membrane potential (Ref. 15) of about 180 mv, one would expect that the gradient of ionized Ca^{2+} concentration across the mitochondrial membrane, as predicted by the Nernst equation, would be about 10^6. This gradient, considering that the ionized Ca^{2+} concentration inside mitochondria probably does not exceed 10^{-4} M (Ref. 2), would correspond to the lowering of cytosolic Ca^{2+} to 10^{-10} M, clearly an improbable event. That the gradient of ionized Ca^{2+} concentration across the inner membrane of energized mitochondria is less than that predicted by the Nernstian equilibrium is indicated by the assays of activities of enzymes known to be dependent on Ca^{2+} inside mitochondria and in the extramitochondrial phase of the cell (Ref. 19) (Table III). The problem has recently been investigated in a number of Laboratories, using also Mn^{2+} as a paramagnetic anologue for Ca^{2+} (Refs. 20 and 21), and considerable deviations from the distribution of ionized Ca^{2+} (and Mn^{2+}) predicted by the equilibrium in the Nernst equation have indeed been found.

TABLE III. Ionized calcium concentration in mitochondria and the cytoplasm

In the mitochondria (from assays of PDHP-phosphatase, PDH-kinase, IDH)	10^{-7} - 10^{-5} M
In the cytoplasm (from assays of α-GP-DH, Phosphorylase kinase, myofibrillar ATPase)	10^{-8} - 10^{-5} M

The simplest way to explain the disequilibrium in the distribution of Ca^{2+} is by postulating that Ca^{2+} "cycles" across the inner mitochondrial membrane, i.e., it is continually taken up on the electrophoretic uniporter, and is continually returned to the extramitochondrial medium by an independent pathway, in which Ca^{2+} flux is partially or completely charge compensated. In the next section, experiments will be described that demonstrate the existence of such an independent release pathway, and characterize its properties in some mitochondrial types.

THE Na^+ - DEPENDENT RELEASE OF Ca^{2+} FROM MITOCHONDRIA

It has been mentioned above that one of the systems for transporting Ca^{2+} across membranes is

by specific Ca^{2+}/Na^{+} exchange carriers. Such systems have now been described in the plasma membrane of both procaryotic and eucaryotic cells, and are best characterized in the cases of the axonal membrane (Ref. 22) and of heart sarcolemma (Ref. 23). It is suggested that the exchange is electrogenic in case of the axonal membrane, i.e., more than 2 Na^{+} enter the axon for each Ca^{2+} that is ejected (Ref. 22), whereas it may be neutral in the case of heart sarcolemma (Ref. 23). It thus seemed reasonable to investigate the possibility that a Na^{+}/Ca^{2+} exchange system could operate also in mitochondria, and be responsible for the release "leg" of the Ca^{2+} cycle. In the experiments to be described, the electrophoretic influx pathway was blocked by ruthenium red at the end of the uptake of a pulse of Ca^{2+}. The efflux pathway could then be studied uncomplicated by the re-uptake of the lost Ca^{2+}. An early experiment (Ref. 24) on the effect of Na^{+} on the release of Ca^{2+} from heart mitochondria is illustrated in Figure 1, in which mitochondria were allowed to accumulate a limited pulse of Ca^{2+}, and

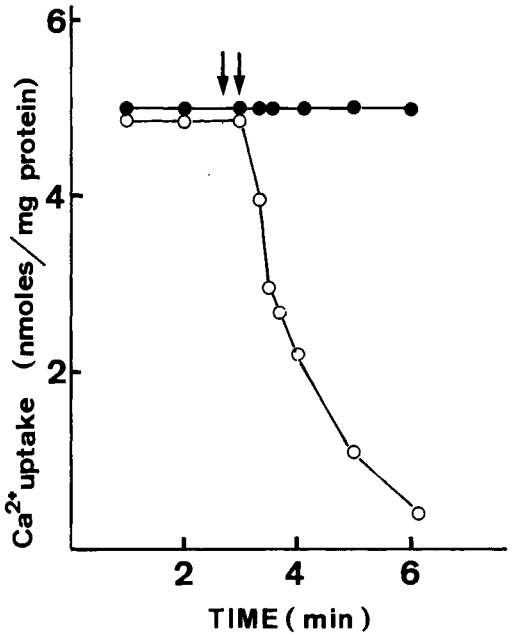

Fig. 1. Release of Ca^{2+} from rat heart mitochondria induced by Na^{+}. Mitochondria were prepared with the method of Pande and Blanchaer (Ref. 25) and incubated (5 mg protein) in 210 mM mannitol, 70 mM sucrose, 10 mM Tris-Cl, pH 7.4, 10 mM Tris-succinate (substrate), and 10 nmoles $^{45}CaCl_2$ per mg of protein, in a final volume of 4 ml, stirred at 25°C. Ruthenium red (2 nmoles per mg of protein) was added at the first arrow, to block the re-uptake of the lost Ca^{2+}. At the second arrow, 50 mM NaCl o—o or 50 mM KCl ●—● were added. The uptake and release of Ca^{2+} were followed isotopically, after rapid filtration through Millipore filters. (Modified from Ref. 24).

were then exposed to Na^{+}, in the presence of ruthenium red to block the re-uptake of the lost Ca^{2+}. A rapid release of Ca^{2+} ensued, which was not seen when K^{+}, instead of Na^{+}, was added at the end of the uptake phase. In the early experiment described in Figure 1, the medium was perturbed with a very large concentration of Na^{+}. In subsequent studies, (Ref. 26), it was possible to establish that a rapid release of Ca^{2+} was already observed upon addition of much lower concentrations of Na^{+}. Moreover, (Figure 2) it was established that the curve relating the rate of Ca^{2+} efflux to the added Na^{+} concentration was strongly sigmoidal, with half maximal velocity of efflux at about 8mM Na^{+}. The maximal rate of efflux observable in heart mitochondria was about 0.2 nmoles of Ca^{2+} per mg of protein per sec., i.e. a rate several times lower than the V_{max} of the energy-linked uptake process. It may also be mentioned that, although the addition of ruthenium red is a convenient means to make the Na^{+}-induced leakage of Ca^{2+} more evident, Na^{+} induces Ca^{2+} efflux even in the absence of ruthenium red, although the extent of release is predictably less since re-uptake of Ca^{2+} occurs (Ref. 26). It was also established that, at variance with ruthenium red, the other specific inhibitor of the uptake of Ca^{2+}, lanthanum, inhibits <u>also</u> the Na^{+}-activated efflux route (Ref. 28).

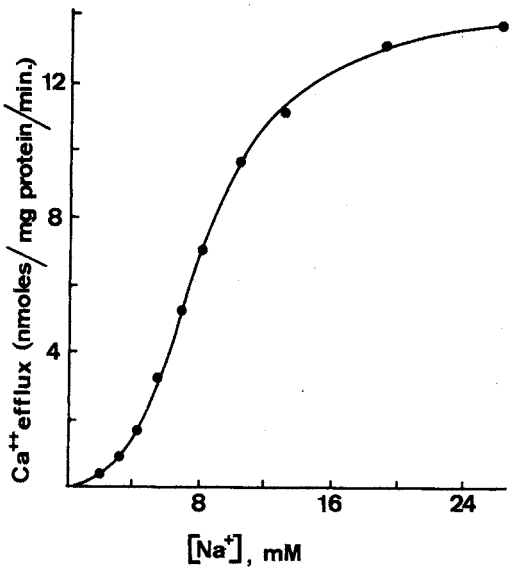

Fig. 2. Kinetics of the Na^+-induced release of Ca^{2+} from heart mitochondria. Rat heart mitochondria were prepared according to the procedure of Scarpa and Graziotti (Ref. 5) which is essentially a modification of the Polytron procedure first introducted by Sordahl and Schwartz (Ref. 27), and depleted of endogenous Ca^{2+} as indicated in Ref 26. The incubation medium contained 120 mM KCl, 10 mM potassium N-(2-hydroxyethylpiperazine-N-2-enthanesulfonate) (HEPES) (pH 7.0), 2 µg rotenone, 60 nmoles of Ca^{2+}, 1 mM K-succinate, and 2.9 mg mitochondrial protein in a final volume of 4.0 ml, stirred at 25°C. 4 minutes after the addition of mitochondria, 0.6 nmoles of ruthenium red were added, followed by the addition of the concentrations of NaCl indicated on the abscissa. The movements of Ca^{2+} were measured with a Ca^{2+}-specific electrode, as indicated in Ref. 26.

The experiments described in Figures 1 and 2 indicate that the efflux of Ca^{2+} from heart mitochondria occurs on a specific Na^+/Ca^{2+} exchange carrier, which is insensitive to ruthenium red, but sensitive to La^{3+}. The sigmoidicity of the release curve (Hill coefficients of about 3 have been obtained) further suggests that the exchange may be electrogenic, i.e., exchange more than 2 Na^+ per 1 Ca^{2+}. The measurement of the uptake of Na^+, and of its stoichiometry to the efflux of Ca^{2+}, was, however, complicated by the existence of a rapidly operating Na^+/H^+ exchange system (Ref. 29), which returned to the extramitochondrial medium the Na^+ that had entered mitochondria in exchange for Ca^{2+}. The existence of a specific exchange-diffusion carrier specific for Na^+ and Ca^{2+} was further supported by experiments in which ruthenium red-insensitive Ca^{2+}/Ca^{2+} exchanges were measured: if a Na^+/Ca^{2+} carrier existed, it would obviously be expected to operate also as a Ca^{2+}/Ca^{2+} exchanger. This was indeed found to be the case (Ref. 28). Heart mitochondria preloaded with radioactive Ca^{2+}, and then exposed to a pulse of non-radioactive Ca^{2+} in the presence of ruthenium red, exchanged the Ca^{2+} pools with a maximal velocity of about 0.1 nmoles per mg of protein per sec. At variance with the Na^+/Ca^{2+} exchange, the kinetics of the Ca^{2+}/Ca^{2+} exchange was hyperbolic, operated with a K_m (external Ca^{2+}) of 13 µM, and with a Ca^{2+}/Ca^{2+} stoichiometry of 1, and was inhibited by La^{3+}. Some of the most important parameters of the $Ca^{2+}/Ca^{2+}/H^+$ exchanges are summarized in Table IV, which shows that the Na^+ H^+ antiporter indeed operated with a V_{max} about 10 times higher than that of the Na^+/Ca^{2+} antiporter, thus preventing the measurement of meaningful Na^+/Ca^{2+} stoichiometries.

The scheme of Figure 3 summarizes the movements of Ca^{2+} in heart mitochondria, and expresses the concept of a "Ca^{2+}-cycle". On this, some comments are in order. Firstly, the end-result of the proposed cycle is the re-entry of H^+ into the mitochondrial matrix in exchange for Ca^{2+}. One could therefore think that a direct coupling between H^+ and Ca^{2+} may exist, making the postulate of a Ca^{2+}/Na^+ exchange as an intermediate step redundant. This reasoning, however, can be ruled out on several grounds. Aside from the fact that Na^+ is indeed required to release Ca^{2+} from heart mitochondria, if a rapid and direct Ca^{2+}/H^+ exchange existed, it would spontaneously operate in Ca^{2+}-loaded mitochondria, where the thermodynamic gradient is evidently in favour of the reentry of H^+. In addition, if an artificial Ca^{2+}/H^+ exchanger (the ionophore A23187) is added to Ca^{2+} loaded mitochondria, Ca^{2+} flows out immediately in the absence of Na^+

TABLE IV. General properties of the Na^+-induced release of Ca^{2+}

Monovalent cation specificity	in addition to Na^+, Li^+ has some releasing effect, K^+, Rb^+, Cs^+, no effect
K_m (Na^+)	about 8 mM
K_m (Ca^{2+}, from outside)	about 13 μM
Kinetics	strongly sigmoidal
V_{max}	about 0.2 nmoles of Ca^{2+} per mg of protein per sec.
Inhibited by	La^{3+} (at high concentrations)
Present in mitochondria from	heart, brain, skeletal muscle, exocrine and endocrine tissues.
Absent in mitochondria from	liver, kidney, lung

(Ref. 30). A second comment on the data contained in Table IV is the following. Since the V_{max} of the process of electrophoretic Ca^{2+} uptake is at least 10 times higher than that of the process of Na^+ induced Ca^{2+} release, it is clear that the rate of the former process must be

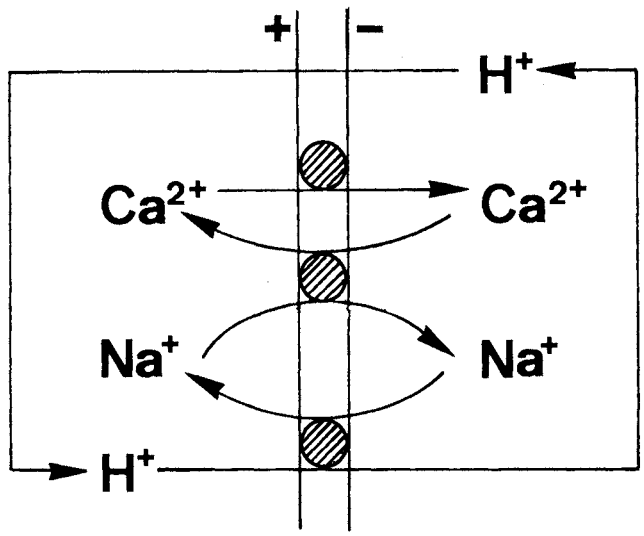

Fig. 3. The Ca^{2+} cycle in heart mitochondria (from Ref. 33)

somehow "limited", if one is to explain the observed considerable deviations from the Nernstian equilibrium with the operation of the Na^+/Ca^{2+} release antiporter. No conclusive indications on ways and means to limit the rate of the energy linked Ca^{2+} uptake are available at the moment, but Mg^{2+} (Ref. 11, 31, 32) may be responsible, at least in part. The Na^+-induced release of Ca^{2+} is not found only in heart mitochondria: Fig. 4 shows that it is present in other mitochondrial types as well. In all these mitochondria, the important characteristics of the process (V_{max}, K_m, sigmoidicity, etc.) are essentially the same as in heart mitochondria. In other mitochondrial types, however, the release of Ca^{2+} has characteristics different from heart, and especially it is not influenced by Na^+. The case of these mitochondria, which com-

prise, among others, those from liver and kidney, is discussed in the next section.

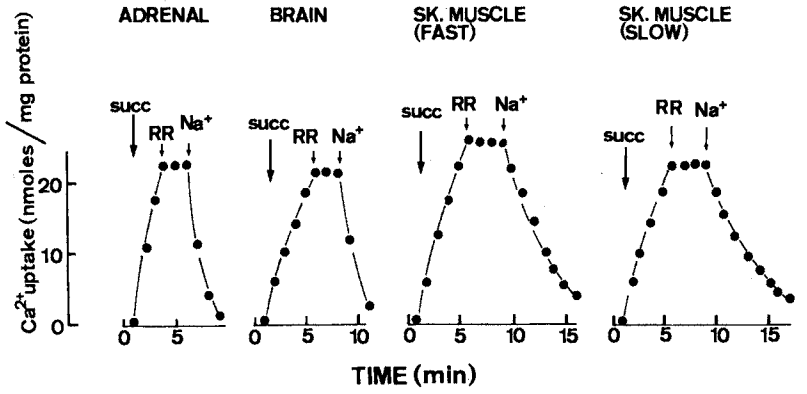

Fig. 4. The Na^+-induced release of Ca^{2+} in mitochondria from various tissues. The preparation of mitochondria from the various tissues indicated is described in Ref. 33. The incubation medium contained, in a final volume of 2 ml, stirred at 25°C, 130 mM KCl, 10 mM K-HEPES (pH 7.0), 1 µg rotenone per mg protein, 30 nmoles of Ca^{2+} per mg of protein, and 1-2 mg mitochondrial protein. Further additions: at the point marked "succ.", 5 µmoles K-succinate, at the point marked "RR" 1 nmole ruthenium red per mg of protein, at the point marked "Na^+" 13.9 mM NaCl. The changes in the extramitochondrial Ca^{2+} concentration were measured with a Ca^{2+}-selective electrode, as indicated in Ref. 33.

THE RELEASE OF Ca^{2+} FROM Na^+-INSENSITIVE MITOCHONDRIA

When liver or kidney mitochondria are made to accumulate a pulse of Ca^{2+}, they release it spontaneously and fairly rapidly, upon addition of ruthenium red (Ref. 33). This is at variance with the case of heart mitochondria where the addition of ruthenium red per se had no effect on the maintenance of Ca^{2+}. In liver and kidney mitochondria, on the other hand, the addition

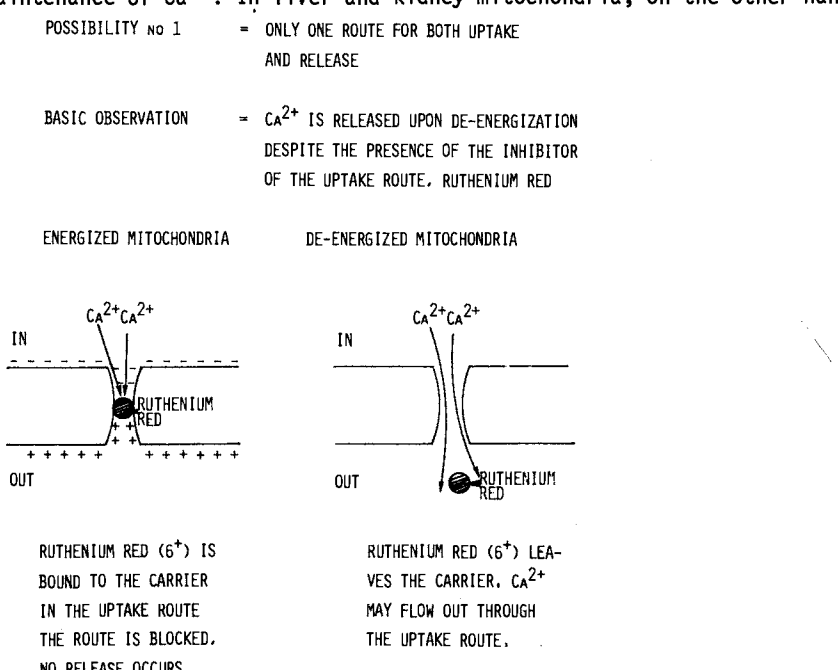

Fig. 5 a. Possibilities for the release of Ca^{2+} from Na^+-insensitive mitochondria

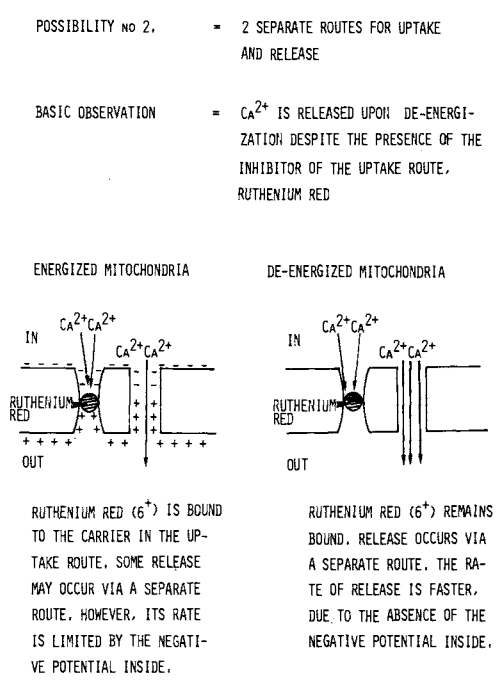

Fig. 5 b. Possibilities for the release of Ca^{2+} from Na^+ insensitive mitochondria

of Na^+ did not induce leakage of Ca^{2+}, nor did it accelerate the spontaneous leakage induced by the addition of ruthenium red. The fact that Ca^{2+} release occurs in the presence of ruthenium red in these mitochondrial types suggests that in this case as well separate pathways for Ca^{2+} influx and efflux exist. Release of Ca^{2+} from liver mitochondria can be induced also

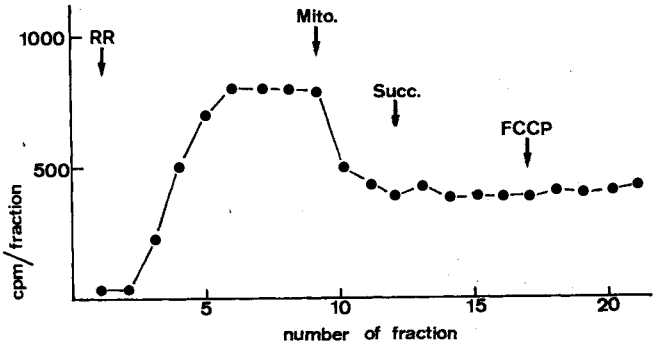

Fig. 6. Binding of ruthenium red to energized and de-energized rat liver mitochondria. Mitochondria were prepared by a conventional differential centrifugation procedure, using 210 mM mannitol, 70 mM succrose, 10 mM Tris-Cl, pH 7.4, and 0.5 mM Na-ethylenediaminetetraacetic acid (EDTA) as the homogenizing medium. The experiment was performed using a flow dialysis method, as described in Ref. 34. The upper chamber of the flow dialysis system contained, in a total volume of 5 ml, at 25°C, 120 mM KCl, and 10 mM Tris-Cl, pH 7.4. At the points indicated by the arrows, the following additions were made: 0.66 µM ruthenium red, labelled with ^{106}Ru-ruthenium red, 11 mg of mitochondrial protein, 10 mM Na-succinate, and 2 µM FCCP.

by other means, for example, by de-energization of the membrane. In this case, the release is only partially inhibited by ruthenium red. This also supports the proposal of a ruthenium-red -insensitive efflux route. However, Pozzan et al. (Ref. 20) have chosen to interprete the leakage of Ca^{2+} that is induced by the de-energization of liver mitochondria in the presence of ruthenium red, with a reversal of the electrophoretic uptake pump. The limited sensitivity to ruthenium red was explained by them with the detachement of ruthenium red (a positively charged molecule) from its specific binding site on the electrophoretic influx carrier, due to the

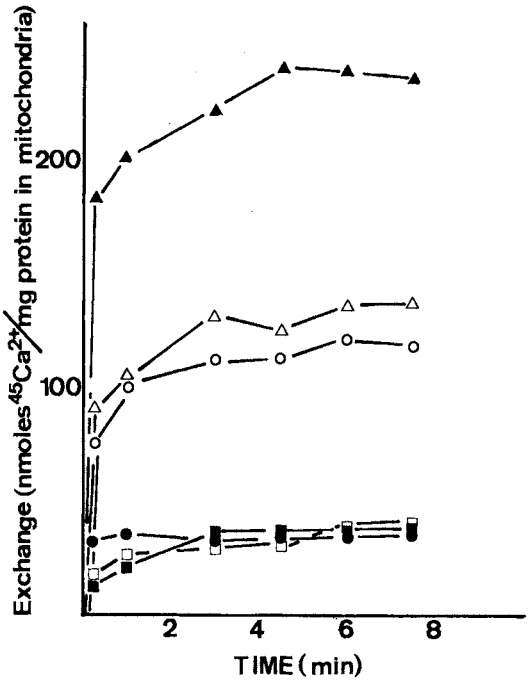

Fig. 7. Ca^{2+}-Ca^{2+} exchange across the rat liver mitochondrial membrane. Mitochondria were prepared as indicated in the legend for figure 6. The incubation medium contained 210 mM mannitol, 70 mM sucrose, 10 mM Tris-Cl pH 7.4, 10 µM rotenone, 125 µM butacaine (to minimize non-specific Ca^{2+} binding to the membrane) 2 mg bovine serum albumin, and 15 mg of mitochondrial protein, in a final volume of 1 ml, stirred at 25°C. After the addition of $^{40}Ca^{2+}$ (100 nmoles per mg of protein), energy-linked uptake was initiated with 25 mM Tris-succinate, pH 7.4. After 2 min. (uptake essentially complete), a pulse of $^{45}Ca^{2+}$ (final concentration, 33 mM), was added (zero time). 50 µl aliquots were withdrawn at the times indicated, filtered through Millipore filters, and washed with 1 ml of ice-cold 140 mM mannitol, 46 mM sucrose, 6 mM Tris-Cl, pH 7.4, 33 mM $^{40}CaCl_2$, and 30 µM ruthenium red (to prevent Ca^{2+} release). The filters were then analyzed for $^{45}Ca^{2+}$. ▲—▲ no additions (additional active uptake of calcium). △—△ 10 µM FCCP, added together with the $^{45}Ca^{2+}$ pulse; o—o 20 µg antimycin A, added together with the $^{45}Ca^{2+}$ pulse; ■—■ 24 µM ruthenium red, added immediately before the $^{45}Ca^{2+}$ pulse; □—□ same as ■—■, but with 10 µM FCCP; ●—● 50 µM A23187, added immediately after the $^{45}Ca^{2+}$ pulse. A23187, a specific Ca^{2+}-ionophore, makes the intramitochondrial Ca^{2+} pool available to the outside medium. The A23187 experiment indicates that the radioacitvity recovered on the filters in the absence of ruthenium red reflected the intramitochondrial Ca^{2+} pool.

disapperance of the transmembrane electrical potential. At this point, then in deciding whether Ca^{2+} leaves de-energized liver mitochondria in the presence of ruthenium red through the influx route, or through a separate route, it was essential to establish whether the inhibitor was still bound to its specific site during the efflux of Ca^{2+}. Clearly, if it could be shown that in the de-energized state ruthenium red becomes detached, and thus "liberates" the influx route, the rationale for postulating separate pathways for Ca^{2+} influx and efflux in liver mitochondria is no longer justified. Conversely, if it could be shown that the inhibitor remains bound to the uptake route in the de-energized state, and blocks it, one would be left with no alternative other than the existence of separate influx and efflux pathways. The 2 alternative possibilities are contrasted in the schemes of Figure 5, a and b.

One approach to the problem is shown in the experiment of Figure 6, in which radioactive ruthenium red was used, and flow dialysis was employed as the method for measuring its binding to mitochondria. The experiment shows clearly that the level of binding of ruthenium red was not influenced by the energetic state of the mitochondrial membrane. The amount bound indeed remained approximately the same before and after the addition of succinate and / or FCCP (Car-

bonyl cyanide-p-trifluoromethoxy phenylhydrazone). It must be pointed out that the amounts of ruthenium red added to the mitochondrial suspension were extremely low (0.25 nmoles per mg of protein), and corresponded to the normal amounts required to inhibit completely the electrophoretic uptake carrier. Essentially all the ruthenium red added was bound, and it is very unlikely that any of it was bound to sites on the membrane different from the uptake carrier.

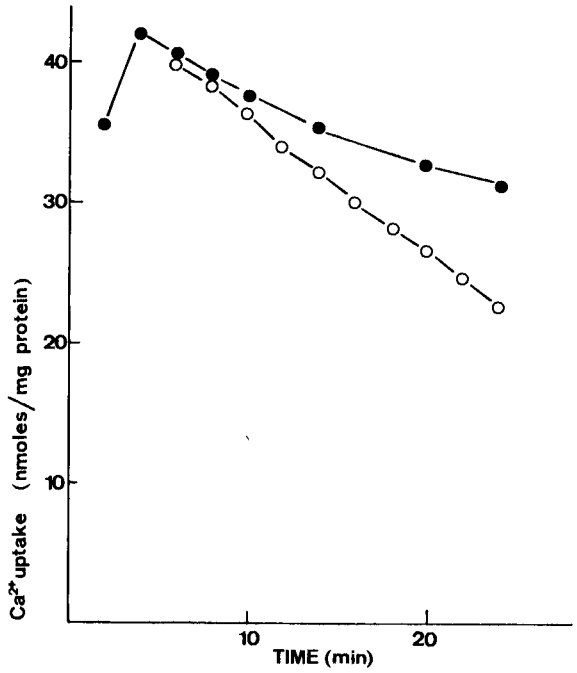

Fig. 8. Release of Ca^{2+} from rat liver mitochondria induced by phosphoenolpyruvate. Mitochondria were prepared by a conventional differential centrifugation procedure. The homogenization medium was 210 mM mannitol, 70 mM sucrose, 10 mM Tris-Cl, pH 7.4, and 100 µM Na-EDTA. The latter was omitted from the final washing. The incubation medium contained, in a final volume of 3.5 ml, stirred at 25°C, 200 mM sucrose, 20 mM HEPES, pH 7.2, 5 µg rotenone, 7.5 mM $MgCl_2$, 750 µM Tris-phosphate, pH 7.2,6 mM Tris-succinate, 210 nmoles Ca^{2+}, (added last) and 7 mg of mitochondrial protein. The movements of Ca^{2+} were followed with a Ca^{2+}-specific electrode (Ref. 40). ●—● 2.5 nmoles ruthenium red per mg of protein 6 min after the addition of Ca^{2+} o—o 2.5 nmoles ruthenium red per mg of protein, and 2.9 mM phosphoenolpyruvate 6 min after the addition of Ca^{2+}.

No other components of the mitochondrial membrane, indeed, have sufficient affinity for ruthenium red (Ref. 35), to compete with the unknown component which binds in the Ca^{2+} uptake system with a K_d of 1 µM or less (Refs 36 and 37). The possibility that the de-energization of the membrane promoted the dissociation of ruthenium red from its specific site of inhibition and its binding to other components elsewhere in the membrane, seemed remote. To rule it out conclusively, however, the experiment shown in Fig. 7 was carried out. Liver mitochondria were allowed to accumulate a pulse of cold Ca^{2+}. At the end of the uptake phase, a second pulse of Ca^{2+}, radioactive, and equivalent to the Ca^{2+} concentration already accumulated, was added. In the de-energized state (FCCP) radioactivity could obviously not become associated with mitochondria through energy-linked uptake, but only through the exchange of intra- and extramitochondrial Ca^{2+}. When ruthenium red was present, the amount of radioactivity associated with mitochondria dropped very considerably, offering compelling indications that a large portion of the exchange of Ca^{2+} occurred through the ruthenium red-sensitive carrier. Most importantly, the inhibitory effect of ruthenium red was not influenced by the presence of FCCP, showing that in the de-energized state the Ca^{2+} influx pathway was still blocked by ruthenium red. Therefore, it must be concluded that also in liver mitochondria (and presumably in the other Na^+-insensitive mitochondria) the influx and efflux of Ca^{2+} occur via 2 separate pathways, of which the second is insensitive to ruthenium red.

The problem is now that of identifying the release pathway in the Na^+-insensitive mitochondrial types. One physiological component which has been shown to promote the efflux of Ca^{2+} from liver mitochondria, is phosphoenolpyruvate (Refs. 38 and 39). Recent experiments (Refs. 40) have however shown that the effect of phosphoenolpyruvate is due to the collapse of the membrane potential across the mitochondrial membrane, and to the reversal of the electrophoretic uptake pathway. It is thus, (Figure 8) as expected, inhibited by ruthenium red, and has probably no physiological significance. More likely candidates as natural inducers of Ca^{2+} release from Na^+-insensitive mitochondria are perhaps endogenous fatty acids. A recent study of the release of Ca^{2+} from kidney mitochondria (Ref. 41) has offered indications that polyunsaturated fatty acids could become liberated within the inner mitochondrial membrane by phospholipases activated by the incoming Ca^{2+}, and play a role in its release.

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SOURCES OF ENERGY FOR MEMBRANE TRANSPORT IN YEASTS

A. Kotyk

Laboratory for Cell Membrane Transport, Institute of Microbiology, Czechoslovak Academy of Sciences, 142 20 Prague, Czechoslovakia

Abstract - Yeast cells are the simplest eukaryotic organisms that are of particular interest in the field of membrane transport and its energization. Primary active transport was demonstrated with certainty in the case of bivalent cations and, somewhat less conclusively, in the case of protons and possibly potassium ions. Secondary, coupled, transport utilizing a proton gradient may serve the transport of some anions, as well as of sugars and amino acids. However, quantitative considerations indicate the inadequacy of a simple proton-motive force being the major energizer of the transport. Even if the transport did draw its energy from the components of the proton-motive force a more intimate coupling within the membrane and/or a more direct requirement for ATP or polyphosphate would be necessary.

INTRODUCTION

Cell membranes are endowed with a host of biophysical, biochemical and physiological capabilities such that it is hardly an exaggeration to say that life - at least in the form we know it - could not exist without biomembranes. It is perhaps reasonable to assume that the earliest and primary function of membranes has been the separation of solutions of different composition which developed, over the two or three billion years of biological evolution, into a highly complex apparatus actively catalyzing the transport of some molecules and ions, passively permitting the passage of some others and completely barring the penetration of others still.

The last 10 - 15 years have witnessed a vigorous effort of molecular biology to unravel the mechanisms underlying the selective permeability of biological membranes and we are now in a position that we can at least sort out the various transport mechanisms on the basis of their characteristic properties. Most transport workers would perhaps agree on the following classification.

I. Simple diffusion
   1. through aqueous pores
   2. through lipid domains of the membrane

II. Carrier transport
   1. facilitated or mediated diffusion
   2. active transport
      a. primary
      b. secondary or coupled

III. Group translocation
   1. by chemical bond forming
   2. by chemical bond breaking

IV. Mechanisms involving a local rearrangement of membrane structure
   1. at the level of individual polymers
   2. at the level of supramolecular complexes

Only cases II.2, III.1 and, in a complicated way, IV, depend on a source of energy, either through direct coupling with an exergonic chemical reaction (II.2.a, III.1) or through tapping the electrochemical gradient of a cation (II.2.b). The molecular basis of the "active" mechanisms is what will probably remain in the focus of interest of biologists, biochemists and biophysicists in the near future. Rather than dwell on the accomplishments of biomembrane kinetics and energetics I will try to emphasize here those points which still await an adequate solution and I will do that by using the example of

transport across the yeast plasma membrane.

In many respects, yeast cells represent the ideal eukaryotic model for studies of biological processes on the molecular level. They are easy to grow under a variety of conditions; they are genetically flexible; they have a complex enough structure to permit comparisons with tissue cells of animals and plants; one can find among the species some "typical" as well as some "extreme" cases of environmental adaptation.

Primary active transport

A clearly established case of primary active transport in yeast is that of some cations, in particular of Mn^{2+}, Mg^{2+}, Ca^{2+}, etc., where an adenosinetriphosphatase is involved (1). However, the classical adenosinetriphosphatase transporting Na^+ and K^+ in animals and plants (2) is absent both in baker's yeast Saccharomyces cerevisiae and in an otherwise highly active species Rhodotorula glutinis (Rhodosporidium toruloides) (Ref. 3). Still, Na^+ and K^+ are unequally distributed at the expense of metabolic energy.

There is no definitive evidence for a proton-translocating adenosinetriphosphatase of the mitochondrial, chloroplast, and bacterial type. Still, protons are actively pumped out of cells, partly in exchange for K^+, partly without any such obligatory partnership, electrogenically. The proton extrusion may serve two purposes: 1. Removal of acid from the cell interior during glycolysis when the anaplerotic sequences involving glycerol and glyceric acid operate (Fig. 1A); 2. maintenance of a pH difference between the cell interior and the outside medium, potentially useful for driving coupled transport of ions and nonelectrolytes (Fig. 1B). The molecular nature of these pumping mechanisms is not yet known - either ATP or polyphosphate might serve as the source of energy, polyphosphate being in immediate equilibrium with ATP owing to catalysis of polyphosphate kinase and, indirectly, of a number of other enzymes (4).

There are reports about an adenosinetriphosphatase from Schizosaccharomyces pombe (5), as well as from Saccharomyces cerevisiae (6,7) that is ouabain, dicyclohexylcarbodiimide and oligomycin insensitive and that might be functional in the proton extrusion. However, if this is the case it would have to depend entirely on glycolytically produced ATP since the proton extrusion (in baker's yeast) is unaffected by respiratory mutations, defects in the ADP-ATP carrier of mitochondria or by anaerobiosis (Fig. 1A).

Secondary active transport

All important organic molecules (with the exception of monosaccharides in Saccharomyces), as well as anions (sulfate, phosphate) are transported actively, often against high concentration differences. The transport mechanisms for amino acids, vitamins, purines and pyrimidines, mono- and disaccharides are of many types: They are constitutive, repressible or inducible (8,9, 10), they work in both directions or inward only (11) and are subject to different types of regulation (12,13). Many of the transport systems were reported to be energized by the pH gradient and/or the membrane potential difference across the membrane (14,15,16). The evidence proferred in this context is either a transient alkalification of the outside medium or membrane depolarization as the presumably "coupled" organic compound was added to the suspension (Fig. 2). The only fairly direct proof was provided in the case of amino sugar transport in Rhodotorula glutinis (17).

I am going to examine this evidence by a quantitative approach and intend to demonstrate that the proton-motive force (combined from the pH difference and the membrane potential terms) is probably not the only driving force in these transports. Schematically, one can depict the coupled transport system as in Fig. 3. One can consider either the complete scheme or any one of the partial schemes enclosed in broken or dash-and-dot lines. Moreover, one can with some justification neglect the movement of either the CA or CS complexes across the membrane so as to obtain single-loop patterns that are easier to manipulate in a steady-state analysis. Such an analysis, or one that proceeds from the assumption of an equilibrium at each of the surfaces (between C, A, and S) will yield appropriate expressions for the half-saturation constant K_T, the maximum flux J_{max}, and the steady-state accumulation ratio ω, either without (18,19) or with (20) the role of the membrane potential included.

The steady-state ratio of a nonelectrolyte is an important parameter for estimating the role played by the proton gradient and the membrane potential difference in nonelectrolyte transport. If the proton gradient is truly the main driving force of a particular nonelectrolyte transport, the membrane po-

tential will also be involved since, by binding the proton, the carrier acquires an extra positive charge and will move preferentially toward the negative (i.e. inner) membrane face.

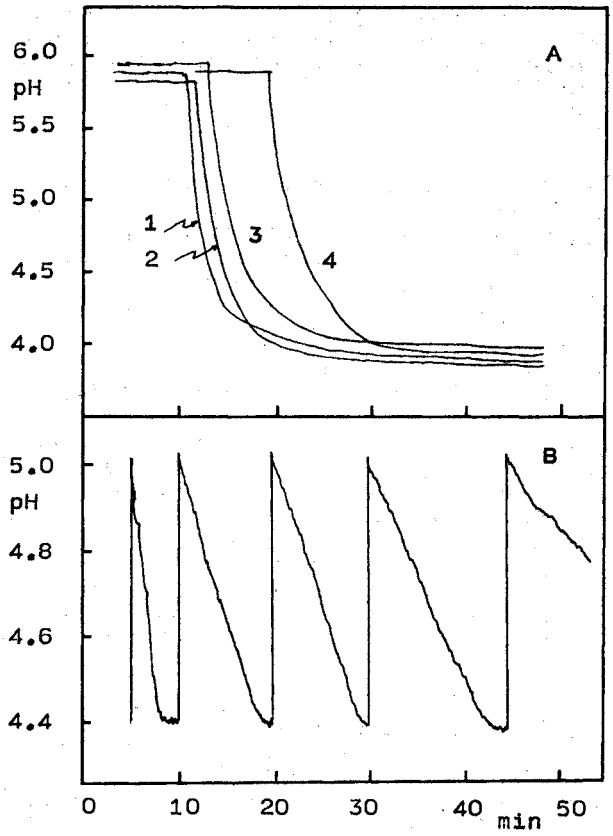

Fig. 1. A. The extracellular pH of Saccharomyces cerevisiae aerobically (1) and anaerobically (2), of a mutant defective in the mitochondrial ATP-ADP translocation (3) and of a respiration-deficient mutant (4). Suspension density was 8 mg dry wt. per ml, incubation took place in distilled water at 30 °C. Glucose to 50 mM concentration was added to elicit the pH drop.
B. The extracellular pH of Rhodotorula glutinis (approximately 10 mg dry wt./ml), incubation in distilled water at 30 °C, aerobically. Potassium hydroxide (1 µmol) was added to 8 ml of suspension at 5, 10, 20, 30 and 45 min. The half-times of pH return to the original value of pH 4.4 were 0.9, 2.5, 3.7, 6, and 15 min, respectively.

Ideally, the energy available from the combination of the two factors is

$$\Delta G = F\Delta\psi - 2.3RT\Delta pH \qquad (1a)$$

and the driving force p.m.f. in millivolts at 30 °C is simply

$$p.m.f. = \Delta\psi - 60\Delta pH \qquad (1b)$$

The maximum accumulation ratio of the proton-driven nonelectrolyte ($\omega = s_{II}/s_I$) cannot exceed the value given, for a neutral substrate, by (cf. Ref. 21)

$$2.3RT\log\omega = -F\Delta\psi + 2.3RT\log(H_o^+/H_i^+) \qquad (1c)$$

However, for an actual carrier system with different affinities for substrate and ion, with different mobilities of the various carrier forms, one arrives at somewhat more complicated formulae for the accumulation ratio.

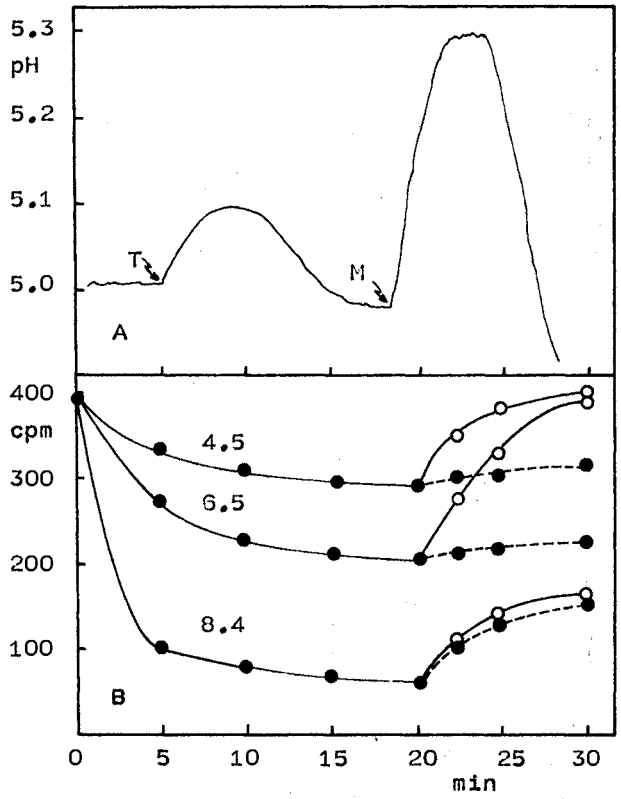

Fig. 2. A. Extracellular pH of a suspension of Saccharomyces cerevisiae, aerobically at 30 °C (approximate dry wt. 7 mg/ ml), in the presence of 20 μM antimycin. 20 mM trehalose was added at T, 20 mM maltose at M.

B. Radioactivity (in counts/min) of a Rhodotorula glutinis suspension supernatant after adding (at time zero) 20 μM labelled tetraphenylphosphonium chloride (TPP^+). Incubation was done at 30 °C, aerobically, in 0.3 M Tris-citrate buffers of the pH shown at curves. After 20 min, either 10 μM carbonyl cyanide m-chlorophenylhydrazone (open circles) or 1 mM 6-deoxy-D-glucose (black circles) was added. The apparent values of membrane potential calculated from the distribution of TPP^+ at 20 min were 66 mV, 86 mV, and 120 mV, respectively.

For the (C + A + S) system we have

$$\omega = \xi^{1/2}(A_I/A_{II})(aK_1\xi^{1/2} + bA_{II})/(aK_1 + b\xi^{1/2}A_I) \tag{2a}$$

and, if K_1 is very large (or CA is immobile)

$$\omega = \xi A_I/A_{II} \tag{2b}$$

For the (C + S + A) system then

$$\omega = (dK_4 + e\xi^{1/2}A_I)/(dK_4 + e\xi^{-1/2}A_{II}) \tag{3a}$$

and, if K_4 is very small or the CS is immobile,

$$\omega = \xi A_I/A_{II} \tag{3b}$$

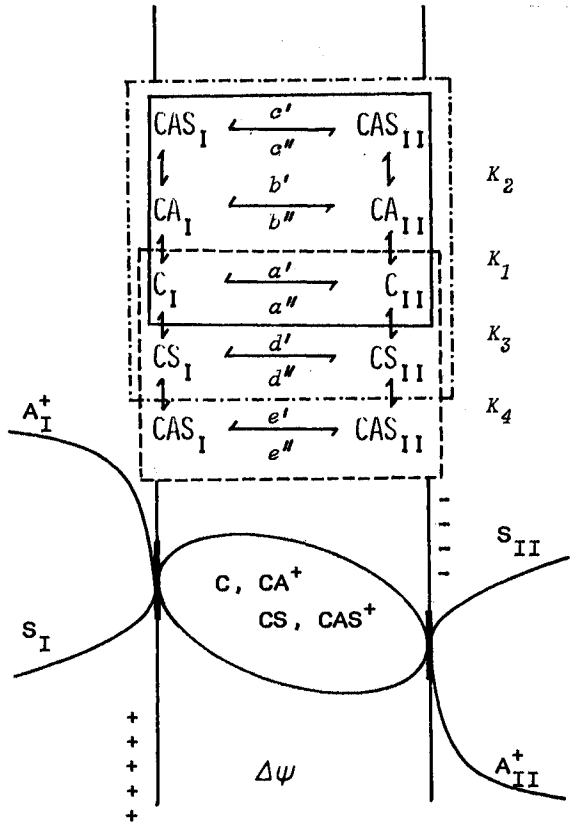

Fig. 3. Schematic representation of carrier transport of a non-electrolyte S and an activating ion A, proceeding from the equilibrium assumption such that the dissociation constants K_1 - K_4 can be ascribed to the individual steps. The hypothetical concentration profiles of S and A at end of incubation are shown below, together with the postulated orientation of the membrane potential.

For the two systems combined (C + S , as well as C + A + S) then

$$\omega = (K_1K_2d + K_3A_Ic\xi^{1/2})(K_1a\xi^{1/2} + A_{II}b)/(K_1K_2d\xi^{1/2} + K_3A_{II}c)(K_1a + A_Ib\xi^{1/2}) \quad (4a)$$

and, if either CAS formation or CS formation is strongly preferred,

$$\omega = 1 \quad (4b)$$

In all the above equations, $\xi = \exp(-F\Delta\psi/RT)$.

The intracellular and the outside H^+ concentrations (A_{II} and A_I, respectively) can be measured from the distribution of a weak acid (e.g., bromophenol blue) (Ref. 22), the $\Delta\psi$ can be assessed from the distribution of a lipophilic cation (e.g., tetraphenylphosphonium ion) (Ref. 23) using the Nernst equation. For the purposes of calculation, values of K_1 - K_4 as well as of a - d may be varied within reasonable limits (10^{-7} - 1^1M for the K's; 1 - 100 s^{-1} for a - d). If the computed values of ω are plotted against A_I (= -antilog pH_o) a family of concave curves are obtained (Fig. 4). Only for a situation where $\omega << 1$ can occasionally a convex curve be obtained. The trend of these curves of course reflects the electrochemical potential of the proton difference across the membrane.

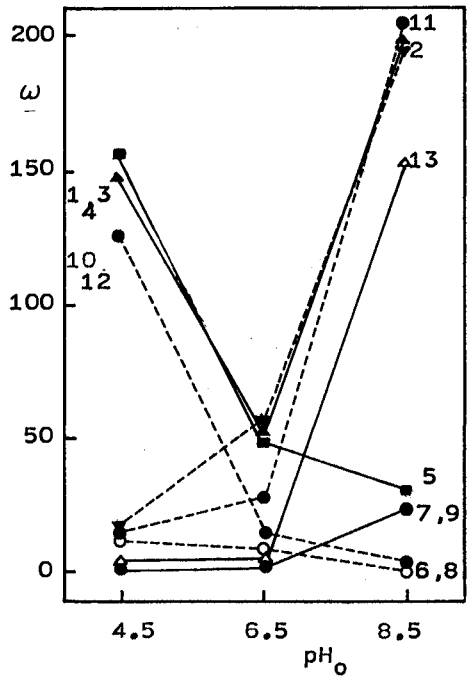

Fig. 4. The accumulation ratio ω plotted against extracellular pH, based on experimental values of the membrane potential and the intracellular pH in Rhodotorula glutinis, and on different values of translocation constants a - d, and dissociation constants K_1 - K_4, according to equation (2a) for curves 1 - 4, (3a) for curve 5 and (4a) for curves 6 - 13. The values used for the curves (a - d in s^{-1}, K_1 - K_4 in mol/liter) were as follows. Curve 1: a = 100, b = 1, $K_1 = 10^{-3}$; curve 2: a = 1, b = 100, $K_1 = 10^{-3}$; curve 3: $a = b$ = 100, K_1 = 1; curve 4: a = 100, b = 1, K_1 = 1; curve 5: d = 1, e = 100, $K_4 = 10^{-7}$; curve 6: a = 100, $b = c = d$ = 1, $K_1 = 10^{-7}$, $K_2 = K_3$ = 1; curve 7: $a = b = d$ = 1, c = 100, $K_1 = 10^{-7}$, $K_2 = K_3$ = 1; curve 8: a = 100, $b = c = d$ = 1, $K_1 = K_2 = K_3 = 10^{-7}$; curve 9: $a = b = d$ = 1, c = 100, $K_1 = K_2 = K_3 = 10^{-7}$; curve 10: $a = c = d$ = 1, b = 100, $K_1 = K_3$ = 1, $K_2 = 10^{-7}$; curve 11: a = 100, $b = c = d$ = 1, $K_1 = K_2 = 10^{-7}$, K_3 = 1; curve 12: $a = b = c = d$ = 100, $K_1 = K_3$ = 1, $K_2 = 10^{-7}$; curve 13: $a = b = c = d$ = 100, $K_1 = K_2 = 10^{-7}$, K_3 = 1.

However, a typical pH dependence of the accumulation ratio of either a mono- or a disaccharide is convex, resembling the enzyme activity dependence on pH generally found with both soluble and membrane-bound enzymes. The theory underlying the occurrence of the optimum is that the enzyme (or carrier) can function only if it is partly protonated, being inactive either in the fully protonated or in the nonprotonated form. An example is shown in Fig. 5. Similar dependences were found in Kaback's laboratory (24) and by Anraku's group (25). However, even if the carrier functioned only in the half-protonated form this would affect the rate of coupled uptake in a way similar to that shown for ω in Fig. 5 but not the accumulation ratio. Since this is identical with the equilibrium constant of a reaction it should not be affected by the mechanism of the reaction.

One is thus forced to modify the recently advanced explanations of a proton symport in yeast. To account for the effect of H^+-ATPase inhibitors dicyclohexylcarbodiimide or Dio-9 on transport of nonelectrolytes one might envisage a proton-extruding system in the membrane next to the nonelectrolyte transport in such a way that protons would be used for driving the transport directly as they are released by the ATPase while the extracellular pH is of no direct importance for the uptake system (somewhat along the lines suggested by Williams, Ref. 26).

There are, to be sure, a number of data militating against a unique role of the proton gradient in nonelectrolyte transport in yeasts.

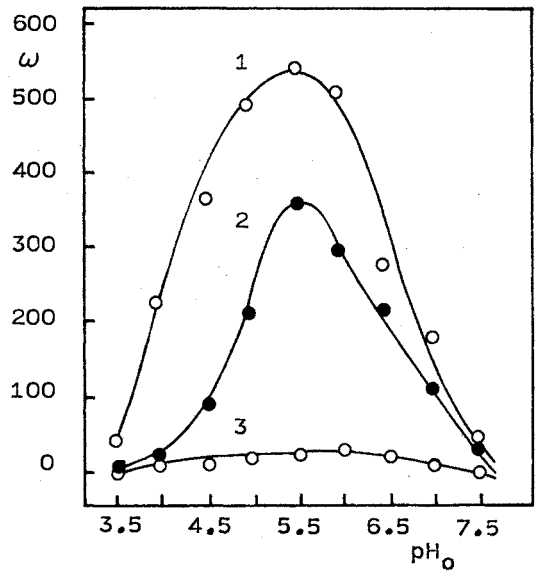

Fig. 5. Dependence of the final accumulation ratio of 6-deoxy-D-glucose in Rhodotorula glutinis on extracellular pH, aerobically at 30 °C. The initial concentrations of 6-deoxy-D-glucose were 0.8 mM (curve 1), 4 mM (curve 2), and 20 mM (curve 3). The suspension density was 7.5 mg dry wt./ml.

1. The amount of insoluble polyphosphate synthesized during preincubation of Saccharomyces cerevisiae with different substrates tallies nicely with the increase of amino acid transport brought about by the preincubation (Fig. 6) (Ref. 27). The same now appears to hold for the uptake of some disaccharides, as well as the phosphate and possibly sulfate anions.

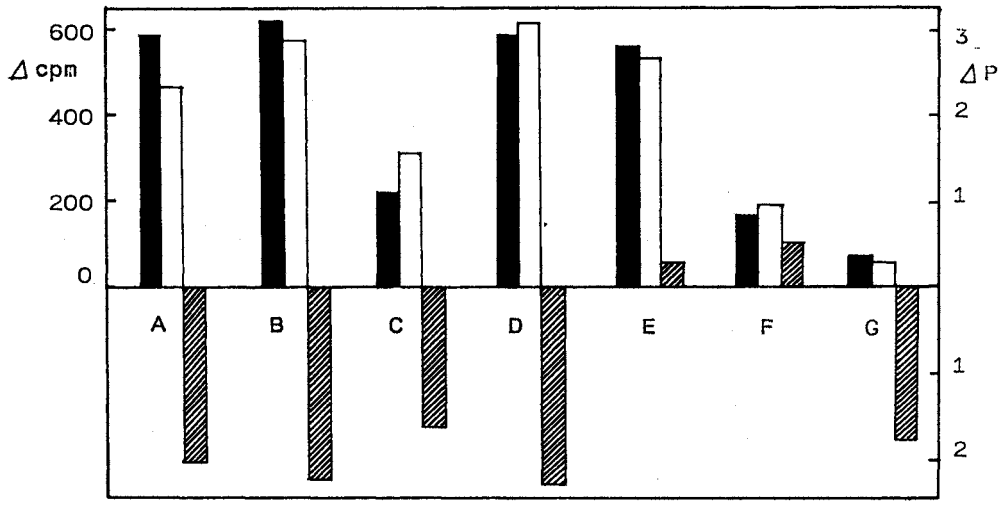

Fig. 6. Comparison of the rate of uptake of 1 mM glycine (solid columns) with the content of acid-extractable high-energy phosphate related to ATP (hatched columns) and of high-molecular weight polyphosphate (void columns). Cells were preincubated for 2 h in the presence of 1% D-glucose (A), D-fructose (B), D-galactose (C), sucrose (D), maltose (E), ethanol (F) and sodium acetate (G). After washing they were resuspended in labelled glycine and incubated aerobically at 30 °C. The difference between a water-preincubated suspension and the substrate-preincubated one is shown. For glycine uptake, it is expressed in counts/min per mg dry wt., for the phosphate fractions in μg P per mg dry wt.

2. Respiration-deficient and ATP-ADP-transportless mutants of Saccharomyces cerevisiae are unable to transport amino acids in the normal way although the pH difference as well as the presumably electrogenic H^+ transport operate normally. Likewise, under anaerobic conditions in baker's yeast, amino acid transport is much reduced.

3. In contrast, in the strictly aerobic Rhodotorula glutinis, anaerobic conditions depolarize the membrane readily while sugar uptake proceeds normally until the very last traces of oxygen (below 1 p.p.m.) are removed, in a convex pH dependence, similar to that in Fig. 5.

4. The very fact that amino acids are transported practically exclusively inward speaks against the operation of a simple proton-driven symport.

5. In Saccharomyces cerevisiae K^+ ions, even more than Na^+ ions, stimulate the uptake of amino acids which is difficult to reconcile with the notion that the exchange of H^+ for K^+ is one of the principal mechanisms of transport of these ions.

6. The observations of alkalification of the outside medium on adding a nonelectrolyte are difficult to reproduce in nonstarved cells and at larger substrate concentrations. The explanation that in energy-rich cells the nonelectrolyte-induced uptake of protons is compensated by the proton-extruding pump is untenable. If a suspension is fed with an alkaline solution at a rate that would correspond to merely one-tenth of the rate of disappearance of protons due to a nonelectrolyte symport (at a 1:1 stoichiometry) there is a pronounced alkalification of the outside medium.

The question may be raised why there is an occasional alkalification observed in the outside medium on addition of transportable substrate and why the membrane is depolarized. One possible explanation would be that the nonelectrolyte transport system draws its energy from the same source as the outward (electrogenic) proton pump. If the availability of this energy source is limited (cf. Ref. 28) such that the total amount P_t at any given moment is equal to $P + PA + PB$, where PB is the energized transport complex of the nonelectrolyte system, PA the same of the proton pump, it can be easily shown that, in this particular case, the outside pH will increase as B is added to the system.

It is unfortunate that at present no isolated active transport systems from yeast are available (although presumably a maltose-binding protein was isolated (Ref. 29) and an arginine-binding protein - a part of the whole system - is readily obtainable from baker's yeast (Ref, 30)) and that work with membrane vesicles is by no means as simple as that with bacterial vesicles. There (31) and with the glucose-Na^+ system of the intestine (32) unequivocal evidence was provided in vitro that an ion symport is involved in sugar uptake. Until this is possible with yeast the evidence for proton-driven symports must be considered as unsatisfactory.

One can only speculate what source of energy could replace the proton gradient in yeast. In bacteria, the noncoupled transports of amino acids utilize ATP or a related compound (33) just as maltose does (34). In yeast, polyphosphate may equally well be involved (4,27).

Group translocation

Now that phosphorylation of sugars during transport in baker's yeast does not appear probable (35,36) there is only one case where a phosphorylative group translocation might operate, that of 2-deoxy-D-glucose in Saccharomyces fragilis (37). However, the source of the phosphate group is still unknown.

To summarize, the problems to which answers should be provided in the nearest future are the molecular nature (1) of the H^+-extruding pump and (2) of the nonelectrolyte transports that are not coupled to a proton gradient. If and when the answers are provided by working with yeast one can more reliably extrapolate them to the highly organized cells and tissues than would be possible when proceeding from work on prokaryotic organisms.

Acknowledgment - I would like to thank my colleagues D. Michaljaničová, L. Říhová and A. Knotková for technical assistance and my postgraduate student V. Vacata for calculations of the theoretical dependences of accumulation ratios on external pH.

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LATERAL MOLECULAR MOTIONS IN MEMBRANES

H. M. McConnell

Department of Chemistry, Stanford University, Stanford, California 94305, USA

Abstract - Lateral molecular motions of membrane components are important for the biological functions of cells. In this paper we cite briefly earlier physical techniques that have been used to study these motions, both in cell membranes, and in reconstituted (model) membranes. We then describe more recent optical and magnetic resonance methods that are currently being used for such studies. Finally, the relationship of molecular motion in membranes to ongoing research in cell surface immunochemistry is discussed, in terms of a single, simple example, the binding of antibodies to hapten-sensitized model membranes

INTRODUCTION

The lateral molecular motion of the components of biological membranes is of great interest in connection with cell structure and function. Membrane-associated functions that appear to have a direct dependence on the lateral motions of membrane components include complement activation (1-4), mast cell degranulation (5-7), and the response to hormones such as insulin (8). At present the lateral movements of a variety of proteins and lipids in intact cells have been observed and studied. For a recent review, see Elson and Schlessinger (9). Some molecular motions, particularly motions of lipids, appear to arise simply from thermal diffusion. Other motions, especially those of some intrinsic membrane proteins, are sometimes slower, and sometimes faster than that expected on the basis of thermal diffusion (9, 10). Much of the research in our laboratory at Stanford University is related to the use of reconstituted membranes ("model membranes") to investigate afferent and efferent responses of the immune system. In other words, the reconstituted membranes are used as "targets," both to stimulate an immune response, and to be subject to attack by this response. We believe that it is essential that such target membranes be characterized as quantitatively as possible, both with respect to chemical composition and physical properties, in order to understand their interactions with the components of the immune system. The present paper describes some of our work in this area. For the sake of brevity, the present paper will be restricted to the lateral motions of phospholipids since at present these motions are better understood than those of the proteins.

LATERAL DIFFUSION IN RECONSTITUTED MEMBRANES

The first studies of the lateral diffusion of phospholipids in lipid bilayer membranes were made by Kornberg and McConnell (11), Träuble and Sackmann (12), and Devaux and McConnell (13), using magnetic resonance methods (nuclear resonance of phospholipids in the presence of spin-label phospholipids), and spin-label paramagnetic resonance. More recently such studies have been carried out using nuclear magnetic resonance methods (14), fluorescence photobleaching (15, 16), and spin-label photochemical methods (17). Again, for the sake of brevity, we only discuss the latter two methods.

Smith and McConnell have recently described a technique of periodic pattern photobleaching that can be used to measure the lateral diffusion of fluorescent-labeled phospholipids in lipid bilayer membranes (16). The technique is illustrated schematically in Figure 1.

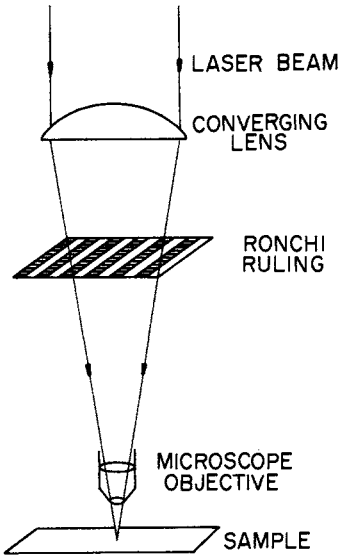
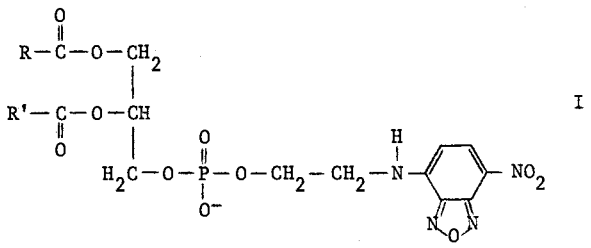


Fig. 1. Diagram of optics used for pattern photobleaching. Light from an argon-ion laser is directed through a Ronchi ruling into the microscope objective which focuses an image of the ruling onto the sample. Details of the microscope optical system have been omitted. Taken from B. Smith and H. M. McConnell (16).

In this example, the light from an argon-ion laser is passed in reverse direction through a microscope, and through a Ronchi ruling onto a sample under the objective of the microscope. The sample is composed of phospholipid multilayers containing a ca. 0.1-1.0 mol % concentration of a fluorescent phospholipid probe such as



I

The fatty acid chains R and R' are derived from egg phosphatidylethanolamine (16). These fluorescent molecules can be photobleached in a striped pattern, such as that illustrated in Figure 2. The time-dependent intensity of these stripes can be recorded photographically,

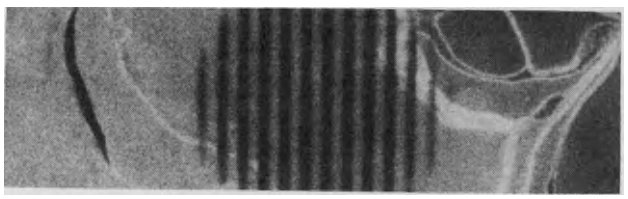


Fig. 2. Fluorescence photomicrograph of a multibilayer sample (dimyristoylphosphatidylcholine doped with NBD-PE) immediately after pattern photobleaching. The period of the striped pattern is 13 μm. Defects in the macroscopic ordering of the sample, which exhibit birefringence when viewed between crossed polarizers, appear as bright lines when viewed by fluorescence. The striped pattern has been placed in a region free of such defects. Taken from B. Smith and H. M. McConnell (16).

and densitometer tracings can then be used to determine the diffusion constants of the fluorescent-labeled phospholipids in coplanar parallel arrays of the lipid bilayers. Data on the lateral diffusion of I in the monoclinic (P_{β}') phase of dimyristoylphosphatidylcholine is given in Figure 3.

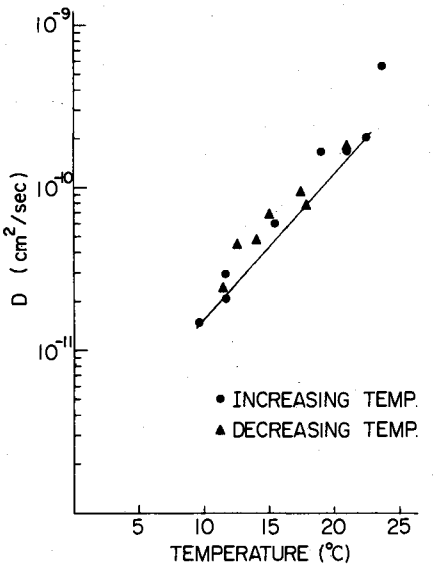


Fig. 3. Diffusion coefficient of NBD-PE in dimyristoylphosphatidylcholine multibilayer samples as a function of temperature. No hysteresis was detected in the diffusion coefficients in the temperature range 9.6-23.7°. The strong dependence of the diffusion coefficient on temperature illustrated by the straight line corresponds to an apparent "activation energy" for diffusion of 36 kcal/mole. Note that the largest diffusion coefficient at 23.7° is in the region of the chain melting phase transition and the diffusion coefficients 1 or 2 degrees higher are $>10^{-8}$ cm^2/sec. ●, increasing temperature; ▲, decreasing temperature. See text. Taken from B. Smith and H. M. McConnell (16).

A second new technique used to measure the lateral diffusion of phospholipid probes in lipid bilayer membranes is illustrated in Figure 4 (17). Light from an argon-ion laser is

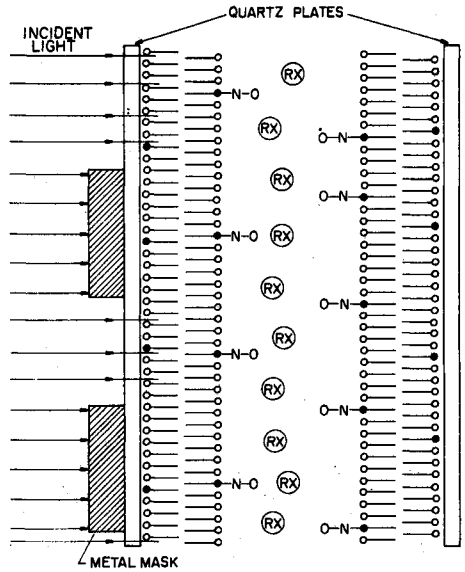
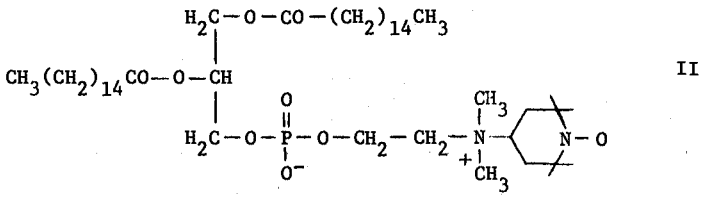


Fig. 4. Schematic diagram of experiment by J. Sheats. -N-O represents the spin label II; RX represents the alkylcobalt complex $[Co(CN)_5CH_2CO_2^-]^{-4}$. Taken from J. Sheats and H. M. McConnell (17).

incident on a phospholipid multilayer sample contained between two optically flat quartz plates having a metal mask in front. The aqueous region between each pair of lipid bilayers contains a photosensitive compound RX that, when activated by light, produces radicals R·. These radicals react rapidly with the nitroxide groups of spin-labeled phospholipids incorporated into the lipid bilayers, such as



II

However, the lifetime of the photoradicals R· is so short that they destroy only the nitroxide radicals that are also in the same illuminated area. Thus, an initial burst of laser radiation destroys exactly 50% of the radicals, if the mask protects 50% of the sample from the radiation. The number of radicals destroyed by a second or third burst of radiation then depends on the grid spacing, and the rate of lateral diffusion of the spin-labeled phospholipids. From measurements of the paramagnetic resonance intensity at appropriate times one can then determine the lateral diffusion constants of spin labels such as II. Lateral diffusion constants of II in the fluid (L_α) phase of dimyristoylphosphatidylcholine are given in Figure 5. Agreement between diffusion constants obtained with the two

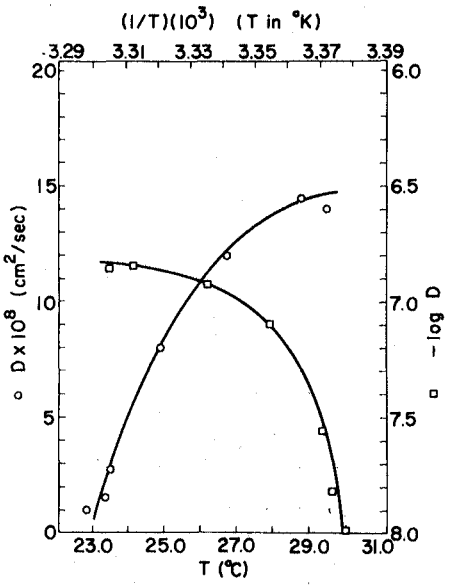


Fig. 5. Preliminary diffusion constants obtained by J. Sheats for the spin label II in multilayers of dimyristoylphosphatidylcholine.

methods is good, when samples are studied under the same conditions (temperature, degree of hydration, etc.).

Extensive studies are in progress in our laboratory to measure lateral diffusion of probes in defined mixtures of phospholipids, since such studies appear promising for determining thermodynamic phase boundaries, and molecular ordering.

MEMBRANE IMMUNOCHEMISTRY

As mentioned in the Introduction, we have been very much interested in the preparation of reconstituted membranes to serve as "targets" for various components of the immune system (1-3, 18-22). As also mentioned in the Introduction, we believe that quantitative chemical and physical characterization of the target membranes is essential for obtaining an understanding of this immunochemistry. To illustrate this point, consider a seemingly very simple reaction, the binding of specific anti-nitroxide rabbit IgG antibodies to lipid membranes

containing 0.5 mol % spin label II in binary mixtures of dipalmitoylphosphatidylcholine and cholesterol. Figure 6 contains data obtained by Brûlet and McConnell (3) showing a sharp increase in antibody binding at cholesterol concentrations above 20 mol %. Also displayed are data of Schwartz and McConnell (23) showing that cholesterol has no effect in enhancing the number of exposed spin-label haptens II in these membranes (multilamellar liposomes).

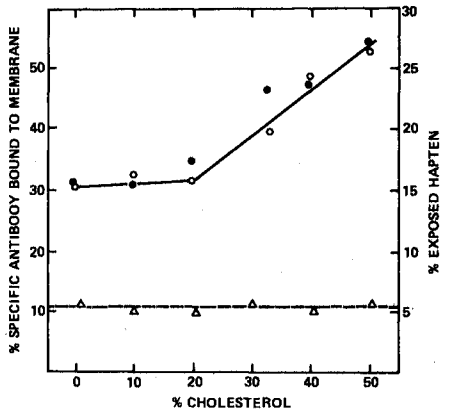


Fig. 6. The solid line represent binding of specific IgG to DPPC-cholesterol membranes containing 0.5% spin label II, taken from Brûlet and McConnell (3). Points O give IgG binding and points ● give corresponding F_{ab} binding. The broken line represents the percent of hapten exposed on the outer surface for similarly prepared membranes. Taken from M. Schwartz and H. M. McConnell (23).

What is then responsible for this enhancement of antibody binding as a function of cholesterol concentration? An important clue is provided by the data of Brûlet and McConnell (2) which show that the binding of specific anti-nitroxide antibodies to spin-label haptens depends strongly on the detailed molecular structure of the haptenic group, i.e., if the "distance" between the NO group of the nitroxide and the phosphorous atom of the phosphodiester group of the phospholipid is increased by three to six atoms, the binding of a given population of specific antibodies to these membranes can be increased by 20% to 100%. This clearly indicates a high sensitivity of antibody binding to local molecular structure.

Many different experiments, some involving spin-label paramagnetic resonance spectra (24), nuclear magnetic resonance spectra (25), and calorimetry (26, 27) have indicated that physical properties of binary mixtures of cholesterol and dipalmitoylphosphatidylcholine do change significantly around 20 mol %. None of these data provide a clear understanding of the enhancement of antibody binding for cholesterol concentrations above 20 mol %, although some of the data are consistent with an enhancement of headgroup motion for this concentration range.

In recent unpublished work, Rubenstein, Smith and the present author have observed a remarkable enhancement in the rate of lateral diffusion of fluorescent probe I for cholesterol concentrations equal to and above 20 mol % (80% dipalmitoylphosphatidylcholine) at 18°. The significance of this enhanced diffusion may simply be a loosening up of the molecular structure, a loosening that also facilitates the accomodation of antibody combining sites to membrane-associated haptens. This may well be a significant or perhaps even major contribution to the extensively discussed issue of why "fluid" target membranes are more effective than "solid" in complement activation and depletion.

Current work in this laboratory includes a number of studies of the interactions of cellular components of the immune system with reconstituted membranes (19). Although these systems are far more complex than the problem of antibody binding described in the present paper, our present discussion does clearly point out the importance of a quantitative understanding of the chemical and physical properties of target membranes.

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APPEARANCE OF PHOTOINDUCED POTENTIAL ON PHOTORECEPTOR MEMBRANE

V. I. Bolshakov, G. R. Kalamkarov and M. A. Ostrovsky

Institute of Chemical Physics, Academy of Sciences of the USSR, Moscow 117334, USSR

Abstract - A photopotential on the photoreceptor cell disc membrane was discovered by two independent methods. One of them was the penetrating ion probes. The artificial lipid membrane was used as a sensitive electrode in this case. It was chosen phenyldicarboundecaborane (PCB^-) as a penetrating anion. The other one was the method of direct measurement of the electric potential on a closed membrane fragment associated with the planar membrane. There was an adhesion of photoreceptor discs or the sonicated membrane fragments to planar membrane by means of the Calcium ions. All experiments were carried out with the fraction of the disks of the cattle rod outer segments. This fraction was sonicated in some cases.
An illumination induced the considerable electric potential difference across the photoreceptor disc membrane in both cases.

INTRODUCTION

At least two coupling stages may be suggested in the photoreceptor mechanism. The first coupling stage is between the visual pigment photolysis and the functional changes in the disc photoreceptor membrane and the second one is a coupling between the light-dependent events in the disc and in the plasma membrane of the photoreceptor cell. The coupling of the rhodopsin molecule photoactivation to control over ionic and enzymic reaction in the disc photoreceptor membrane is one of the most important questions of the visual reception problem.

The light-induced alterations of the photoreceptor membrane electric properties should play the key role in this coupling mechanism.

Some data about the photoinduced conductance alterations of the artificial lipid membrane (ALM) modified by the photoreceptor membrane fragments were obtained (1,2). Recently, the photoinduced potential in the photoreceptor membrane was registrated in our laboratory by two independent methods. One of them was the penetrating ion probes (3) and the other one was the measuring of the photopotential by a conventional electrometer technique.

It's impossible to register the potential alteration from the single photoreceptor disc by the usual microelectrode technique because of the small size of it.

USING OF PENETRATING ION PROBES

The sonicated and unsonicated photoreceptor discs suspension was investigated. There was shown in the special experiments that the fraction obtained by the lysis of rod outer segments in the distillated water, and the fraction obtained by the sonication of outer segments had closed osmotically active membrane structures.

All experiments were made with the pure fraction of the photoreceptor membranes. The fraction purity was estimated by the value of the optic absorption A_{278}/A_{500} which was 2,1-2,3. Only the fresh suspension was used in the experiments. The suspension of the cattle retina rod outer segments was isolated by the method described in the work (4). The additional procedures of the discs fraction obtaining were described earlier (3).

The method of the penetrating anions had been successfully used to discover the membrane potential alteration in mitochondria, photophosphorylating bacterias and their fragments (5).

The membrane was formed on a bore of 2 mm in a partition deviding the Teflon cell. The mixing of the solution was carried out by a magnetic mixer. The Teflon cell was placed into a thermostatic house serving also as an additional electrostatic screen. A potential difference across ALM was measured by an electrometer ITN-6 and KSP-4 recorder.

The illumination was realized by the visible light with a thermofilter. The switching of the light was carried out by a photoshutter (opening time-10 msec). ALM was formed from the cattle brain lipid dissolved in octane; the lipid concentration was 15-20 mg/ml. We chose phenyldicarbaundecaborane anion (PCB^-) as a penetrating anion. It had been demonstrated (6) that the diffusion potential of this anion was the same as Nernst-potential in a wide concentration range. The value of the diffusion potential was measured in every experiment after the adding of PCB^- into the cell. In the presence of the penetrating ions the characteristic value of the ALM resistance was 10^9 ohms, i.e. an order lower than in their absence.

In the darkness the diffusion potential appears on ALM (75-80 mV) after the adding of the fraction of the discs (F.D.) into a compartment of the cell containing the penetrating ions. It is a result of the ions absorption by the photoreceptor membrane lipid matrix. The appearance of the diffusion potential of a definite value is the additional test for the ALM sensitivity to the penetrating ions.

The illumination of the cell by the visible light (λ = 400-600 nm) results in the light-induced potential generation on ALM, as shown in Fig. 1.

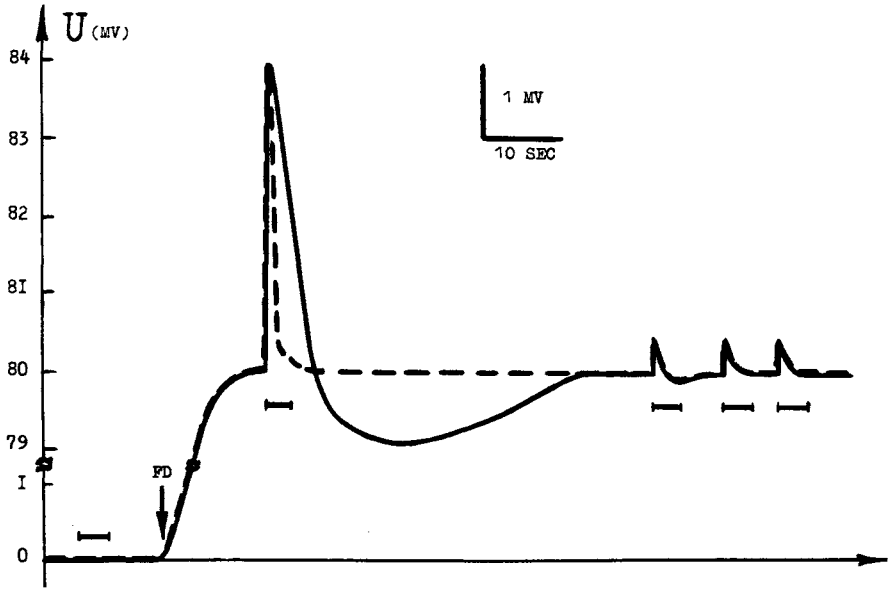

Fig. 1. There are shown photoelectric responses of ALM separating two compartments containing a solution of 100 mM NaCl, 250 mM sucrose, 30 mM Tris-HCl (pH=7.2), $1x10^{-7}$ M PCB^- (————); the photoresponses of ALM with protonophorous uncoupler-CCCP with $5x10^{-7}$ M concentration (- - - - --); the fraction of discs (F.D.) was added in one of the compartments of the cell with the concentration of rhodopsin 0,15 mg/ml (↓). The switching of the visible light was realized by a photoshutter with 10 msec opening time (⟷).

This effect is due to the shift of the ions concentration in the compartment of the cell containing F.D. The marked effect is caused by the ionic concentration shift exactly in the volume of the cell but not by a photoreaction of ALM on which the FD fragments are probably absorbed.

Indeed, on one hand, the value of the effect shown by special tests did not depend on the ALM resistance as well as an applied voltage. On the other hand, if the mixing of the solution in the cell was not provided the value of the photopotential fell considerably. Moreover, when there were no penetrating ions in the cell the ALM photoreaction was not observed.

One should pay attention to the fact that the photoresponse has a bicomponent character. The first, relatively fast component has the characteristic time of approximately 1 sec. The second one is slow (with the characteristic time about 10 sec). All subsequent switchings of the light result in the generation of the considerably less signals (the second-type effects).

The common form of the photoresponse both in the sonicated and unsonicated FD are the same. In our conditions of the sonication (frequency - 22 kHz; the value of an output current - 0,3 a) the process of the sonication results in the formation of the inturned closed fragments of the photoreceptor membrane.

The intensive raising of the membrane proton conductance by a protonophorous uncoupler (e.g. trichlorcarbonylcyanide phenylhydrazone - CCCP) alters essentially the characteristic time and the general form of the photoresponse (Fig. 1). You can see on the picture the slow component practically disappears in the presence of the uncoupler. It is likely that the cause of the form signal alteration is the decreasing of the photoreceptor disc membrane time constant. We used a concentration of the protonophorous which did not result in the diffusion ptential changing on ALM.

The same our results were obtained with the other penetrating anion - tetraphenylborate.

One could suggest that the light absorption by rhodopsin and it's photolysis result in a generation of the electric potential with a positive charge sign on the internal side of the disc membrane.

But the method of the penetrating ions has some restrictions. The time permission of the this method is limited by the diffusion time of the anion in the cell. This method doesn't allow to except the possibility of the anions redistribution in a membrane lipid matrix.

DIRECT MEASUREMENT OF ELECTRIC POTENTIAL

Recently an attempt to choose the condition of the discs or the closed vesicles adhesion to the planar lipid membrane and to measure the electric potential by a conventional electrometer technique was made. Such approach to study an electric potential in the different coupling membranes had been developed and employed in the work (7). This method is assumed to be the most direct way to register an electric activity of the closed membrane fragments.

As a planar membrane we used a porous filter saturated by the phospholipids solution where azolectin was dissolved in decane. The lipid concentration was 100 mg/ml.

The membrane filter blocking un a hole with 4-10 mm diameter was clamped between two Teflon cells (volume - 1,5 ml). The potential difference was measured by means of the chlor-silver electrodes. The registration technique was just the same as in the penetrating ions experiments. After the addition of the photoreceptor discs or sonicated vesicules suspension into one of the compartments of the cell the solution was mixed intensively by a magnetic mixer for 2-3 hours. The adhesion of discs was achieved by a presence of Calcium ions in the solution. The association of the discs with a planar membrane was found to be irreversible, for the photoresponse could be also registered when the disc-containing incubation mixture was substituted by a solution without photoreceptor discs after 2-3 hours of incubation.

The illumination of the membrane system by the visible light resulted in the generation of the photoresponse on the planar membrane, as shown in Fig. 2. We had no photoresponse on the following flashes if the whole rhodopsin was bleached in the cell after the first illumination. The illumination of the cell without disc suspension did not result in any observed potential. The photoresponse depends essentially on the choose of the filters and of the porous diameters. We have registered the photoresponse using the filters: Sortorius SM13306 (0.45 μm); Schleicher and Schüll BA 85

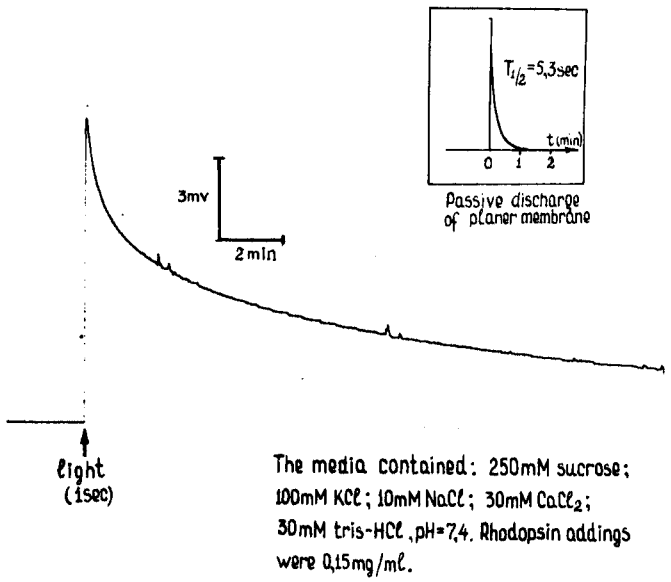

Fig. 2. There is a photoelectric response of the planar membrane associated with the photoreceptor discs. It was used Synpor filter (diameter or the porous: 0,6-0,9 μm). The switching of the visible light was realized by a photoshutter (↑).

(0,45 μm); Synpore (0,6-0,9 μm) (diameter of the porous is given in the brackets).

As shown in Fig. 2 the photoinduced potential about 15 mv can de registered. There are a fast component with the characteristic time about 1 sec and at least two slow components with the characteristic times about 10 sec and 100 sec. A sign and an amplitude of the photopotential don't practically depend on whether we deal with sonicated or unsonicated fraction of the photoreceptor discs. One should pay attention to the curve of a passive discharge of the planar membrane. Apparently the complicated form of the photosignal can't be accounted for this discharge.

In Fig. 3. there is given an equivalent electric scheme suggested in the work (8). In accordance with equivalent scheme a sign of electric potential on the internal side of the closed membrane fragments coincides with a charge sign on the opposite free side of the planar membrane.

It should be point out that in a contrast to the method of penetrating ions the time parameters of a registered photoresponse within the bounderies of an equivalent scheme correspond to the actual time of the processes on the photoreceptor disc membrane. The method of a direct registration of the electric potential permits to investigate the electric reaction in the photoreceptor membrane with the characteristic time less than one second. But we didn't consider the rapid reaction in this paper.

A comparison of the photoresponses registered by two independent methods makes it obvious that the first rapid component has the similar time and electric parameters. The next slow components have also an analogical general form.

Thus the photopotential on ALM in the presence of the penetrating ions and the same photoresponse on the planar membrane associated by the photoreceptor discs were registered. This effect was directly resulted by the rhodopsin photolysis.

Apparently the photoresponse is the result of the membrane potential appearance on the photoreceptor disc membrane. This photopotential is likely to be the cause of the ions transfer by rhodopsin through the membrane. However, it's impossible to except the other mechanisms of the membrane poten-

tial difference rise. For example such phenomena as a photoinduced conductance alteration in the photoreceptor membrane (2, 9, 10) and a rhodopsin molecule dipole moment change, which had been measured by different physical methods (11-13) may result in the electric potential alteration on the photoreceptor membrane.

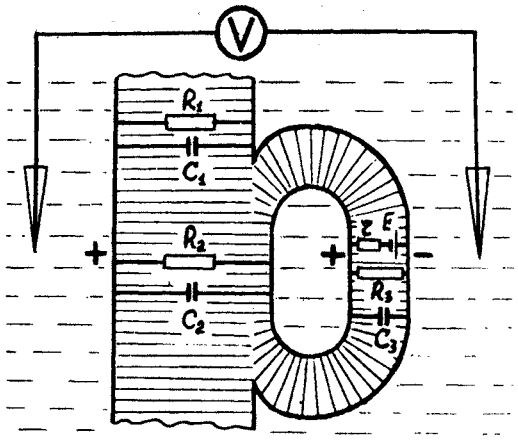

Fig. 3. There are shown an equivalent scheme for the discs associated with a planar membrane; a photoactivated source of energy (E) with internal resistance (r); resistances of a planar membrane, of the fusion region of a planar membrane with a disc and of a disc respectively (R_1, R_2 and R_3); corresponding capacities (C_1, C_2 and C_3); a voltmeter (V).

Further investigation of the photoelectric phenomena expounded above is now on the list; particularly the correlating of the photoelectric reaction components with various phases of a rhodopsin photolysis is a point of a special interest.

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THE STUDY OF IONOPHORIC EFFECT OF SOME NEW DERIVATIVES OF CYCLIC POLYETHERS ON ARTIFICIAL AND BIOLOGICAL MEMBRANES

B. A. Tashmukhamedov, A. I. Gagelgans, A. V. Shkinev, U. Z. Mirkhodjaev, M. V. Zamaraeva and A. K. Tashmukhamedova*

Institute of Biochemistry of the Uzbek Academy of Sciences, Tashkent, USSR
**Institute of Bioorganic Chemistry of the Uzbek Academy of Sciences, Tashkent, USSR*

Abstract - Membrane activity of about 50 newly synthesized 2,3,11,12-dibenzo-18-crown-6 derivatives, containing alkyl-, acyl- or ω-hydroxialkyl-radicals in benzene rings with different length of chain (2-8 carbon atoms) has been studied. Ionophoric activity of cyclopolyethers was tested on bilayer phospholipid membranes, mitochondria and sarcoplasmic reticulum.

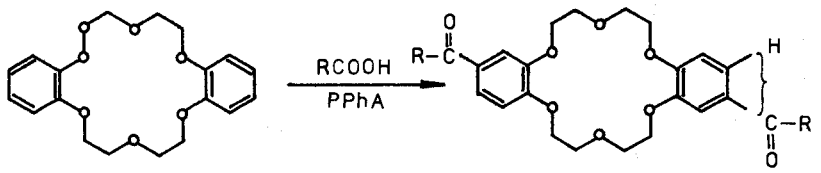
It was shown that dialkyl- and di-ω-hydroxialkyl-derivatives of 2,3,11-12-dibenzo-18-crown-6 at low concentrations mainly induced potassium conductivity of biological and artificial membranes, promoting in some cases H^+-permeability at higher concentrations. Diacylderivatives of 2,3,11,12-dibenzo-18-crown-6 are characterized by ability to increase permeability of mitochondrial membranes and sarcoplasmic reticulum for bivalent cations. The most effective are diacylderivatives of 2,3,11,12-dibenzo-18-crown-6 containing 3-5 carbon atoms in side chains. Dibutiryl-, divaleryl- and dihexanoylderivatives in concentration 5×10^{-5}M stimulated 5-6 folds respiration of mitochondria in the presence of 5 mM Mg^{2+}; at higher concentrations these ligands increased respiration unspecifically due to induction of marked permeability for hydrogen ions.

The synthetic membrane-active compounds together with their natural prototypes to study of which M.M.Shemyakin's school has made a valuable contribution, have a wide application in pure and applied fields of science (Ref. 1). Synthesis of cyclic polyethers differing in size of macrocycle, side chains and complex forming characteristics and other parameters (Refs. 1-4) is well represented in literature, however their membrane-active properties are less studied (Refs. 1,5-9). Nevertheless it is clear that in principle macrocyclic polyethers can act as ion carriers across the membranes, the efficacy and selectivity of which depend on both diameter of the macrocycle and the nature of side chains (Refs, 1,5, 6,9). In particular, it was demonstrated that polyethers with macrocycle containing 4 oxygen atoms show preference to Li^+, with 5 O-atoms - to Na^+, with 6 O-atoms - to K^+ (Ref. 3). Non-modified macrocycles possess lower membrane activity in comparison with their dibenzo- or dicyclohexylderivatives (Refs. 1,6-9). Alkylation of benzene rings results in the rise of ionophoric activity of cyclic polyethers (Refs. 1,5,9).

The state of substituents in the benzene rings may affect ionic selectivity of dibenzo-18-crown-6 derivatives. This was demonstrated by comparison of free diamino-dibenzo-18-crown-6 with the same molecule included into polymeric chain (Ref. 10). In general dependence of membrane-active properties of macrocyclic polyethers from the structure of the side chains required further study.

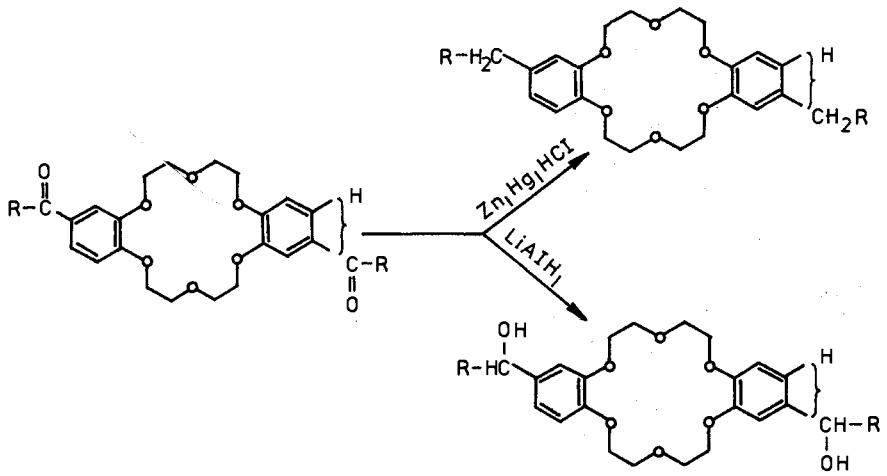
In the present work the synthesis and comparative analysis of ionophoric activity of about 50 dialkyl-, di- ω-hydroxyalkyl- and diacylderivatives of 2,3,11,12-dibenzo-18-crown-6, the linear side chain in which contained from 2 to 8 carbon atoms are described.

Synthesis of 2,3,11,12-dibenzo-18-crown-6 derivatives. In order to obtain derivatives of 2,3,11,12-dibenzo-18-crown-6 (dibenzo-18-crown-6), possessing higher ionophoric activity, we have developed a new synthesis of diacylderivatives of dibenzo-18-crown-6, which may be applied to the other aromatic polyethers (Refs. 11,12). According to this method the aromatic polyether is acylated by carbonic acids in the presence of polyphosphoric acid (PPhA):



where R = CH_3; C_2H_5; C_3H_7; C_4H_9; C_5H_{11}; C_6H_{13}; C_7H_{15}; C_6H_5.

The resultant mixture of geometrical isomeres was separated by fractional crystallization. The structures of isomers are proved by chemical and spectral methods. It has been shown that the low-melting isomer (which is the main reaction product) has "trans"-configuration and the high-melting isomer has "cis" configuration. Diacylderivatives of dibenzo-18-crown-6 were used as a starting material to obtain dialkyl- and dihydroxialkylderivatives. The former were obtained by Klemmensen reduction as described earlier (Ref. 12) and the later ones - by reduction in the presence of lithium aluminium hydride (Ref. 13):



The compounds synthesized have been characterized by elementary analysis IR-, NMR- and Mass-spectra.

Diacylderivatives of dibenzo-18-crown-6. The membrane activity of dibenzo-18-crown-6 derivatives was studied on mitochondria, sarcoplasmic reticulum and bilayer phospholipid membranes.

Rat liver mitochondria were isolated by conventional methods (Ref. 14). Their functional characteristics were studied polarografically (Ref. 15), Permeability of inner mitochondrial membranes for different ions was measured by registration of energy-independent swelling of pellets at 520 nm as described by Brierley (Ref. 16). Permeability for sodium, potassium, magnesium, calcium and barium ions was studied in isoosmotic solutions of nitrates of respective chlorides buffered by 10 mM Tris-nitrate (pH = 7.4) and containing inhibitors of respiratory chain - Antimycin A and Rotenone (0.33 ug/ml). Permeability for hydrogen ions was measured in isoosmotic solutions of ammonium nitrate, buffered by 10 mM Tris-nitrate (pH = 7.4). As nitrate is a penetrating anion, passive swelling of mitochondria is limited only by the rate of transmembrane transport of investigated cation. The cyclopolyethers were dissolved in

dimethylsulphoxide (DMSO) and added in volumes 15-150 µl into cell after mitochondria. Samples with equal volumes of solvent were used as controls.

Sarcoplasmic reticulum was isolated from rabbit skeletal muscles, as described in Ref. 17, with the incubation medium, containing 10 mM potassium chloride, 10 mM Tris-chloride (pH = 7.4), 5 mM magnesium chloride, 2 mM ATP and about 100 µg protein per ml. Potassium oxalate and calcium chloride was added during the experiment. The system was calibrated by exact amount of hydrogen chloride.

Artificial phospholipid membranes were formed from beef brain lipids by Mueller's method (Ref. 18). Membrane parameters were measured by means of Ag/AgCl-electrodes, connected to electrometer. The cation selectivity constants ($K_{K,Na}$) were calculated from the value of biionic potentials at 10 mM potassium and sodium concentrations.

18-crown-6 and dibenzo-18-crown-t at a concentration of $5x10^{-5}$ M did not affect substantially the permeability of inner mitochondrial membranes, measured by means of the rate of energy-independent swelling. Diacylderivatives showed notable ionophoric activity but dipropionyl-, dibutiryl- and divaleryldibenzo-18-crown-6 were the most effective. These compounds caused increase in permeability of mitochondrial membrane for calcium and magnesium and some rise in transfer of monovalent cations. The most selective was dibutirylderivative which increased several folds the transfer of bivalent cations in the concentration of $1x10^{-5}$ M while the permeability for potassium and sodium ions changed insignificantly even in the presence of higher concentration of cyclopolyethers (Fig. 1). Simultaneously this compound induced some proton permeability reaching ca. 20% of the calcium one. The rise in permeability of inner mitochondrial membranes for different cations in the presence of dibutirylderivatives was proportional of its concentration (Fig. 1).

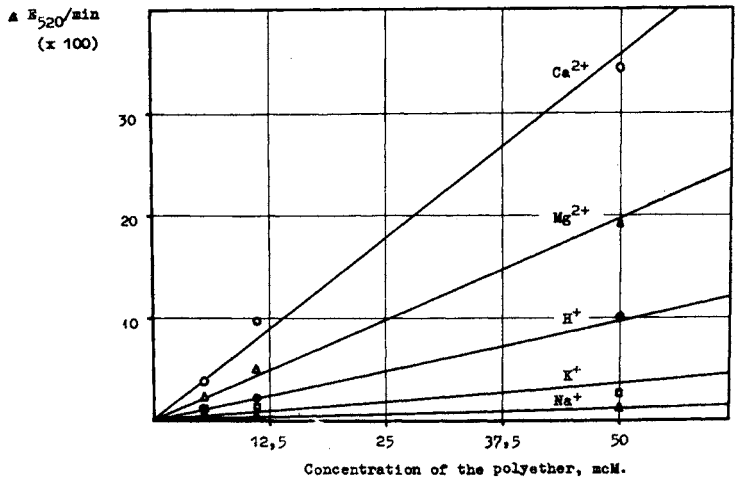


Fig. 1. The effect of dibutiril-derivative of dibenzo-18 crown-6 on the rate of energy-independent swelling of rat liver mitochondria in the presence of mono- and bivalent cations.

Dibutiryl-, divaleryl- and dihexanoylderivatives of dibenzo-18-crown-6 in $5x10^{-5}$ M concentration stimulated 6-7 fold the respiration of mitochondria in the presence of 5 mM of magnesium. At higher concentrations these compounds increased the respiration unspecifically essentially due to induction of hydrogen conductivity, which correlated with uncoupling of oxidative phosphorylation.

Diacylderivatives of dibenzo-18-crown-6 decreased the transport ratio of Ca^{2+}/ATP of rabbit muscle sarcoplasmic reticulum with the same dependence

on the length of substitutes in side chains as on mitochondria (Fig. 2), i.e. dibutiryl- and divalerylderivatives were the most active ionophores.

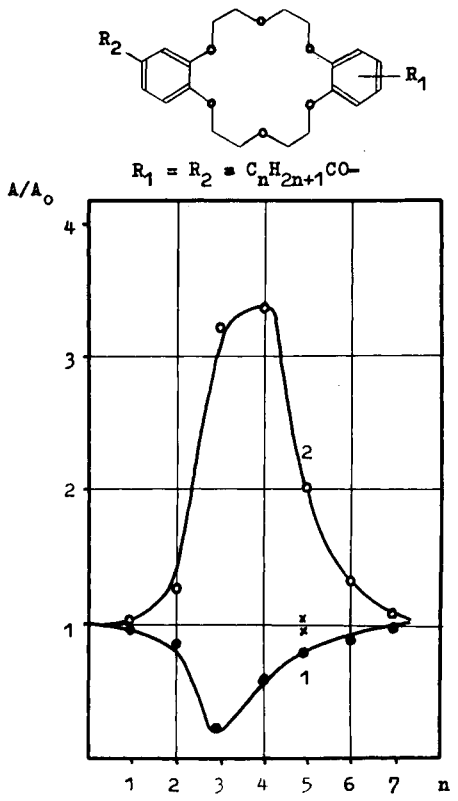


Fig. 2. The effect of side chain length (COC_nH_{2n+1}) of dibenzo-18-crown-6 in diacyl derivatives on the relative change of Ca/ATP ratio in sarcoplasmic reticulum (1) and permeability of liver mitochondria for Ca^{2+} (2). A refers to data obtained in the presence of macrocyclic polyether. A_0 refers to control. The concentration of polyethers was $3x10^{-4}$ (1) and $5x10^{-5}$ M (2). * mark the activity of dibenzoyldibenzo-18-crown-6.

However the effective concentrations of cyclopolyethers in experiments on retuculum were several times higher in comparison with the mitochondria. This may be due to the differences in surface charge of membranes, their dielectric constants, unequal density of lipids and protein packing, etc.

The effect of dibutirylderivative on the calcium pump of reticulum is prevented by hydrophobic anions when added in cell prior to the cyclopolyether. The effect of lipid-soluble anions is not present with the carboxylic ionophore A 23187.

Thus the data obtained indicate that some diacylderivatives of dibenzo-18-crown-6 effectively induce the transfer of bivalent cations through mitochondrial and sarcoplasmic reticulum membranes. The chain length of 4-5 carbon atoms in benzene ring substituents is optimal for the membrane activity. Shortening or lengthening of the chain and also its substitution by benzoyl (dibenzoyldibenzo-18-crown-6) reduces the selectivity for bivalent cations. Removal of the keto-group from the α-postion or its substitution by a hydroxyl as will be shown below essentially reduces or completely excludes the membrane activity associated with the bivalent cations. The effect of length of the side chains can be related to solubility of cyclopolyethers in the lipid.

Di-ω-hydroxyalkylderivatives of dibenzo-18-crown-6. The membrane activity of a number of dibenzo-18-crown-6 derivatives has been studied, the substitutes of which in benzene rings range from ω-hydroxyethyl to ω-hydroxyoctyl.

In comparison with diacylderivatives, these cyclopolyethers are highly soluble in ethanol and their effects can be observed both on biological and artificial phospholipid membranes. Membrane activity of di-ω-hydroxyalkylderivatives as estimated from their minimal effective concentration is by 3-4 orders of magnitude higher than for the original dibenzo-18-crown-6. Experiments on bilayer phospholipid membranes show that di-ω-hydroxyethyl-dibenzo-18-crown-6 is the most efficient amont this group of cyclic polyethers. At $1x10^{-5}$ M in the presence of 10 mM potassium chloride it increases 15 fold the conductivity of bilayer phospholipid membranes. The cation specificity constant of di-ω-hydroxyethylderivative $K_{K,Na}$ measured from the biionic potentials is 8.1. With lengthening of the hydrocarbon chain the ability of cyclopolyethers, to induce potassium permeability of bilayers gradually falls, however the value of $K_{K,Na}$ increases. So, di-ω-hydroxyheptyl derivatives ($1x10^{-5}$ M) in the presence of 10 mM of potassium chloride reduce the resistance of bilayers only less than twofold, while the coefficient $K_{K,Na}$ is 14.5. At the same concentration di-ω-hydroxyoctyl-dibenzo-18-crown-6 does not affect the conductivity of artificial membranes in the presence of potassium or sodium. All the compounds of this group do not induce proton conductivity which together with the above data allows to consider them as ionophores with pronounced potassium selectivity.

agrees
In general this conclusion with the results obtained on mitochondria, but in this case the specificity of membrane effects depends essentially on concentration of cyclopolyether. Among the cyclopolyethers tested di-ω-hydroxyamyl-dibenzo-18-crown-6 was the most effective. This compound at $1x10^{-6}$ M concentration increased twice the rate of energy-independent swelling of mitochondria in potassium nitrate, practically not affecting the kinetics of swelling due to the transport of other mono- and bivalent cations.

Thus the presence of hydroxy-group in ω-position of the side chains results in the rise of potassium ionophoric activity of cyclopolyethers while the keto group in the same postion makes divaleryl-dibenzo-18-crown-6 a calcium ionophore. However high concentrations of di-ω-hydroxyalkylderivatives of dibenzo-18-crown-6 ($5x10^{-5}$ M) cause less specific change in permeability of the inner mitochondrial membranes. The highest selectivity is shown by the compounds with 6-7 carbon atoms side chains carrying hydroxy-group in ω-position. For instance the permeability ratios of mitochondria treated with di-ω-hydroxyheptyl-dibenzo-18-crown-6 are: $K^+:Na^+:H^+:Ca^{2+}:Mg^{2+}:Ba^{2+} = 1:0.4:0.15:0.07:0.7:0.09$.

Some discrepancy in effects induced by di-ω-hydroxyalkylderivatives in mitochondria and artificial membranes is perhaps due to the peculiarities of their chemical composition, surface charge, dielectric parameters, etc.

Dialkylderivatives of dibenzo-18-crown-6. As shown by experiments on isolated mitochondria and bilayer phospholipid membranes, dialkylderivatives of dibenzo-18-crown-6 are very effective membrane-active compounds. For example, dibutyl-dibenzo-18-crown-6 at a concentration of $5x10^{-7}$ M increases 2-3 fold potassium permeability of mitochondrial inner membranes. This compound at concentration $1x10^{-5}$ M increases 150 fold the conductivity of artificial membranes in the medium containing 10 mM of potassium chloride. The cation specificity constant of dibutylderivative, $K_{K,Na}$, measured by biionic potentials equals 10. In addition to potassium this cyclopolyether induces some although 10 times smaller proton permeability. Cyclopolyethers with linear substitutes containing 2-5 carbon atoms were the most powerful ion carriers in mitochondrial and phospholipid membranes. Further lengthening of alkyl-groups resulted in the decrease of membrane activity, however the K/No selectivity of these compounds increased up to 11-12.

At low concentrations ($5x10^{-7}$ - $5x10^{-6}$ M) diethyl- and dipropylderivatives increased mainly the potassium permeability of mitochondrial membranes; higher concentrations of these derivatives (up to $1x10^{-4}$ M) caused total rise in membrane permeability which was less specific towards the ion

species. On the other hand, the potassium selectivity of dihexyl- and diheptylderivatives remaind high in a wide range of concentration.

Thus, alkylation of benzene rings of dibenzo-18-crown-6 rises the membrane activity of cyclopolyethers so that at low concentrations they behave as powerful potassium ionophores. Similar results were obtained by Harris et al. (Ref. 9), when they inserted tret-butyl radicals into benzene rings of dibenzo-30-crown-10. The effect of substitutes in benzene rings of polyethers can be also demonstrated in the case of dibenzo-24-crown-8 which at a concentration of 1×10^{-3} M increases 3-4 fold potassium permeability of mitochondria. Dibutyl-dibenzo-24-crown-8 also induces analogous permeability changes but at significantly lower concentration (5×10^{-6} - 1×10^{-5} M). At the same time the cation specificity constant $K_{K,Na}$ of this compound in comparison with the original one remains practically unchanged.

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HEAVY WATER EFFECTS ON EXCITABLE MEMBRANES

V. Vasilescu and E. Katona

Department of Biophysics, Medical Faculty, Bucharest, Romania

Abstract - Studies concerning heavy water (^2H_2O) effects on the kinetics of excitability processes and energy metabolism in nerve, muscle, heart and retina are reported. An analysis of data suggests the possible role of protons both in maintaining the functional supramolecular structure of excitable membranes and in the dynamics of the cellular energy processes. Deuteriation is rendered evident as an excellent non-destructive tool for investigating the membrane function and the role of water therein; the molecular mechanisms of heavy water effects on excitable membranes are also discussed.

INTRODUCTION

The structure and function of biological membranes constitute one of the problems of paramount importance in molecular biology. The comprehensive area of this problem was the object of intensive investigations for many years and in spite of the large number of different techniques used to scan its diverse facets, many questions remained unanswered (1, 2). As major constituents of biological membranes were revealed lipids, proteins, oligosaccharides and water, but how the individual species in any specific membrane are arranged in some steady state is not precisely known till now (3). The understanding of the molecular architecture of membranes being poor, it is not surprising that uncertainties about many mechanisms associated with the membrane function persist. Though a considerable body of data has been acquired, the mechanisms whereby the physical characteristics of membrane components influence various functions, the way in which energy transport molecules are organized and details at the molecular level of the nerve impulse remain open questions. Thus membrane biophysics continues to be an active field of investigation in which exciting results can be expected. On the other hand a great deal of experimental studies have been devoted to the effects of heavy water (^2H_2O) on various biological systems (4), this keen interest being at least partly accounted for by the assumption that the replacement of light water by heavy water might disclose the contribution of water to various biological processes (5, 6). However no molecular understanding of deuterium isotope effects is so far available; large discrepancies and debates continue with respect to the significance of certain data (7), and consequently the problem still deserves attention.

The study of heavy water effects has been for many years the major concern in our Laboratory and the present work is a synthesis of more recent data and at the same time of essential aspects disclosed by previous investigations and interpreted through a new outlook.
Starting from the valuable achievements in molecular biology it must not be overlooked the inframolecular level of the mechanisms of biological processes and therefore it is time to revalue them in the light of the role of protons and possibly to speak today about a proton biology.
Our studies regarding ^2H_2O-effects revealed deuteriation as an excellent non-destructive tool of investigating various membrane functions. As H_2O - - ^2H_2O exchange is due to only the diffusional forces, it does not impose any stress upon the tissue. The method has a twofold advantage: on the one hand the investigation of the kinetics of ^2H_2O for H_2O substitution in the tissue can provide data on the distribution and state of water in biological systems; on the other, deuteriation modifies all the functions involving proton participation or depending on the presence of certain water stabilized conformational states of biopolymers and therefore enables to elucidate some essential aspects of the role of water and protons in the evolvement of the fundamental mechanisms in membrane functioning.
We investigated the molecular mechanisms of ^2H_2O effects by following up the time course of its influence on the function and energy metabolism of isolated frog nerves, muscles, heart and retina.

^2H_2O PERMEATION INTO TISSUES

Considering that the discrepancies in the interpretation of data concerning $^2H_2$ effects are at least partly due to the ignorance of the time course of its incorporation into each biological object, we investigated the kinetics of ^2H_2O permeation into tissues (8, 9). A somewhat more detailed analysis of kinetic data regarding heavy water intake in tissues, in terms of an appropriate model, enabled us to disclose the various tissue water compartments too (10). Similar investigations were reported on the smooth and striated muscles (11, 12) and on frog ovarian eggs (13), but using a different technique and/or within a rather different conceptual framework.

Methods and computations

The kinetics of H_2O-^2H_2O exchange in tissues was studied by us both gravimetrically and by infrared photometry at $\lambda = 1.47\mu m$. The basic principle of the method using infrared photometry is illustrated in Fig. 1.
A sample of freshly prepared tissue was immersed in 99.7% ^2H_2O-Ringer solution of volume V_o. At $\lambda = 1470$ nm H_2O has one of its maxima of absorption while ^2H_2O does not absorb at all, so that the extinction E is proportional to the water concentration in the external solution, that is: $E(t)=\varkappa w(t)/V_o$ where w(t) is the quantity of ^2H_2O entering the whole tissue, equal to the quantity of H_2O leaving the tissues and $\varkappa$ is the calibration factor. We followed up the extinction increase in time for 60-90 min. on pairs of objects taken from the same animal, at different temperatures, usually T and T' = T + 10K. The optical absorption of the solution was recorded each minute except for the first minute needed to handle the tissue and adjust

the apparatus.

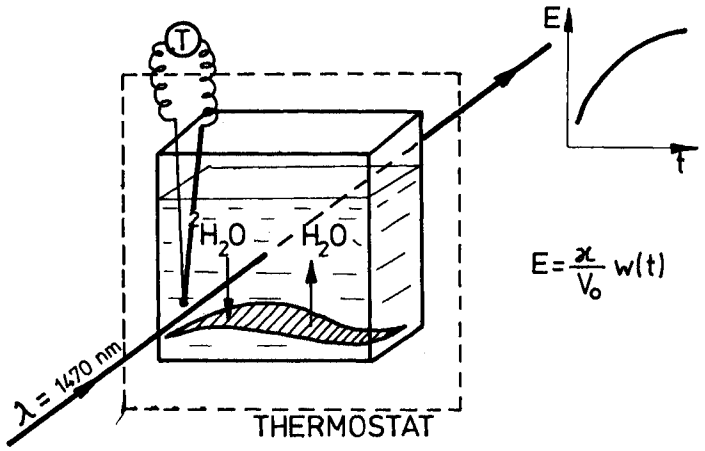

Fig. 1. The basic principle and the main experimental assembly of the method of following up by infrared photometry the H_2O-^2H_2O exchange between tissue and the bathing solution.

The fact that ^2H_2O-Ringer and H_2O-Ringer solutions are of different densities allowed us to study H_2O-^2H_2O and ^2H_2O-H_2O exchanges also by immersion weighing methods, the rate of a change in the apparent tissue weight being linearly related to the flows. Parallel experiments performed by these two methods show a very close fit of the results. The crude experimental data consist in the time course of either the extinction in the external solution or the apparent weight of the immersed tissue. These data multiplied by the calibration factors are directly converted into the values of ^2H_2O quantities entering the tissue, w(t). In Fig. 2 one can see a typical multiexponential curve indicating the time dependence of the experimental values of extinction E(t). Practically E(t) is accurately expressed as a biexponential function:

$$E(t) = A_1 \left[1-\exp(-\alpha_1 t) \right] + A_2 \left[1-\exp(-\alpha_2 t) \right]$$

whose parameters can be obtained by computer data fitting or by graphical analysis. This means that the time-dependence of w(t) is also at least biexponential. This multiexponential character of the time increase of ^2H_2O intake shows the presence of more than one simple process.
The kinetic parameters A_1, A_2, α_1, α_2 indicate the rate of H_2O-^2H_2O exchange and of the tissue space accessible to ^2H_2O.
With the view of a proper data fitting we used a bicompartmental theoretical model for tissue. The basic assumptions of the model are: no electrical gradient or chemical coupling is directly involved in water movements; ^2H_2O inflow into each compartment is continuously counterbalanced by an equal H_2O outflow; the various water compartments are defined solely on the basis of a "diffusion accessibility" criterion, this water accessibility

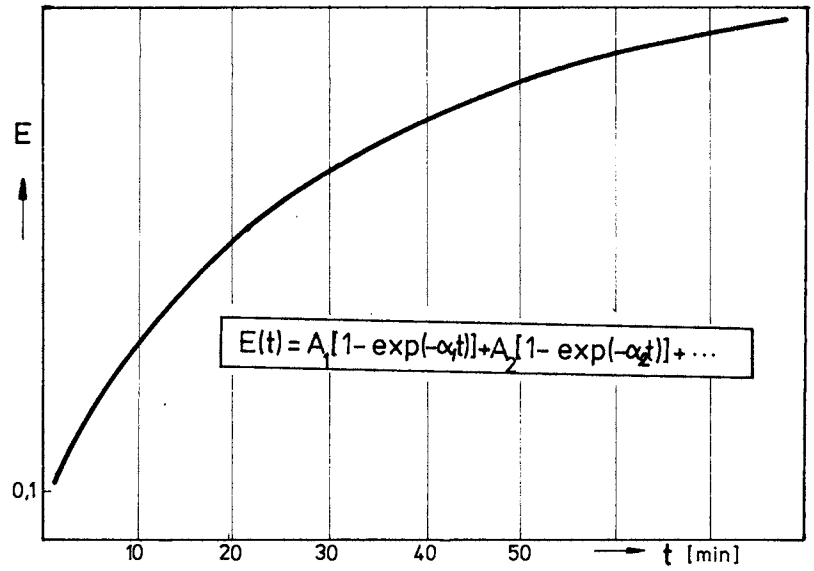

Fig. 2. Extinction increase at λ = 1.47 μm in 1.6 ml ^2H_2O-Ringer solution in which 430 mg frog muscle was immersed.

for diffusion exchange being dependent on the height of energy barriers separating the compartments, more precisely on the apparent free energy change in water passage from one compartment into another (ΔG_{ij}). In Fig. 3. a

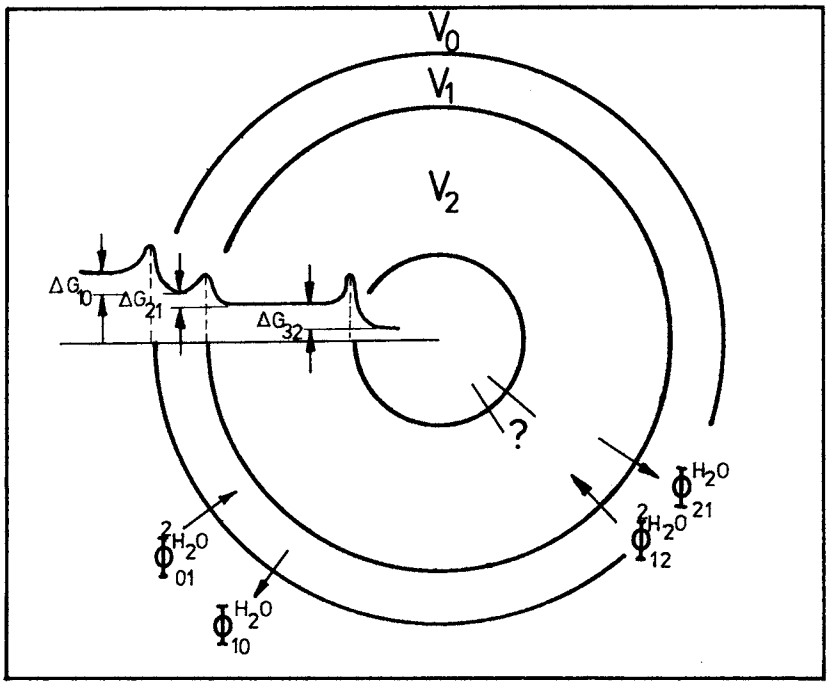

Fig. 3. Series array of tissue water compartments and the apparent free energy barriers for ^2H_2O passage through them. $\Phi^{H_2O}_{ij}$ and $\Phi^{^2H_2O}_{ij}$ are the net fluxes from one compartment into another, ΔG_{ij}, the apparent free energy changes and V_i, the volumes of the compartments.

series array of water compartments is represented. Only three compartments are considered because the experimental data can be confidently resolved into two exponentials. The outer solution is the 0th compartment, the most accessible tissue water constitutes the 1st compartment, the less accessible the 2nd compartment and the remainder of the tissue water which cannot be replaced by ^2H_2O, the 3rd compartment. V_1 and V_2 are the volumes of the two experimentally revealed compartments whose sum is smaller than the total water content of the tissue (V_t) and V_o is the volume of the external solution bathing the tissue. In this way the data fitting in terms of the model just described discloses the existence of at least 3 distinct aqueous tissue compartments with gradually decreasing ^2H_2O accessibility. The volumes of these aqueous tissue compartments as well as the apparent free energy changes of water passage through them have been calculated from the kinetic parameters A_1, A_2, α_1, α_2 of the experimental curves recorded on pairs of objects at two different temperatures.

The formulas giving the volumes of the two compartments V_1 and V_2 computed within the limits of assuming symmetrical energy barriers separating the compartments and the apparent free energy changes for heavy water intake in the first tissular compartment from the outer one (ΔG_{1-0}) and into the second compartment from the first one (ΔG_{2-1}) are those listed below:

$$V_1 = V_o(A_1\alpha_1+A_2\alpha_2)/\,(\varkappa-A_1)\alpha_1-A_2\alpha_2$$

$$V_2 = V_o\varkappa\, A_2(\alpha_1-\alpha_2)/\left\{[(\varkappa-A_1)\alpha_1-A_2\alpha_2](\varkappa-A_1-A_2)\right\}$$

$$\Delta G_{1-0} = \frac{RTT'}{T'-T}\ln\frac{(A_1\alpha_1+A_2\alpha_2)\,[(\varkappa-A_1')\alpha_1'-A_2'\alpha_2']}{(A_1'\alpha_1'+A_2'\alpha_2')\,[(\varkappa-A_1)\alpha_1-A_2\alpha_2]}$$

$$\Delta G_{2-1} = \frac{RTT'}{T'-T}\ln\frac{A_2(\alpha_1-\alpha_2)(A_1'\alpha_1'+A_2'\alpha_2')(\varkappa-A_1'-A_2')}{A_2'(\alpha_1'-\alpha_2')(A_1\alpha_1+A_2\alpha_2)(\varkappa-A_1-A_2)}$$

In view of inherent differences among biological objects, the fitting procedure by computer or graphical analysis has been performed for each pair of corresponding curves and only the final values of V_1, V_2, ΔG_{1-0} and ΔG_{2-1} have been mediated.

The time course of water replacement by heavy water is significantly expressed by the quasi-saturation times τ_i defined by the condition: $1 - \exp.(-\alpha_i\tau_i) = 0.95$, so that

$$\tau_i = (-\ln 0.05)/\alpha_i \qquad i = 1,\ 2$$

Taking into account the uncertainties in assessing the areas of each compartment, the permeability coefficients for water diffusion into the compartments are defined as the ratios of the fluxes (in each compartment) to the tissue mass:

$$P_i = \alpha_i V_t/m \qquad i = 1,\ 2$$

Representative data

Our data concerning water distribution and state in frog (Rana temporaria) nerve, muscle and retina are listed in Table 1.

TABLE 1. Volumes of tissue water compartments and the apparent free energy changes in water passage through them

TISSUE	V_t ml/kg	V_1 ml/kg (%)	V_2 ml/kg (%)	$1-(V_1+V_2)/V_t$ %	ΔG_{1-0} kcal/mol	ΔG_{2-0} kcal/mol
Nerve	763	163 (21)	382 (5o)	29	4.7	2.1
Retina	900	189 (21)	585 (65)	14		
Muscle	798	18o (23)	258 (32)	45	2.1	5.9

V_t is the volume of the total water obtained by heat drying and expressed with respect to the initial fresh weight of tissue. V_1 and V_2 are expressed in the same way as V_t, and also as % of V_t.

Each listed value is the mean of 6-16 determinations and in all cases the sample standard deviation lies within ± 12% of the mean.

Table 2 contains the values of quasi-saturation times and permeability coefficients calculated for the two water compartments whose volumes are shown in Table 1.

TABLE 2. Quasi saturation times and permeability coefficients of the faster (τ_1,P_1) and slower (τ_2,P_2) exchangeable tissue water compartments

TISSUE	τ_1 (min)	τ_2 (min)	P_1 (cm^3/min.g)	P_2 (cm^3/min.g)
Nerve	1.4	22.1	1.97	0.15
Retina	4.1	15.4	0.81	0.24
Muscle	24.6	147.6	0.12	0.02[a]
	5.6	64.4	0.53	0.06[b]

Each listed value is the mean of 6 determinations and in all cases the sample standard deviation lies within ± 25% of the mean.

Obviously these kinetic parameters strongly depend on the volumes of tissue water and on the size of the external compartment. The given values have been calculated for a ratio of $r = V_t/V_o = 0.125$ and $r = 0.1$ in the case of nerve and retina respectively, while in that of muscle $r = 0.03$ for (a) and $r = 0.3$ for (b).

Although in our model the tissue water compartments are defined only on the basis of the water accessiblity for diffusion exchange, taking into account the rapidity of the exchange between the external solution and the first, faster exchangeable tissue water compartment, as well as the rather low value of the free energy change for ^2H_2O intake into it, we can infer that this compartment is extracellular, namely that it represents the intercellular space. The second, a slower exchangeable tissue water compartment, most probably is the intracellular water, more precisely the greatest part of it. The third compartment, "invisible" from the viewpoint of our technique, may be the water somehow "obstructed" or/and "bond" of the tissue including the hydration water of biopolymers too. These conclusions are in very good agreement with similar investigations (11) and with certain NMR measurements on muscle (14, 15, 16) and nerve (17).
The overall process of ^2H_2O permeation into tissue is a rather slow one, the deuteriation of various tissue water compartments taking place gradually in time. As a practical indication arises that in the investigation of the physiological effects of ^2H_2O on different biological objects, when deuteriation is obtained by simple immersion in heavy water, one should not overlook the time period needed until ^2H_2O replaces H_2O in different tissular compartments.

^2H_2O EFFECTS ON EXCITABILITY

Experiments carried out on nerve, muscle, heart and retina showed that the substitution of heavy water for light water leads to substantial changes in the bioelectrogenesis processes. An alteration of several excitability parameters - progressive rheobase, latency and potential duration increase and conduction velocity decrease - occurs, the changes observed reaching a gradual and partially reversible abolishment of the various physical manifestations accompaning the excitability processes.
In spite of a great amount of work in this line, although it is sure that heavy water influences almost all the excitation processes, a strictly coherent description of its influence could not be elaborated as yet. Parallel studies of ^2H_2O electrophysiological and energetical effects and of its incorporation in tissues are desirable in order that the alteration of various functions be correlated with the deuteriation of different tissue-compartments or/and with a diminution in the energy pools due to the deuteron for proton substitution.

^2H_2O effects on nerve

Researches concerning ^2H_2O influence on nerve activity revealed a 20% reversible decrease of the conduction velocity in the frog sciatic nerve (18) and squid giant axon (19), modifications of the time course of the action potential (AP), a 30-40% increase in the AP anterior and posterior fronts and a 30-40% increment of the excitation threshold (2o, 21). The most obvious changes induced by ^2H_2O in nerve trunks, observed by us (22, 23), were the progressive rheobase increase and conduction velocity decrease as the degree of nerve deuteriation advanced (Fig. 4, 5).

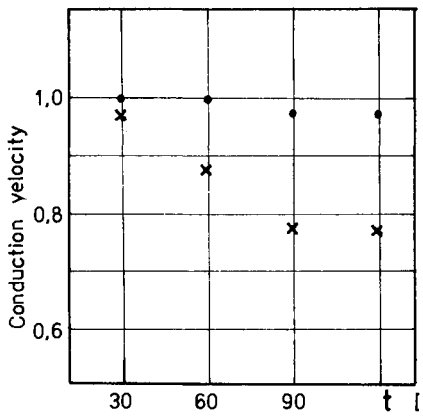

Fig. 4. ^2H_2O effect on the conduction velocity of the nerve impulse.

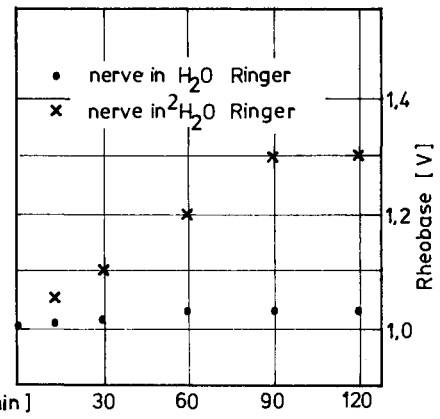

Fig. 5. ^2H_2O effect on the rheobase of the stimulated frog nerve.

^2H_2O effects on retina

We studied the effect of ^2H_2O on the frog retina bioelectrogenesis (24, 25) in order to obtain information about the role of protons in the genesis of the ERG and new data regarding the nature of different components of ERG as well. Experiments were performed on oxygenated retina placed in thermostated box at 22°C and stimulated every 5 minutes with rectangular stimulus (white light, 42 Lx, 1 min duration). It was found that in frog retina a substantial increase in time of the OFF-response latency occurs under deuteriation. Fig. 6. shows ^2H_2O effect on OFF-response latency.
At the same time the latency of ON-response is almost unaffected by the substitution of deuterium for hydrogen, a fact indicating an essential difference in the starting mechanisms of the two responses. On the contrary the ON response itself is much more sensitive to the level of deuteriation than the OFF response; it vanishes during the first 15 minutes while the OFF response is considerable even for over 1 h.
In Figs. 7 and 8 ^2H_2O-effects on the amplitude of "a" and "b"-waves of the retina ON-response are illustrated. It may be observed that in the ON response the "a"-wave is less sensitive to ^2H_2O than the "b"-wave; at a constant level of stimulation (white light, 42 Lx) its vanishing is slower while its recovery, faster. This shows a difference in the development mechanisms of the two processes. For all it is known the "a"-wave is generated by the receptor cells while the "b"-wave, by the cells in the neural part of retina. The processes underlying "a"-wave generation seem to be morphologically related to the interstitial water because they are depressed immediately after the saturation of the Ist compartment. The decrease of the "b"-wave might be caused by a depression in the primary events underlying "a"-wave, as well as by a direct effect of ^2H_2O on "b"-wave generating cells. All these ^2H_2O effects observed were partially reversible when we replaced ^2H_2O-Ringer by H_2O-Ringer.
As the amplitude decreases of the ON response, leading to its partially

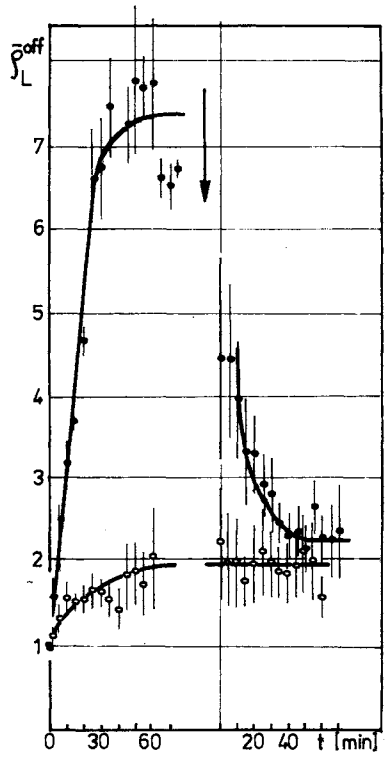

Fig. 6. Time evolution of the OFF-response latency in ^2H_2O (•) and control experiments (o).
The arrow points to the moment when the bathing solution is replaced by normal Ringer. $\bar{\rho}_L^{off}$ is the average relative latency of the OFF-response, that is the mean of the ratios between the latencies at a given moment and those at t=1 min.

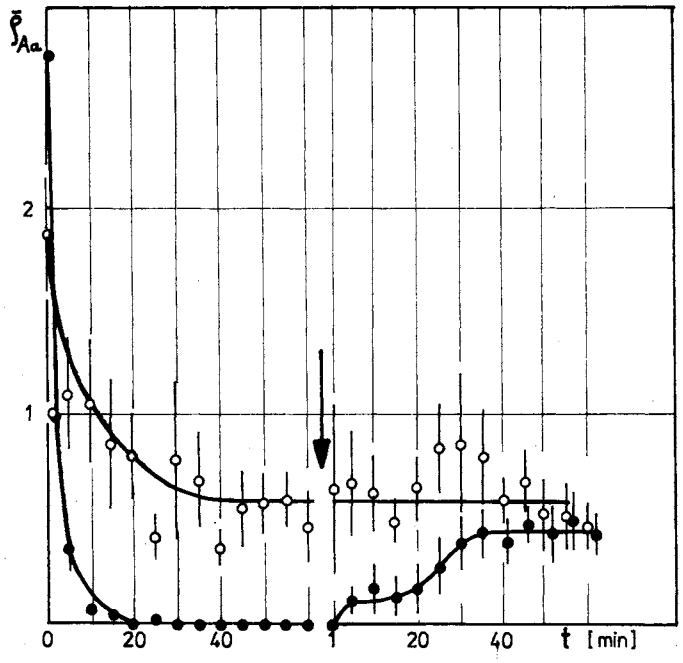

Fig. 7. Time evolution of the amplitude of the ON-response "a"-wave in ^2H_2O (•) and control experiments (o).
The arrow and $\bar{\rho}_{Aa}$, the average relative amplitude of the "a"-wave, signify the same as in Fig. 6.

reversible vanishing has been seen at a constant stimulation level, they might be the result of a change in the excitation threshold under deuteriation. Further experiments are desirable to be made at various stimulation levels in order to check this possible threshold dependence on the deuteriation degree.

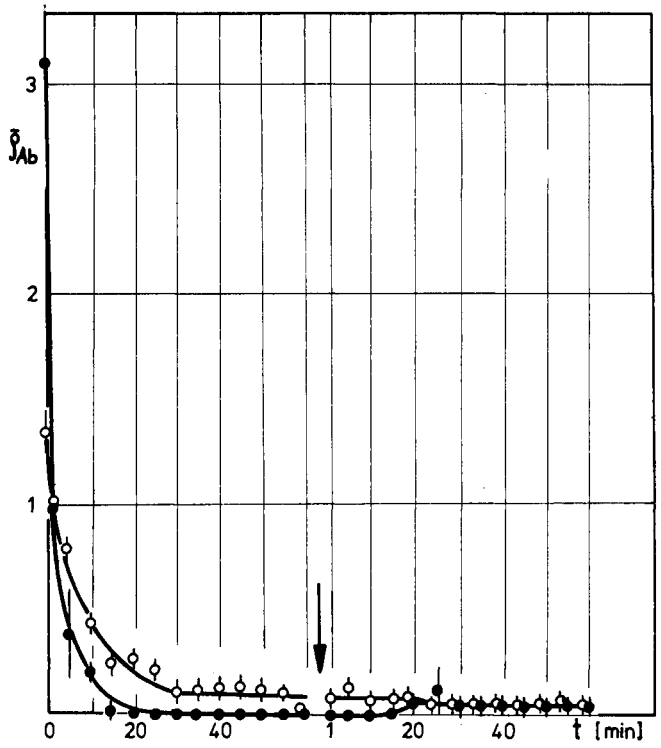

Fig. 8. Time evolution of the amplitude of the ON-response "b"-wave in ^2H_2O (•) and control experiments (o).
The arrow and $\bar{\rho}_{Ab}$, the average relative amplitude of the "b"-wave, signify the same as in Fig. 6.

^2H_2O effects on heart

From experiments performed in vivo and on isolated hearts it appears that the cardiac stimulating system is particularly sensitive to deuterium and that acute ^2H_2O intoxication death is caused essentially by acute cardiac failure (26).

Our results (27) disclose ^2H_2O as a strong inhibitory agent both of bioelectrogenesis and of contractibility of isolated frog heart, it proving to be also a factor capable of uncoupling the electrical function from the mechanical one. By comparing the modified mechanograms and electrocardiograms following ^2H_2O perfusion of an isolated frog heart a much stronger inhibition of the mechanical than the electric activity is observed. Although a continuous decrease both in the action potential and in the rhythm of the ^2H_2O perfused isolated frog heart is found, the electrical activity can still continue for a relatively long time while the mechanical one colapses within a much shorter interval (Fig. 9.).

The inhibitory effects can be tentatively explained in terms of

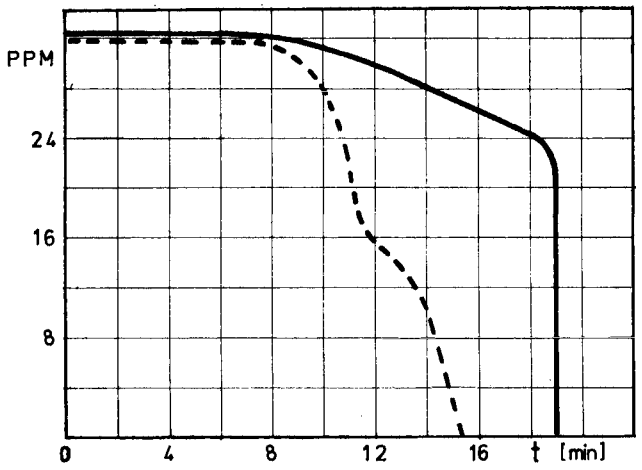

Fig. 9. Typical diagram showing the activity of an isolated frog heart perfused with ^2H_2O.
- solid line: electrical activity; broken line: mechanical activity; PPM: pulse per minute.

conformational changes caused by ^2H_2O in membrane enzymes and in the contractile actomyosin system and also in terms of energy pool diminution induced by deuteriation. The greater complexity and higher energy requirement of the contraction function might account for the ^2H_2O uncoupling effect.

^2H_2O EFFECTS ON ENERGY METABOLISM

In order to check a possible impact of ^2H_2O on the energy metabolism we followed up the dynamics of ATP pool in isolated nerves and retina.

Methods

ATP concentrations in tissues were measured by Strehler's bioluminescence method (28) modified by us (29)with the view of an increased sensitivity. Strehler's method is based on the bioluminescence reaction in fire flies , according to which luciferase in the presence of luciferin, oxygen, magnesium and ATP catalyzes the synthesis of adenyl-luciferin from ATP and luciferin. Adenyl-luciferin is oxidized by the atmosphere oxygen and converted into adenyl-oxyluciferin, a process which takes place with light emission. After De Luca and McElroy (3o) the reaction scheme now accepted is:

$$E + LH_2 + ATP \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} E \cdot LH_2 - AMP + PP$$

$$E \cdot LH_2 - AMP + O_2 \xrightarrow{-CO_2} P^+ - E \cdot AMP \longrightarrow h\nu + \text{products}$$

where E = luciferase, LH_2 = luciferin and P^+ represents an electronically excited reaction-product emitting light.
ATP determination implies the measurement of the relative intensity of

light emitted by luciferase solution a few seconds following the addition of ATP solution (in the presence of suitable quantities of Mg^{++}, oxygen and luciferin). As the intensity of the emitted light depends on the type of buffer solution in which luciferin-luciferase extraction from fire fly tails was performed, by modifying the extraction conditions namely by using a glycyl-glycine buffer the sensitivity of the method becomes one order of magnitude higher than that of the original one (Fig. 10.).

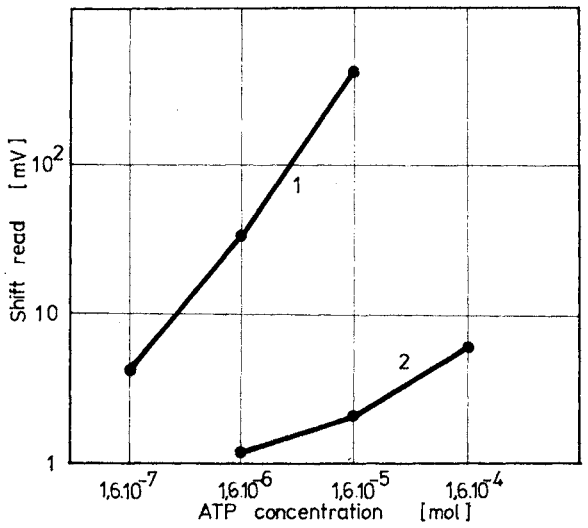

Fig. 10. Calibration curve: the shift read on the oscilloscope as a function of the ATP concentration.
1 - extraction in buffer; 2 - extraction in water.

We used for our experiments symmetric sciatic nerves taken from the same frog and immersed for 90′ in ^2H_2O-Ringer and H_2O-Ringer respectively. Measurements were made on resting and continuously stimulated (by electric pulses with a 50 Hz frequency) nerves (31). The dynamics of ATP pool was followed up also on pairs of frog retinae immersed for various time periods at dark in ^2H_2O-Ringer and H_2O-Ringer respectively and then continuously illuminated with white light for 10′ therein (32).

Representative data

In Figs. 11.and 12.ATP-concentrations in unstimulated (a) and continuously stimulated (b) deuteriated nerves in comparison with ATP concentrations in control pairs are represented. A decrease of ATP content in the deuteriated nerve against control can be observed in both cases, which decrease is more pronounced in ceaselessly stimulated nerves, the ratio between the ATP concentrations in deuteriated nerves and those in controls being 0.78 for unstimulated nerves and 0.65 for those undergoing a continuous stimulation for 90′ in ^2H_2O-Ringer and H_2O-Ringer respectively.
Fig. 13. illustrates the ATP concentration in deuteriated retina (immersed in ^2H_2O-Ringer 30′at dark and then illuminated 10′ therein) as compared to the ATP concentration in the control pair. A marked decrease (more than 50%) of ATP pool in the deuteriated retina against control may be seen, the

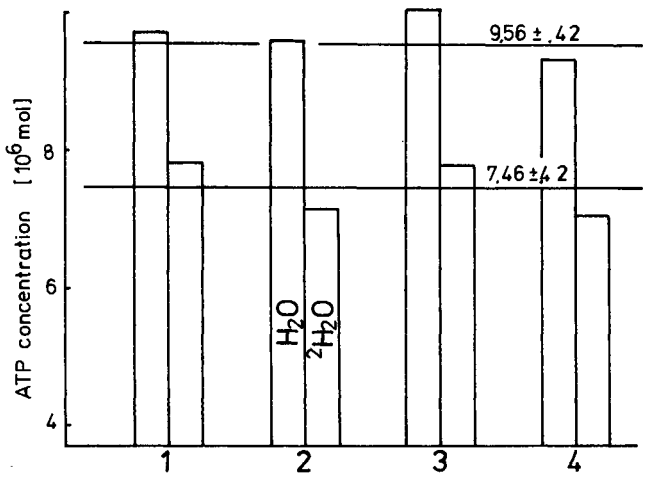

Fig. 11. ^2H_2O effect on cellular ATP content in unstimulated nerves.
In each group of experiments the mean values for 5-7 pairs of symmetric sciatic nerves (immersed 90′ in ^2H_2O-Ringer and H_2O-Ringer respectively) are represented. The sample standard deviations lie in all cases within ± 25%. The figures on the diagram are the population mean values with their standard errors.

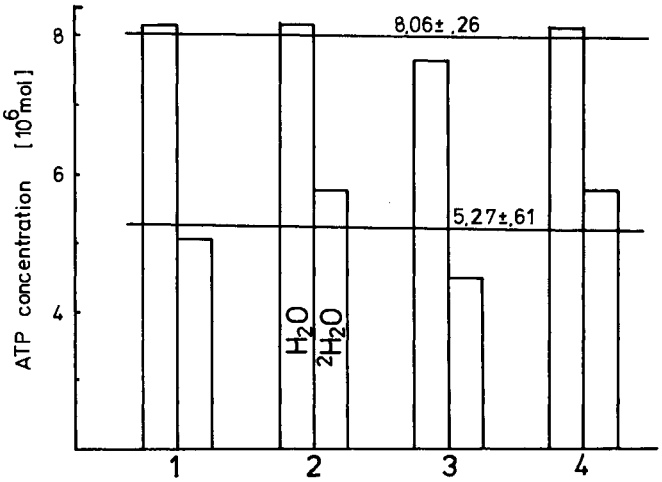

Fig. 12. ^2H_2O effect on cellular ATP content in stimulated nerves.
In each group de experiments the mean values for 5-8 pairs of continuously stimulated symmetric sciatic nerves are represented. The sample standard deviations lie in all cases within ± 20%. The meaning of the figures on the diagram is the same as in Fig. 11.

ratio between the population mean values of the ATP concentrations in deuteriated and control retinae being 0.406. Therefore an obvious diminution of ATP concentration in the deuteriated tissue as compared to the control

appears when both are continuously stimulated in the same way. Measurements effected on retina at various time intervals of immersion discloses a greater decrease in the ATP concentration when immersion periods are longer. Systematic investigations are necessary to check the dependence of the ATP pool diminution in the continuously stimulated tissue on the degree of tissue deuteriation.

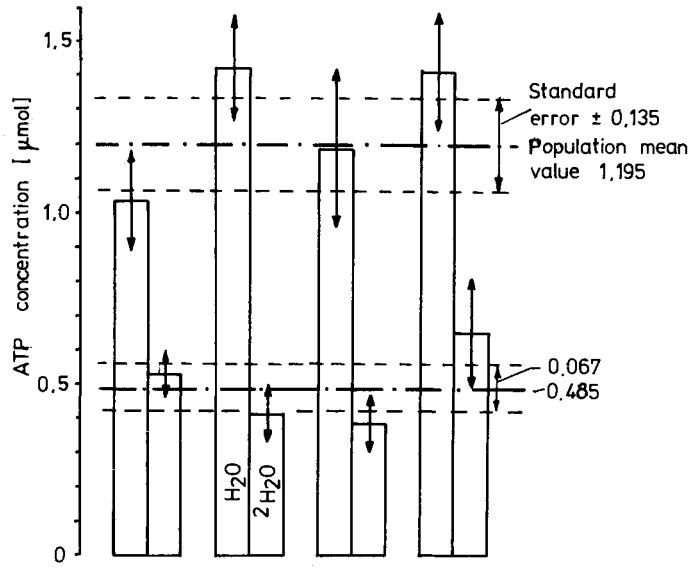

Fig. 13. $^{2}H_2O$ effect on cellular ATP content in retina. In each group of experiments mean values and sample standard deviations for 5 pairs of retina are represented.

Possible role of protons in bioenergetics

The observed changes in the cellular ATP pool are possible to be accounted for by the differences between proton and deuteron quantum properties, mostly as concerns the strength of intermolecular forces and the mobilities of ions in $^{2}H_2O$ and H_2O respectively.

$^{2}H_2O$ substitution can affect the mechanisms of ATP hydrolysis and its production as well. William's idea (33) that ATP synthesis is promoted by a relatively "dry" proton offers a clue to explain how deuteriation can influence ATP synthesis. Indeed the deuteron keeping more strongly water molecules is less "dry" than the proton and consequently leads to a slower ATP synthesis.

On the other hand this pronounced decrease in the ATP concentration could be partially explained by a change of the ATP $\rightleftharpoons$ ADP equilibrium under the influence of heavy water. After George et al. (34) the equilibrium equation might be written as follows:

$$ADP(aq) + P_i(aq) \overset{ATP+water}{\rightleftharpoons} ATP(aq) + H_2O(l)$$

where (aq) indicates that the species is in aqueous solution, therefore hydrated, and H_2O represents water in liquid state. As a function of water affinities of the species involved in reaction the equilibrium position,

then the reaction sense may be modified (35), namely by increasing the hydration degree the equilibrium is shifted to left, therefore to ATP hydrolysis.
By replacing a large part of water in biological systems with heavy water a change in the hydration degree of different species occurs: as deuteron bond is stronger than hydrogen bond, the hydration degree will increase. ADP $\rightleftharpoons$ ATP equilibrium should correspondingly undergo a marked shift to left, then to ATP hydrolysis.

CONCLUSIONS

1) The existence in excitable tissues of at least three water compartments having different physical properties is revealed by kinetical studies of H_2O-^2H_2O exchange; through these compartments whose size depends on tissue, the water passage takes place without surpassing high energy barriers.
2) ^2H_2O permeation into tissues is a slow process, the deuteriation of different tissue compartments having continuously water accessibility for diffusion exchange proceeds gradually.
3) Deuteriation is an excellent non-destructive method for studying the possible role of water and protons in biological systems.
4) ^2H_2O substitution for tissue water modifies the kinetics of excitability processes, namely it causes a substantial slowering in bioelectrogenesis processes. If the excitable tissue is continuously stimulated, the ^2H_2O substitution leads to a gradual and reversible abolishment of the various electric manifestations of excitability processes.
5) ^2H_2O substitution for tissue water induces the transition of biopolymers into a more compact conformation, leads to changes in the membrane structure and in active sites of membrane enzymes, thus interfering with the tissue energetic mechanisms and increasing the ATP demand.
6) In view of the differences between proton and deuteron physical properties, ^2H_2O substitution may affect the mechanisms of ATP-synthesis.
7) The changes in the various physical events related to different tissue functions may be correlated with the deuteriation of different tissue compartments or/and with a diminution in the tissular energy pools also caused by deuteriation.
8) Water protons appear to have a role not only in determining the supramolecular architecture of excitable membranes, but also in establishing the dynamics of cellular processes by means of the energy-excitation coupling.

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MECHANISMS OF CELL ADHESION TO CELLULAR AND NON-CELLULAR SURFACES

Ju. M. Vasiliev and I. M. Gelfand

Oncological Scientific Center of the Academy of Medical Sciences of USSR; Laboratory of Molecular Biology and Bioorganic Chemistry of the Moscow State University, Moscow, USSR

Abstract - Adhesion of cells to various surfaces is mediated by special active cellular reaction, pseudopodial attachment reaction. Only the membrane of pseudopodia and not that of other cell parts is able to form specialized contact structures. Mechanisms of pseudopodial reactions involved in adhesion of normal and neoplastic tissue cells and of blood platelets are discussed.

Mechanism of cell adhesion to various surfaces is a major unresolved problem of molesular and cellular biology. A number of data suggests that adhesion is a result of active cellular reaction involving surface movements of the two types: alteration of shape of the whole cell surface and movements of plasma membrane receptors within the plane of the membrane. An active cellular reaction leading to adhesion, so called pseudopodial attachment reaction (Refs.1,2) consists of the three stages:
a) The contact of the cell membrane with another surface induces an extension of pseudopodium in the nearby area of the surface.
b) The membrane of pseudopodium forms specialized contacts with another surface.
c) Alteration of submembraneous cortical layer, especially, formation of the bundle of actin microfilaments leads to development of centripetal tension acting on the contact.
Two groups of facts obtained in the experiments with cultured epithelial and fibroblastic cells (see review in Ref.2) support the suggestion that pseudopodia play special role in the formation of cell contacts with other cells and with the non-living supporting substrata of the culture:
1) Cultured epithelia of various types form coherent cellular sheets in culture. Pseudopodia are formed only on lateral surfaces of these sheets but not on their upper surfaces. Inert particles such as carmine particles, red blood cells etc are easily attached to the surfaces of marginal pseudopodia but not to the inactive upper surface. Formation of contacts with homologous epithelial cells is also observed only in the marginal areas.
2) Fibroblasts and epithelial cells contacting non-living substrates (glass, plastics etc) spread on these substrata. Numerous focal cell-substrate attachments are formed in the course of this spreading. Analysis of this process shows that cell contact with the substrate induced formation of pseudopodia on the cell surface. Focal attachments with associated bundles of microfilaments are formed only in the pseudopodia.
These experiments suggest that specialized cell contacts with other structures can be formed only by the surface of pseudopodia but not by the surface of other cell parts. What are possible mechanisms of special adhesion properties of pseudopodia?
Besides their different adhesiveness, active and non-active parts of the cell surface have also different ability to be cleared from the membrane receptors cross-linked by external ligands. This was shown in the experiments with mouse kidney epithelium treated with different ligands such as Concanavalin A or cationized ferritin (Refs.1,3). Initial distribution of the receptors of these ligands on the surface of epithelial cells was diffuse. Treatment of living cells with one of the ligands induced redistribution of corresponding receptors. In epithelial cells, like in other cell types (Refs.1,3), redistribution of cross-linked surface receptors

had two components: collection of these receptors into microscopically visible groups (patching) and selective removal of the patched receptors from certain parts of the surface (capping or clearing). Patching was observed on all the parts of the upper surfaces of epithelial sheets. In contrast, only the pseudopodia and lamellar cytoplasm of the marginal cells of the sheets were cleared from patched receptors; clearing was not observed on the upper surface of the inactive central cells of the sheets. Selective clearing of pseudopodia from cross-linked surface receptors was also observed in other cell types (Refs.1,3-7). Patching is usually regarded as a passive aggregation of molecules diffusing in a fluid lipid bilayer. In contrast, capping is an active energy-requiring process. Several theories of the mechanism of this process had been proposed. One plausible suggestion (Ref.4) is that the capping is a result of anchoring of the patched receptors to some cortical structures, eg., to microfilaments; these cortical structures may then actively translocate the patches in the plane of themembrane. Developing this hypothesis we (Ref.1) assumed that anchoring of the patches can take place only in active parts of the cell surface. This suggestion explains not only selective clearing of the patched receptors from these parts of the surface but also their special adhesive properties. Formation of adhesive structure between the cell membrane and another surface may be somewhat similar to patching and capping. The contact structure may be regarded as a group of membrane receptors linked from the outside to the molecules of the other surface directly or via some intermediate molecules. This group of receptors is equivalent to a patch formed by external ligand. Specific ligands and receptors forming different types of contact structures are still to be indentified. The "contact patch" will be unstable in a sense that it may be easily displaced in the plane of the fluid membrane or even detached from this membrane. This displacement and detachment can be caused by the tension from the contacting cell (Refs.8,9) or by any other mechanical stress acting from the contacting surface. The anchoring of the patched membrane molecules to the underlying cortical structures may stabilize the contact. Non-adhesiveness of inactive epithelial and endothelial sheets may be due to the inability of their surface to anchor adequately the membrane receptors attached to the external ligands.

The facts and hypotheses summarized above provided the basis for planning of a number of new experiments. In particular, two series of experiments recently performed by our group should be mentioned: experiments testing adhesiveness of epithelial surface for blood platelets and experiments testing adhesiveness of the surfaces of artificial lipid films for blood platelets and fibroblasts.

Our experiments had shown that upper surface of cultured mouse kidney epithelium is non-adhesive not only for tissue cells and for inert particles but also for rabbit blood platelets: the average number of platelets attached to epithelial surface was 8-12 times less than that attached to the same area of the glass within the same culture (Ref.10).

The endothelial sheet covering the internal surface of the blood vessels is similar in structure to the epithelia sheets. Therefore, these experiments give reason to think that non-adhesiveness of endothelium and of epithelium may have similar mechanisms, that is, in both cases non-adhesiveness may be due to the absence of pseudopodial activity on the upper surface of these shests (Ref.11). Non-adhesiveness of endothelium for platelets is essential for prevention of blood clotting in the vessels; therefore its mechanism certainly deserves further study.

Adhesive properties of articial lipid membranes are interesting because their surfaces may imitate to some degree the surfaces of cell membranes. Fibroblasts and blood platelets cannot attach themselves to the surfaces of the films made from phospholipids which are in a liquid-cristalline state at 37°C. In contrast, the cells of both types are attached to the surface of the lipids which are a cristalline state at that temperature (Refs.12,13). As suggested by Maroudas (Ref.9) non-adhesiveness of the fluid lipid films may be due to their inability to withstand tension from contacting cellular pseudopodia. According to our hypothesis presented above non-adhesiveness of the inactive cellular surface may have similar explanation.

Another very important aspect of the studies of cell adhesiveness is an analysis of the mechanisms of deficient attachment reactions of neoplastic cells. Deficiency of spreading on the non-living substrates is a characteristic feature of neoplastic transformed cells in culture; formation of cell-cell adhesions is also deficient in the cultures of these cells (see review in Ref.2). Several types of molecular alterations may be respon-

sible for the deficiency of pseudopodial attachment reactions of neoplastic cells:
a)deficient formation of extracellular ligands participating in the cell-cell and cell-substrate adhesion. The major glycoprotein of the fibroblastic cell surface (so called LETS or fibronectin) may possibly play a role of such ligand; the quantity of this glycoprotein in the cultures of fibroblasts often decreases considerably after neoplastic transformation (Refs. 14-16).
b) deficiency of cellular receptors participating in adhesion, especially, deficient formation of the carbohydrate portions of these receptors.
c)deficient anchoring of the membrane receptors by cortical components; absence of microfilament bundles in the substrate-attached neoplastic fibroblasts (Refs.17,18) may be a morphological manifestation of this deficiency.
d)abnormality of the lipid layer leading to alterations of receptor movements.
Further studies are necessary before we be able to select between these possibilities.

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BIOELECTROCATALYSIS AS A NEW PHENOMENON

I. V. Berezin and S. D. Varfolomeev

Department of Chemistry and A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 117234, USSR

Abstract - The paper describes a theory for constructing electrocatalysts based on immobilized enzymes. High-rate enzymatic electron-transfer reactions in solutions and heterogeneous are analyzed. Macrokinetics of electrocatalysis with enzymes anchored to equiaccessible surface electrodes is discussed, and a number of expressions interrelating electrode potential and current output on the one hand, and kinetic and microkinetic characteristics of the system on the other, are derived.
Electron transfer from the enzyme active center to the conducting matrix is discussed. Two mechanisms are considered: the direct electron transfer mechanism and the mechanism involving intermediate carriers (mediators).
The technological potential of immobilized enzyme electrocatalysis is surveyed (bioelectrochemical converters, biophotolysis of water, specific electrosynthesis, biochemical sensors).

INTRODUCTION

Much progress has been made in the last decade in the development of catalysts, especially for hetero-catalytic, homogenous catalytic, and enzymic reactions. However, catalytic electrochemical transformations have not been well studied. Theoretical and experimental studies in this field have been, as a rule, confined to the simplest possible systems, such as electrochemical hydrogen oxidation or oxygen reduction.

A variety of redox reactions, which involve electron transport in biological systems, are ensured by a set of redox enzymes and protein electron carriers and proceed at very high rates. Therefore by combining electrode and enzymatic processes, the study of enzyme properties in electrochemical systems becomes a challenge because of the high specificity and catalytic activity. Recently it was discovered that enzymes can act as the catalysts of electrochemical reactions.

Electrocatalysis by enzymes is very attractive from the practical point of view.

Enzymes as catalysts of electron transport may be useful in chemical-to--electrical energy converters. Biochemical fuel cell research can proceed along two lines (Ref. 1).

(a) the use of enzymes for oxidation of organic substrates such as alcohols and carbohydrates may lead to electrocatalysts for oxidation of organic fuels;

(b) the development of electrochemical enzyme converters of highly specific characteristics and power output may be possible if certain macrokinetic problems can be overcome.

Hydrogenases, enzyme systems which oxidize methane and methanol, glucoseoxidase, and dehydrogenases seem promising.

A biocathode may be developed through studies of enzymes which activate molecular oxygen. Especially promixing is cytochrome C-oxidase, which catalyzes the reaction of organic compounds with oxygen in aerobic respiration, and laccase, a similar enzyme of microbic origin. These enzymes catalyze four electron oxygen reduction without peroxide intermediates.

Enzymes and electron carriers are rather effective catalysts of electron

transports. The rates of enzymatic reactions as functions of substrate concentrations are usually represented as an equation of the form

$$v = \frac{k_{cat} E_o S_o}{K_M + S_o} \qquad (1)$$

where k_{cat} is the rate constant of the limiting process stage, and K_M is the Michaelis constant.

Statistical analysis of the rates of various redox processes with enzyme catalysts reveals that processes where k_{cat} is about 2×10^2 sec^{-1} are rather widespread. The maximum enzyme distribution density that has been found is compatible with such high reaction rates (Refs 7,6). If an enzyme whose high but quite feasible concentration is 5×10^{-3} M is introduced into a process with the enzyme saturated with substrate ($S_o \gg K_M$) the process goes at a rate of 1 mole/liter·sec. This rate of electron transport processes is associated with intermolecular microcurrents totaling about 10^5 ampere/liter. If the process could be made macrokinetic and if the electrochemical potential difference of two electrodes amounted to one volt, then the total power of this energy converter would be approximately 100 kW/liter, an extremely high value. Obtaining one per cent of that value would be major contribution to energy converter theory and development. These estimates stimulate bioelectrocatalysis research.

Making good use of the electrosynthetic potential of immobilized enzymes would also be an important step. The development of reversible immobilized enzyme-based electrodes could entail electrosynthesis. A number of compounds synthesizable with energy consumption (carbohydrates, aminoacids, and steroids) can in a number of cases be obtained by using immobilized enzymes and electric power. Another problem of this kind is electrochemical cofactor regeneration (Refs 2-5).

The high specificity of enzymes makes it possible to develop detectors of various compounds for quantitative analysis. The use of bioelectrocatalytic effects produced by direct electron transport from the enzyme active center to the electrode may significantly simplify the development of such systems and lead to miniature sensors which would be efficient because of the high rates of enzymatic reactions.

The promise of immobilized enzyme-based electrocatalysts is obvious; and the fundamentals now are under study. The subsequent discussion in this paper will be devoted to analysis of the fundamental problems in this field and approaches to their solution.

THE KINETIC THEORY OF BIOELECTROCATALYSIS

Any analysis of acceleration of electrode processes by enzymes should first of all account for the heterogenous nature of the system. Thus in one idealized model (Ref. 8), the enzyme was assumed to be uniformly distributed over the electrode surface with a surface concentration of E_S (moles/cm^2). The rate limiting step was assumed to be the reaction of ES in the enzymatic reaction sequence

$$E + S \underset{}{\overset{K_M}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E(ne) \longrightarrow E + ne \qquad (2)$$

where ES is the enzyme-substrate complex and E(ne) is the state of the enzyme active center which has accepted (or lost) the electrons. The surface with the immobilized enzyme was assumed equiaccessible. With due regard for the existence of a diffusion layer of thickness δ_d at the solid-moving liquid interface, the specific current may be written:

$$i = -\frac{nF\, k_{cat}\, E_S}{1 + \left\{ \frac{\frac{S}{K_M} - (1+\theta)}{2} + \sqrt{\left[\frac{\frac{S}{K_M} - (1+\theta)}{2}\right]^2 + \frac{S}{K_M}} \right\}^{-1}} \qquad (3)$$

θ is a dimensionales parameter found through the equation

$$\theta = \frac{k_{cat} E_s \delta_d}{K_M D} \qquad (4)$$

where D is the substrate diffusion coefficient, S^{δ} is the substrate concentration in the fluid volume.

The detailed analysis of these equations are given in Ref. 8 .

The parameter θ and the ratio S^{δ}/K_M determine whether the system will act under diffusion or kinetic control. Depending on these parameters the ratio S^o/S^{δ} can be either near or very much smaller than unity.

In the extreme case, with saturating substrate concentrations, $S^{\delta} \gg K_M$ the current from the electrode is

$$i_{max}^{cat} = - nF k_{cat} E_s \qquad (5)$$

The maximal specific diffusion current

$$i_{diff} = - nF DS^{\delta}/\delta_d \qquad (6)$$

The above analysis of the model leads to several conclusions:

(a) The maximum catalytic current which is expected with $\theta \ll 1$ depends on the surface concentration of the enzyme and the effectiveness of the enzyme catalytic action. With a monomolecular layer of medium size protein molecules covering the electrode surface, the enzyme surface concentration would be about 1×10^{-11} moles/cm^2. If the limiting rate constant of the enzyme catalyzed conversion was about 10^3 sec^{-1}, then the maximum catalytic current would be 1 milliamp/cm^2. Under kinetic control no concentration polarization occurs.

(b) Equation (6) can be used to estimate the value of the maximum diffusion current. Assume that the diffusion layer permeability is 10^{-3} cm/sec, the maximum diffusion current which should be observed with the electrode operating under diffusion control ($\theta \gg 1$, $S^{\delta} \ll K_M$) is 0.1 milliamp/cm^2 (with $S^{\delta} \sim 10^{-3}$ moles/l). This estimate emphasizes the importance of macrokinetic electrode characteristics.

Analysis of the macrokinetic model of enzymatic action in an electrochemical system shows that high current densities can be used. The most important requirement is that no concentration polarization occurs under the catalytic control. The electrode characteristics can be considerably improved by using macrokinetic factors. High porosity carbon carriers of specific surface about 100 m^2/g may make it possible to have electrodes featuring very favourable electrochemical parameters.

ELECTRON TRANSPORT FROM THE ENZYME ACTIVE CENTER TO THE ELECTRODE

The most important assumption in bioelectrocatalysis is that the active center of enzyme can have a fast exchange of electrons with the electrode. This process is a prerequisite for obtaining electrocatalytic effects by using enzymes. Two essentially different active center-to-electrode transport mechanisms are possible:

(a) Use of a mobile low molecular weight electron carrier or mediator. In Ref. 9 have been formulated a set of basic requirements for the mediator to be maximally effective. The mediator should be a reasonably specific substrate of the enzyme and electrolytically active at an electrode of a given material. The redox potential should be nearly that of the fuel or the oxidant. The mediator should be resistant to possible destructive processes.

The mediator mechanism of transport is extensively used in enzymatic electrochemical reactions. Suzuki et al.(Ref. 10) described an electrochemical process with lactatedehydrogenase, flavinmononucleotide and phenasinmetasulphate being used as mediators. Peroxidase activated by manganese ions in the presence of quinone-hydroquinone can catalyze the oxygen reduction on carbon electrodes (Ref. 11). Varfolomeev et al. (Ref. 8) have analyzed in detail the mediator properties of methylviologen and its use in electrochemical hydrogen ionization caused by bacterial hydrogenases.

(b) Experimental studies of recent years have revealed that enzymatic processes without the use of a mobile mediator are possible. Thus, in an oxygen atmosphere and in the presence of Poluporos versicolor adsorbed on electrodes of different materials, a potential is established which is close to the thermodynamic potential of oxygen. Also, laccase catalyzes the electrochemical four-electron oxygen reduction through electron transfer from the electrode to the active center (Ref. 12).

CONDUCTING AND SEMICONDUCTING ORGANIC MATRIXES FOR IMMOBILIZED ENZYMES

One general approach to provide direct electron transfer from the enzyme active center to the electrode is by using conducting and semiconducting matrixes for enzyme immobilization. Enzyme introduction into a polymeric matrix featuring high electric conductivity may ensure conditions for electron transport and electron transfer to the electrode having a low activation barrier. Ways to immobilize enzymes with electrically conducting carriers are now available. One of most promising materials for immobilized enzyme-based electrocatalysts is carbon.

Organic polymeric semiconductors represent a large class of potential carriers for bioelectrocatalysts. Electric conductivity of semiconducting polymers can vary from 10^{-15} Ohm^{-1} cm^{-1} to 10^{4} Ohm^{-1} cm^{-1} and approach that of metals (Ref. 13). Chemical modification of organic semiconductors leads to carriers of very high capacities, as far as the amount of immobilized enzyme is concerned. Enzymes can be attached to polymer semiconductors either by enzyme polymer bonding or by adsorbing the enzyme into a semiconducting water impermeable organic gels. Let us consider two examples.

Thermal anaerobic treatment may make polyacrylonitryl a conductor because of formation of poly-conjugated naphtiridine domains form. By the proper selection of the treatment temperature the size of the domains can be optimized and the resulting polymer conductivity may be made as high as 10^{-4} $Ohm^{-1}cm^{-1}$. For immobilization the polymer was oxidized by concentrated nitric acid, treated by hydrazine-hydrate and reduced by tin dichloride. The amino groups, which formed, were bonded to the enzymes by a standard procedure involving glutaraldehyde. The capacity of the electron conducting carrier was 10-100 mg of protein per gram of the polymer.

Varfolomeev et al. (Ref. 14) showed that enzymes can be immobilized in a conducting water impermeable organic "metals". The enzymes were immobilized by co-precipitation with poly-1-propargylpyridinium bromide and the semi-reduced form of tetracyanoquinodimethane (TCNQ). The mediator capacity was 500 mg of protein per gram. In this case the conductivity was obtained both by electron transport along the conjugation chain of poly-1-propargylpyridinium bromide and by transport between TCNQ molecules in the charge transfer complex.

IMMOBILIZED HYDROGENASE-BASE HYDROGEN ELECTRODE

Hydrogen electrodes, which ensure electrochemical ionization of hydrogen, are model systems which were used in developing many basic concepts of today's theoretical electrochemistry. Hydrogen electrodes are of considerable theoretical and applied interest. Their catalysts are chiefly platinum or metals of Group VII. Therefore studies of a hydrogenase-based hydrogen electrode is a promising line of research.

In Ref. 8 has been studied electrochemical hydrogen ionization under the action of hydrogenase from Thiocapsa reseopersicina. The electrode and electrochemical processes were coupled using methylviologen (MV^{2+}) as mediator. Methylviologen is a classical substrate of hydrogenases. The equilibrium and kinetics of its electrode reactions on carbon electrodes were studied. The normal redox potential of the pair MV^{2+}/MV^{+} is 0.011 volts, which is nearly the equilibrium potential of a hydrogen electrode. The electrode process is completely reversible; in other words, both electrochemical reduction and oxidization of the reduced methylviologen form can be observed. The electrochemical process

$$MV^{+\bullet} \longrightarrow MV^{2+} + \bar{e} \qquad (7)$$

was studied by using a rotating disc electrode; and oxidation-reduction of this compound was shown to proceed reversibly under diffusion control with the disc electrode rotating at rates up to 600 radians/sec. Electrochemical oxidation-reduction of this mediator is independent of the nature of the electrode used and is equally effective with platinum, amalgamated gold,

and pyrographite. The kinetics and mechanism of hydrogenase action with methylviologen (MV^{2+}) as the electron acceptor was studied in Refs 8,16 . The overall process sequence can be represented as

Enzymatic reaction

$$
\begin{aligned}
H_2 + E &\rightleftharpoons EH_2 \\
EH_2 &\rightleftharpoons EH^- + H^+ \\
EH^- + MV^{2+} &\rightleftharpoons EH + MV^{+\bullet} \\
EH &\rightleftharpoons E^- + H^+ \\
E^- + MV^{2+} &\rightleftharpoons MV^{+\bullet} + E
\end{aligned}
\qquad (8)
$$

where EH_2, EH^-, EH and E^- are intermediate electron states of the enzyme active center.

The following reaction proceeds electrochemically

$$
MV^{+\bullet} \overset{\text{fast}}{\rightleftharpoons} MV^{2+} \qquad (9)
$$

(pyrographite electrode)

The sequence (8) - (9) results in electrochemical ionization of hydrogen. With high enzyme concentrations the reaction changes to diffusion control with a current density of 0.4 mA per square centimeter of smooth surface, which is close to the theoretical maximum. Hydrogen ionization proceeds at equilibrium conditions on carbon electrodes, on which the reaction proceeds with great overvoltage if there is no enzyme.

Yaropolov (Ref. 16) studied the process of immobilization of hydrogenase on carbon black and the possibility of developing a porous gas diffusion electrode in this way.

It would be of essential importance to demonstrate the feasibility of a hydrogen electrode, operating without a diffusionally mobile mediator. Varfolomeev et al. (Ref. 14) described electrocatalysis by hydrogenase immobilized in a gel made of polypropargyl pyridine and the lithium salt of tetracyanoquinodimethane. Suspension electrodes were used to study the reaction of hydrogen evolution with anodic polarization of the electrode. The process was greatly accelerated by hydrogenase immobilized in this semiconducting organic "metal". The specific hydrogenase activity for an enzyme immobilized in such a gel was approximately five times that of the enzyme in solution. Further study of enzyme properties in TCNQ-based gels have demonstrated the promise of this method in development of high-porosity electrodes.

IMMOBILIZED LACCASE-BASED OXYGEN ELECTRODE

The development of biochemical fuel cells is dependent on the oxygen electrode for those cells where oxygen reduction is the cathodic reaction. The creation of reversible oxygen electrode is one of the most important problems in traditional electrochemistry. The equilibrium potential of the oxidation-reduction pair O_2/H_2O of 1.23 volts is obtainable only on specially treated platinum and in especially pure solutions (Refs 17,18). Exchange currents on platinum are as low as 10^{-11} amp/cm^2.

There are a few enzymes which smoothly reduce oxygen to water via a four electron mechanism without hydrogen peroxide formation as an intermediate. These enzymes include cytochrome C-oxidase, ceruloplasmine, ascorbatoxidase, and laccase. For bioelectrocatalytic purposes these enzymes may be regarded as potential catalysts of cathode oxygen reduction. Yaropolov et al. (Ref. 11) and Berezin et al. (Ref. 19) have described systems where peroxidase and cytochrome C-oxidase were the catalysts of oxygen reduction. Mediators transfered electrons from the electrode to active centers. The electrode potential, equal in this case to the ratio of the reduced and oxidated mediator forms, was 0.6-0.8 volts, below the equilibrium oxygen potential.

Berezin et al. (Ref. 12) studied an oxygen electrode where electrons were directly exchanged with the active center. The catalyst was Poluporos ver-

sicolar laccase a sample of which was kindly supplied by Professor B.Malmström (Sweden, Göteborg).

Electroreduction of oxygen in neutral or weakly acid solutions considerably shifts the stationary potential towards positive values and accelerates electroreduction of oxygen.

Electrochemical measurements were made on electrodes of carbon black, pyrographite, carbon glass, or gold, laccase was adsorbed directly on the electrode that has been kept in the enzyme solution for 24 hours. The electrode potential increased in the presence of oxygen and laccase. The maximal potential value of 1.207 volts near equilibrium, was obtained on carbon black electrodes that had been kept in a 10^{-5}M laccase solution for 24 hours.

Enzyme adsorption on electrodes may be practically irreversible. Following the immobilization of the enzyme, the electrode retains catalytic properties in the absence of laccase in the solution. The enzymic nature of electrocatalysis was proved by specific inhibition of electrocatalytic effects by fluoride ion, by heat inactivation, by comparison of pH dependence of electrocatalytic effects, and by catalytic activity in the oxidation of the ferricyanide ion by oxygen. The electrode stationary potential depends on the partial oxygen pressure and pH. To ascertain the nature of the stationary potential on an electrode with immobilized laccase, φ_{st} was studied as a function of the partial oxygen pressure (P_{O_2}) and the solution pH. It was found that $\partial\varphi_{st}/\lg P_{O_2}$ amounts to 10-12 mV and $\partial\varphi_{st}/\partial$ pH, to 60 mV. These results were close to coefficient values for the Nernst equation for the O_2/H_2O system. In a special experiments on a rotating disc electrode no detectable hydrogen peroxide was found in the solution in the case of electrochemical oxygen reduction by immobilized laccase. All available experimental data suggest that the observed stationary potential on an electrode with immobilized laccase is determined by the four electron oxygen reduction to water. The current output with a specified potential depended on the amount of immobilized laccase and attained a limiting value as predicted by the kinetic theory of biocatalysts. The above results suggest the feasibility of enzymatic electrocatalysis of oxygen reduction using direct electron transport without mediators along the electrode - active center - oxygen molecule chain.

CONCLUSIONS

An attempt has been made to formulate basic problems that face the use of enzymes for power generation. The attention is focused on a new phenomenon - bioelectrocatalysis - acceleration of electrode processes by enzymes. The use of enzymes makes it possible to formulate and theoretically solve the problem of chemical energy conversion into the electric power. There are some model systems which have been experimentally tested and are being optimized. On the other hand, only theoretical approaches to engineering implementation of these processes are now available. However the basic research completed thus far gives hope that new biocatalytic power units will become a reality.

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