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The Major Histocompatibility Antigens in Various Species

(Comparative aspects of major histocompatibility systems)

Pavol Iványi

With 10 Figures

Contents

"The plasticity of such a gene suggests that its complexity is itself the quality which has been selected for;"

L. C. DUNN, 1956

I. Introduction

A review aiming to give a short survey of comparative aspects of major histocompatibility systems (MHS) in different species would certainly be much too lengthy if it did not limit itself to selected aspects. The main object of this review is simply to point out that, in all species which have been examined in sufficient detail, the genetic systems show remarkable similarities. The second aim of this review is to try to point out the possible general significance and/or the common evolutionary forces which could have led to the development of these genetic complexes. This review goes into many hypotheses and presumptions, a number of them being the expression of the author's personal conception, which may serve as a working hypothesis. We shall give more space to recently published data. These data will be discussed in detail, altough some of them are not yet fully understood or confirmed, as they may serve for alternative explanations.

The review follows previous articles on comparative aspects of cellular alloantigens published by IVÁNYI (1966a) and SNELL (1968).

Before beginning, it is necessary to elucidate at least two terminological points.

1. Antigenic complexity of the system. Many analyses of histocompatibility systems are based on the serological approach. Antigenic specificities are determined by monospecific sera. Nevertheless, in a complex system where the final result depends on the number of sera tested and their origin (from one or more populations and from selected, or randomly chosen, typing reagents) and on the number and origin of cell samples tested, after a certain limit of simplification, situations arise in which the definition of an antigenic specificity is not absolute. That is, segregating entities (antigens) can be further subdivided without a clear outlook of the final solution. The Rh 1 (Rh_0, D) antigen

serves as the best example[1]. Doubts arise about the definition of monospecificity, which remains operational (WALFORD and TROUP, 1967; WALFORD et al. 1967; KLOUDA et al,. 1969). Defined ("monospecific") sera are recognized as relatively, operationally or effectively monospecific. The serological findings in the human HL-A system reopened the problem for the definition of an alloantigen (PAYNE et al., 1964; BODMER and PAYNE, 1965; IVÁNYI and DAUSSET, 1966; DAUSSET et al., 1965a, 1967c; MICKEY et al., 1967a, b; VREDEVOE et al., 1966; TERASAKI et al., 1966).

All this makes communication difficult from the point of view of terminology. In this review, following some aspects of a hypothesis about the nature of the complexity of alloantigenic systems (IVÁNYI and DAUSSET, 1966), we shall use the terms: a) antigenic specificity as a neutral descriptive term, b) antigen and c) antigenic factor. The differentiation between antigen and antigenic factor is operational and is applied to certain situations, if it is necessary, or possible, to differentiate between a less exactly defined complex and the existing, but not defined, subunits composing it. The term, "antigen", is used for a complex of antigenic factors associated more closely with each other than with other antigenic factors in a *given population*. The term, antigenic factor", is used for the designation of units composing the complex. It is preferred to the terms, "determinant, component, partigen, subfactor, etc.", because it is more neutral and thus serves better for operational use.

[1] "Further problems arise when more than two components of a complex are involved in the variation of the antigenic mosaic. The Rh 1 antigen is the most studied example of such a situation. The variants of the Rh 1 antigen which do not react with all anti-Rh 1 sera and the Rh 1 individuals producing 'anti-Rh 1' antibody were interpreted as mosaics lacking one or more factors present in all normal Rh 1 antigens (UNGER et al., 1959). WIENER and UNGER (1962) analysed the specificity of 'anti-Rh 1' sera produced in Rh 1 individuals and recognized among them reagents detecting antigens (blood factors) Rh 13, Rh 14, Rh 15 and Rh 16 (Rh^A, Rh^B, Rh^C, Rh^D). These antigens are associated with the antigen Rh 1 and are present in almost all Rh 1 individuals. When a rare individual, lacking one or more of these antigens, becomes sensitized with red cells bearing a complete mosaic of the Rh 1 plus the associated antigens, it can produce an antibody against the absent antigen (antigenic factor). Since this factor is present in almost all Rh 1 individuals, the antibody against it resembles as to its specificity the usual anti-Rh 1 serum and differs from it only in negative reactions with the red cells of the individual who has produced it and with red cells of some other rare Rh 1 individuals. However, this method of analysis of the Rh 1 antigen failed to explain conclusively all features of the Rh 1 variants. The specificities of sera produced by Rh 1 individuals differ from each other even if the individuals who produced them lack the same antigen (e.g. Rh 13) in their mosaic. It was impracticable to extend the number of defined associated antigens beyond certain limits because of the impossibility of determining whether a new serum contains antibody(ies) against one or more associated antigens (WIENER and UNGER, 1962). Moreover, with the increasing number of such sera, the specificity of previously studied sera becomes doubtful. The practical impossibility of complete resolution of the Rh 1 complex is recognized by WIENER and UNGER (1962), ROSEFIELD et al. (1962) and TIPPETT and SANGER (1962). The resolution of antigenic specificities by usual methods becomes impracticable as the complexity of antigens under study exceeds certain limits" (DÉMANT et al., 1967).

2. The most serious obstacle to communication is the double sense of the term, "allele". As stated by SNELL et al. (1964), "The word allele has been employed in mouse immunogenetic terminology in two rather different connotations. It has been used to designate a particular H-2 combination behaving as a unit transplantation studies. Thus H-2^h is one of 18 alleles of H-2. It has been used also to designate alternative forms of the hypothetical loci believed to determine individual specificities."

In the present work, the term, "allele", will be used only for the designation of a chromosomal locus responsible for the determination of a set of antigenic specificities. We would like to emphasize by this that at the present time we do not believe that it is possible to determine which antigenic specificities in the framework of a complex system are determined by the alternative subregions of the locus. Antigenic specificities which behave as though determined by alternative subregions will be designated as antigenic specificities with alternative distribution or simply alternative antigenic specificities. We do not think, for example, that HL-A.1 and HL-A.2 are specificities determined by allelic subregions of the HL-A gene but antigens alternatively distributed in the given population. In the populations studied, the HL-A alleles $1+2-$, $1-2+$ and $1-2-$ are rather frequent, while the HL-A allele $1+2+$ is rare or absent. Nevertheless, this "rare" HL-A allele can possibly be found frequently in other populations.

II. Estimates of the Number of Histocompatibility Systems

A great number of findings provide evidence that there is one Major Histocompatibility System (MHS) for each biological species. This term is a general one and implies that the system represents a "strong" histocompatibility system, a term about which SNELL and STIMPFLING (1966) wrote: "Once pairs of congenic resistant lines with single histocompatibility differences were available, it became possible to characterize individual histocompatibility loci in a way that had been quite impossible when all information was based on comparison of inbred strains with multiple differences or the segregating generations derived therefrom. Not surprisingly, the different loci showed marked individuality.

"One of the first differences that became apparent was the magnitude of the barriers which different loci oppose to transplants. Some of the barriers can be easily transgressed, others are transgressed only with great difficultics. This has been referred to as differences in the "strength" of the loci (COUNCE et al., 1956). This is not an altogether happy expression but the usage is now well established and, in the absence of any better alternative, we shall follow it here" (SNELL and STIMPFLING, 1966). The same is true of the term, "MHS", which thus becomes an *operational* concept. Nevertheless, the great number of similar characteristics shared by the MHS of different species leads us to the presumption that common or similar developmental events (forces) played an important role in the course of their development.

The data originating from studies in chickens, rats, mice, and humans, supplemented by preliminary data from studies in primates, rabbits, dogs, cattle and pigs, will be discussed in this review.

A. The Number of Histocompatibility Loci in Different Species

Skin grafts, which are sensitive indicators of histocompatibility, are generally rejected when transplanted between unrelated, randomly chosen individuals. Even skin grafting experiments using donor-recipient pairs from non-inbred populations are not sufficiently explored. Beginning with MEDAWAR'S work on rabbits (1944, 1945), and including hundreds of grafts performed in the last few years by various investigators in humans, the author of this review does not know of any permanently surviving graft between randomly chosen individuals. While this could be basically explained by one sufficiently polymorphic system (and we shall see in the chicken and in the humans that this is not far from being the truth), this finding strongly indicates the existence of a large number of segregating histocompatibility loci. An approach to this number was obtained by grafts transplanted in sib-mating or backcross populations of mice, rats, guinea-pigs, chickens and rabbits (BARNES and KROHN, 1957; PREHN and MAIN, 1958; CHAI and CHIANG, 1963; BAILEY and MOBRAATEN, 1964; BAILEY, 1967; BILLINGHAM et al., 1962; BAUER, 1960; HÁLA, 1967a; CHAI, 1968). The number of segregating loci in guinea-pigs and chickens was estimated as 4 to 6 and 7 to 10, respectively, while in rats and mice the numbers from similar experiments were 14 to 17. It was argued (GILMOUR, 1964) that these estimates had been too precise. The fiducial limits of the above numbers for mice were 10—32 (95 %) or 9—37 (99 %). BAILEY and MOBRAATEN (1964) obtained the number 29—32 as a minimum estimate of the number of histocompatibility loci (hereafter H loci) differences between BALB/c and C57BL/6 strains. The numbers obtained should be considered as limits which could be obtained by the approaches used in the given experiments, i.e. not more than 30 segregating H systems could be estimated by the skin grafting method in the given mouse populations. In all these experiments the numbers were calculated on the basis of a relatively small number of surviving grafts with the presumption of no linkage, no selective advantage in favour of heterozygotes, and an absence of recessive H genes. "Such estimates are severely restricted in that they include only those H loci at which the two parental strains carry distinctive alleles and only those H loci at which the alleles determine antigens strong enough to elicit graft rejections during the observation period (usually 200 days). How many other H loci there are has remained undetermined". (BAILEY, 1967).

The low number of segregating loci in experiments on chickens and guinea-pigs may reflect a common origin of the lines tested, similar to the results obtained in Syrian hamsters, where not more than three loci are necessary to account for the rejection of skin homografts interchanged between members of three different strains (BILLINGHAM et al., 1960).

The conclusion from the above data is that not less than 30 segregating loci exist in mice and possibly in other species. Finally, it should be pointed out that the term "segregating loci" is operational, and based on premises not clearly defined. The present state of knowledge indicates that the chromosomal regions representing H loci can be subdivided, as demonstrated by a recombination of 0.3—0.5 % in the H-2 system (STIMPFLING and RICHARDSON, 1965; SHREFFLER, 1965). A gene for a serum group Ss is "inserted" in the H-2 region, between the subregions E and K (SHREFFLER, 1965). This illustrates the difficulties in defining *segregating* versus *linked* or *closely linked* H loci. In a different approach, the minimum value of genetic sites at which the determinants of tissue antigens are located was 430 (BAILEY, 1967). This number represents the result in a model situation and further experimental data are needed for a more precise estimation of this number.

B. The Number of Strong Histocompatibility Loci in Different Species

Allogeneic skin graft survival varies from 8 days (single strong H-2 difference or possibly multiple weak H differences) to more than 200—300 days (single weak H difference). The term strong transplantation antigen defines operationally an alloantigenic difference which results in a short survival time (for convenience 13—15 day survival time). Skin grafts between inbred strains with different H-2 alleles never survive beyond this time. It is presumed that probably all histocompatibility systems are more or less polymorphic. The difference produced by different alleles of the same system can represent strong or weak histocompatibility differences. In some weak systems the existence of strong differences cannot be excluded, though until now all weak systems have been characterized by a survival time longer than 15 days.

Strong histocompatibility *systems* are operationally defined as systems which generally or very frequently produce strong histocopatibility differences, while weak histocompatibility systems produce predominantly weak histocompatibility differences. This means that the finding of KLEIN (1966) that some H-2 combination reduced to a difference in one H-2 specificity allows late skin graft rejection (some grafts surviving even longer than others in weak systems) does not invalidate the definition that skin grafts between mice with different H-2 alleles are, as a rule, rejected by the 15 th day, while in weak systems the rejection occurs later[2].

With congenic strains of inbred mice the strength of 11 H loci could be measured individually (SNELL and BUNKER, 1965; GRAFF et al., 1966; SNELL et al., 1967). From H-1, 2, 3, 4, 7, 8, 9, 10, 11, 12, 13 and the sex-linked X

[2] This question requires some caution. It is possible that if in the future the polymorphism of H-2 is extended by a greater number of further alleles probably derived from wild populations of Mus, a great number of "minor" differences will be observed. This could diminish the sharp difference as it is presently given by the unique features of the H-2 system. There are preliminary data indidating the existence of such minor differences (SNELL et al., 1953; GORER, 1956; SNELL and STIMPFLING, 1966).

(BAILEY, 1963) and Y (EICHWALD et al., 1958) loci only H-2 could be classified as a strong system. The median survival times for the various loci ranged from 15 to more than 300 days, whereas skin grafted between mice of different H-2 alleles is rejected in less than 15 days. Further experiments with new congenic lines could discover a second locus comparable in strength with H-2 but this

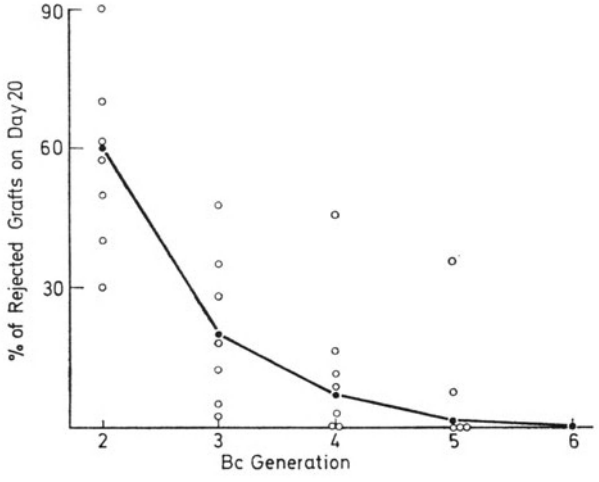

Fig. 1. Absence of a second strong histocompatibility difference between inbred mouse strain A versus C57BL/10ScSn(B10). (A × B10) F_1 hybrids were backcrossed to B10 females. Skin grafts were transplanted from a number of Bc1 individuals to B10 mice. H-2 compatible ($H\text{-}2^b/H\text{-}2^b$) mice whose skin was rejected within less than 15 days were selected for further Bc matings with B10 recipients and *all* those of their progeny whose skin was rejected within less than 20 days were further backcrossed to B10 females. This was repeated as long as any individual in the successive generation could be obtained whose skin was rejected within less than 20 days. Absence of such individuals in Bc 3—5 generation indicates with high probability the absence of a second strong H difference in the example thus studied (IVÁNYI, 1969). *o* mean values from the progeny of the seven animals selected at Bc 1 generation

is not likely. Experiments were carried out to test the possibility that H-2 represents the only strong H system in mice (IVÁNYI, 1969). When (A × B10) hybrids were successively backcrossed, the mice whose skin grafts were rejected by the 20th day on the parental (B10) strain were selected for further mating. After *eliminating* the H-2 difference in Bc_1 individuals, no strong histocompatibility difference could be found after 3—5 Bc generations (Fig. 1). Obviously (H-2 compatible) skin grafts were rejected by the 20th day (in the first few Bc generations), due to the synergic effect of weak H loci. The existence of a synergic effect of weak loci has been demonstrated previously (MCKHANN, 1964a; KLEIN, 1965; GRAFF et al., 1966b).

There are no similar analytic data in other species. The number of H loci calculated from parental skin graft survivals on F_2 hybrids in rats was 14—16. From the survival curves it was concluded that "the products of relatively few, 3 or 4, are singly sufficient to elicit homograft rejection within 14 days,

and that the majority appear to determine weak antigens of varying potency" (BILLINGHAM et al., 1962). Nevertheless, an alternative possibility is that there is only one strong locus (the rat H-1 system, see later), while the synergic effect of weak loci is responsible for rapid skin rejections. The finding that the rat H-1 systems basically decides kidney (WHITE and HILDEMAN, 1968) and tooth germ (IVÁNYI, D., 1966, 1968) survival strongly supports the second explanation. A similar observation with ovarian skin grafts (HICKEN and KROHN, 1960; LINDER, 1961) argues in favour of the unique position of H-2 in mice.

The existence of only one strong histocompatibility system in every species can be proved directly only with difficulty or not at all (in humans, for example) by the grafting technique. However, a quantity of indirect evidence supports this possibility in other species.

III. The Strong Histocompatibility Loci in Different Species

A. The B System in Chickens

Twelve blood group systems were described in chickens (for review see GILMOUR, 1960; MCDERMID, 1964). SCHIERMAN and NORDSKOG (1961) demonstrated that the blood group system B is a strong histocompatibility system. Skin grafts transplanted between 16-day-old chickens survived in B blood group incompatible donor-recipient combinations for 12 days only, while in B-compatible combinations all skin grafts survived for 40 days, 20% of them were rejected and the remainder survived for a longer time. Several approaches (HAŠEK et al., 1961), including lymphoagglutination by anti-B sera (SCHIERMAN and NORDSKOG, 1962), proved the presence of B antigens on white cells. Immediately after this it was also found that chorioallantoic membrane foci production, as well as splenomegaly after inoculation with adult leucocytes was principally due to B locus incompatibility (JAFFE and MCDERMID, 1962; SCHIERMAN and NORDSKOG, 1963). Congenic lines differing only at the B system were produced (HAŠEK et al., 1966; HÁLA et al., 1966); skin grafts were rejected between them in less than 15 days.

It should be pointed out that chickens represent the only species in which

1. the second histocompatibility system has been serologically well defined. This is the C system, which is nevertheless a weak H system, as the incompatible skin grafts survive longer than 60 days (SCHIERMAN and NORDSKOG, 1965).

2. Testing of congenic strains differing at the A blood group system only has shown that A blood group incompatible skin grafts can survive permanently in spite of pre- or postimmunization leading to anti-A haemagglutinin formation (HÁLA et al., 1966; HÁLA, 1969).

The B system is a polymorphic immunogenetic system. As the population studies on H antigens were done only in this animal species, more details will be presented here.

The B system in chickens is a serologically well defined blood group and H system. Chickens are excellent producers of anti-B haemagglutinins. As a rule, after 2—3 injections of 1—2 cc. of blood, they produce B-haemagglutinins reacting in a relatively high titre in simple saline agglutination. In the course of random immunization in randomly bred chicken populations predominantly anti-B sera are produced. This is the routine procedure by which the products of several B *alleles* are distinguished.

Table 1. *The MHS in chickens; the B system*

The complex nature of the B system antigens in chicken

Type strain	B alleles	Antigenic specificities												
		A	B	C	D	E	F	G	I	J	L	X	Y	Z
CA[a]	B^1	−	−	−	−	−	−	−	−	−	+	−	+	+
CD[a]	B^2	+	+	+	+	+	+	+	+	+	−	+	−	+

Type strain	B alleles	Antigenic specificities														
		A	B	C	D	E	F	G	H	I	J	K	L	M	O	P	Y
RIR[b]	B^6	−	−	−	−	+	−	+	−	+	+	−	−	−	+	−	−
	B^8	−	−	−	−	−	+	−	−	−	−	+	−	+	−	−	−
	B^9	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−
WLBa[b]	B^{11}	−	+	−	−	−	+	+	+	+	−	−	−	−	−	−	+
	B^{12}	+	−	−	−	−	−	−	−	−	+	−	+	+	−	+	−

[a] CA and CD are congenic lines differing at the B system only, with B^1 and B^2 alleles respectively. Anti-B1 and anti-B2 reagents were prepared by reciprocal immunization and analysed by absorption experiments by use of blood samples from outbred individuals. B1 animals produced antibodies against at least 10 specificities of the B2 complex and B2 animals recognized at least 2 specificities of the B1 complex (from HÁLA et al., 1966).

[b] R.I.R. and W.L.Ba are not inbred chicken lines. The specificities A—Y were determined on birds homozygous for the B system. The specificities were approached from the reaction pattern and absorption experiments with a set of anti-B reagents (from OOSTERLEE and BOUW, 1966).

On the other hand, the B system represents perhaps the only thoroughly studied H system in which the whole antigenic product of an allele (which obviously produces a number of serologic specificities) is today designated as "antigen". Only recently preliminary data were published describing the individual antigenic specificities produced by one B allele (HÁLA et al., 1966; OOSTERLEE and BOUW, 1966). In the earlier stages of the work on the B system attempts were made to distinguish between antigens: complex products of alleles and their individual antigenic specificities. Monospecific sera were produced by absorption experiments but when these were tested on different populations it was found that they remained in fact polyvalent. After unsuccessful experiments to define the discrete serological specificities produced by B alleles, the nomenclature was changed and the term antigen was used for the whole product of an allele, though it was clear that this can easily be sub-

divided into many factors whose definition (requiring monospecific sera and the possibility of reproducing the *same* typing reagent in different donor-host combinations) is unattainable[3].

Recently HÁLA et al. (1966), HÁLA and KNIŽETOVA (1969) and OOSTERLEE and BOUW (1966) reopened this question. Without trying to give a final resolution of the B antigens they attempted to obtain insight into the complexity of individual B alleles. Inbred and congenic lines were used to obtain reproducible sera analysed by absoption experiments. The first steps in the construction of a B-chart, representing at least a model of the complex situation, were published (Table 1).

What is the reason for this complex situation and what are the consequences for the situation in non-inbred populations? The number of different B alleles is obviously very high and the same is true of the number of individual antigenic specificities produced by different alleles. Practically all chicken breeds, provided that they have no common origin, differ at the B allele, and in non-inbred populations hardly any two identical individuals have been found.

[3] It is generally accepted that the respective specific anti-B sera widely cross-react. The nature of these cross-reactions is not fully explained. It is interesting to read that in the original work of BRILES et al. (1950) the term *antigen* was used "only in referring to the total antigenic product of an allele, while the term antigenic factor will be used in referring to the 'serological components' of which the antigen seems to be composed. For example, the total antigenic product of the allele B^{15} is referred to as antigen B_{15}, which is composed of antigenic factors B_1 and B_5". Already in this work it was pointed out that "these designations are made for the sole purpose of convenience in discussion and should not be viewed necessarily as representing natural units". Later "the concept that antigenic product of a blood group gene was composed of a group of more or less discrete antigenic factors has been completely abandoned" (BRILES et al., 1957). This change, reflected also in the terminology used, was discussed in greater detail by GILMOUR (1960): "A distinct antigen, determined by a separate allele, was defined as one which had a distinct pattern of cross-reactions with all the 16 reagents available. It should be noted that the authors abandoned the terminology introduced by BRILES et al. (1950), in which an antigen inherited as a unit was labelled according to its reaction with several serially numbered reagents. Instead, each antigen of the 21 identified among the 12 inbred lines was assigned a separate serial number B_1, B_2, B_3 . . . etc., and the reagent specific for it was labelled similarly B1, B2, B3 etc. These numberings replaced the Texas numbers mentioned above, and were not homologous with them. No attempt was made to give cross-reaction information in the label assigned to a gene, as had been done in the 1950 work, and in the terminology of cattle blood-group workers (e.g. STORMONT, OWEN and IRWIN, 1951). Such labelling was considered too cumbersome because of the multiplicity of possible cross-reactions among the many antigens and antibodies used in this study". According to GILMOUR's view resulting from his own absorption experiments "we may regard the cross reactivities as resulting from the fact that each antigen is made up of a large number of antigenic factors eventually identifiable by ordinary serological methods, or suppose them to be comparable with the more discontinuous type of cross reactivities . . .". In any case "the number of such factors is too large for them to be easily sorted out". Until now there is no precise information on how far these two possibilities overlap in different situations. "To some extent these two explanations may eventually be found to be equivalent" (GILMOUR, 1959).

Indicative of the extent of polymorphism of the B system is the finding of DROBNÁ (1969) and DROBNÁ and HÁLA (1969) that many pure anti-B sera react with almost 90—100% of 110—150 individuals from different randomly bred chicken populations. These sera are highly polyvalent while all antibodies obtained in them react with specificities of one complex system only. Absorptions of such sera by individual randomly chosen blood cells diminish their reaction frequency to different degrees but almost never to zero. The frequency of production of such sera and their patterns during absorption analysis can be explained only by assuming that a) the frequencies of individual B alleles are, in a panel of unrelated birds, very low; b) the products of individual alleles are very different, they probably overlap, but only in part. On the basis of absorption experiments DROBNÁ presumed that at least 7 specificities were produced by the B1 allele, 6 by the B2 allele, 11 by the B9 and 6 by the B10 allele (Prague nomenclature for B alleles; DROBNÁ, 1969). Some immunogenetic aspects of such a situation were illustrated by computer simulation of complex immunogenetic systems (DÉMANT et al., 1967; DÉMANT and IVÁNYI, 1967a, b). This represents a situation similar to that in humans where possibly no identical HL-A alleles are found in a medium-sized sample of unrelated individuals of one nation (DAUSSET et al., 1967; CEPPELLINI, 1967; CEPPELLINI et al., 1967). It can be said that in chickens we have a situation where the polymorphism of one genetical system is so extensive that it seems to secure the individuality of a great part of unrelated individuals. On the other hand, the difficulties of the pure serological interpretation were evident from the finding that pure anti-B sera produced in identical donor-recipient combinations did not react identically and had not the same reaction frequency (HÁLA et al., 1966; DROBNÁ, 1969).

There is an abundant literature about the significance of the B system for selective advantage of some genotypes (see reviews by GILMOUR (1960), BRILES (1964) and MCDERMID (1964). There are mainly two lines of evidence for the significance of the B system. First, there is the continuing segregation of blood group antigens during inbreeding. Under these conditions, after more than 20 brother-sister matings or with computed inbreeding coefficients of 80—99 per cent, continous segregation of the B locus was found. "The general conclusion seems justified that the B locus showed clear evidence of continuing segregation but this was not obvious with other loci" (GILMOUR, 1960). STRATIL et al. (1969) found no segregation of serum groups in highly inbred chicken lines.

Parameters of several aspects of fitness (fertility, hatchability, viability, egg production, egg weight, rearing mortality, adult mortality, etc.) were measured in different experiments and corelated with respective B genotypes. Positive results were obtained by different workers (see above-quoted reviews and BRILES and ALLEN, 1961; BRILES, 1964; MCDERMID, 1965, 1966) but the effect varied for each cross probably, due to the different results of gene interactions in the hybrids.

Table 2. *The MHS in rats; the H-1 (Ag-B) system*

Type strain	H-1 allele	Antigenic specificities										
		1	2	3	4	5	6	7	8	9	10	11
AVN	a	1	2	—	4	—	—	7	—	—	—	—
BP	b	—	2	3	—	—	—	—	8	9	—	—
CAP	c	—	—	—	—	—	—	—	—	9	10	—
BD V	d	—	—	—	4	—	—	—	8	9	—	—
BD VII	e	—	2	—	4	—	—	—	—	—	—	—
AS 2	f	—	—	—	4	—	—	—	8	—	—	—
LEW	l	—	—	—	—	5	—	7	8	—	—	—
BN	n	—	—	—	—	—	—	—	—	9	—	11
WP	w	—	—	—	4	—	6	—	—	9	—	—

B. The H-1 (Ag-B) System in Rats

After preliminary data (BOGDEN and APTEKMAN, 1960, 1962; PALM, 1962; KŘEN et al., 1960 1962), had shown the possibility of serological determination of H antigen in rats, PALM (1964) demonstrated the existence of one strong locus segregating in F_2 hybrids of two inbred strains. Different alleles of this locus were described in further strains (RAMSEIER and PALM, 1967). The production of congenic lines differing at the strong system on the Lewis background was initiated (IVÁNYI, 1966b). In a series of papers ŠTARK et al. (1966), ŠTARK and KŘEN (1967a—e), ŠTARK et al. (1967a, b), ŠTARK et al. (1968a, b), ŠTARK and HAUPTFELD (1969) exhibit a detalied serological analysis of 8 alleles. With the help of typing sera obtained by different cross absorption and sera produced in different hybrids, a situation analogous to the H-2 was progressively obtained, showing that the strong H locus in rats is the MHS. The nine analysed alleles and their antigenic make-up are presented in Table 2. The designation H-1 (RtH-1) was proposed for this system by ŠTARK et al. (1967a) and Ag-B by PALM (1964).

As a rule, skin grafts between rats differing at the H-1 system are rejected by the 15th day. Skin grafts between congenic rat strains differing at H-1 are rejected also within 15 days. A number of grafts exchanged between individuals not differing at H-1 survive considerably longer than 15 days. The cause of rejection of H-1 compatible grafts in some H-1 compatible combinations within 15 days can be explained by the synergic effect of weak loci.

The transplantation of H-1 incompatible grafts evokes a strong secondary response and leads to the formation of circulating antibodies, which react both with donor's erythrocytes and with lymphoid cells in the cytotoxic test. The most suitable serological tests used in mice for H-2, i.e. the cytotoxic test with lymphoid cells and agglutination of erythrocytes in dextran or PVP medium, proved to be also the most suitable fer rats (PALM, 1964; ŠTARK et al., 1967a). In contrast to the situation on mice, even the trypsin test is suitable (IVÁNYI, 1966b).

(from ŠTARK *and* HAUPTFELD, *1969)*

Antigenic specificities										Other strains with probably identical alleles
12	13	14	15	16	17	18	19	20	21	
—	13	14	—	—	17	—	—	20	21	DA
—	13	—	—	—	—	18	—	—	—	
—	13	—	15	—	17	—	—	—	—	Y 59
12	13	14	—	16	—	18	—	20	21	BD X
—	13	14	—	—	17	—	—	—	21	
—	13	—	—	—	—	18	19	20	21	
—	—	—	—	16	17	—	—	—	—	AGA, CDF, AS, BS, HS
—	13	—	15	—	—	—	—	—	—	
—	—	—	—	—	17	—	—	—	—	LEP, WR, VM, BD II

Although the anti-H-1 antibodies are not the only ones which originate by alloimmunization of two lines of rats, they play a predominant role. They appear most frequently, earliest and are the strongest ones. As a matter of fact, in many sera, using certain immunization schedules and serological methods for detection, only anti-H-1 antibodies could be found.

The complexity of H-1 has been gradually elucidated. No population studies have been carried out and there are neither data about the possible complexity of individual antigenic specificities, nor about recombinations between different H-1 alleles. Nevertheless, there is no doubt the complexity of this system, as represented by 21 specificities found in about 25 strains tested to a sufficient extent. The products of the H-1 alleles are asymmetric, like those of the H-2 system. Regarding the distribution of 21 specificities, some alleles produce 10, some only 4 specificities (ŠTARK and HAUPTFELD, 1969), (Table 2).

SILVERS et al. (1967a, b), WILSON (1967), ELVES (1968) described that blast transformation in mixed cultures of lymphoid cells can be obtained only in the presence of H-1 differences, while even multiple weak differences exhibit zero values of thymidine incorporation. Similarly, the normal spleen-cell transfer assay resulted in the identification of a single strong histocompatibility system responsible for the induction of graft-versus-host reactions in the rat (ELKINS and PALM, 1966).

There are two exciting reports on pronounced selective forces acting in rats which are probably both linked in some manner with the H-1 system. MICHIE and ANDERSON (1966) found an almost complete elimination of homozygotes in a highly inbred strain (F 72!), in which obviously one strong (unidentified) H system remained segregating. There was no corresponding reduction of litter size. This finding favoured the hypothesis of selective fertilization. RAMSEIER and PALM (1967) found a deficiency of Ag-B_4 homozygotes in F_2 population of two inbred lines (derived from F_1 hybrid progeny of Lewis females and DA males); this was not observed in F_2 hybrids derived from F_1 hybrid progeny of DA females and Lewis males.

Table 3. *The MHS in mice; the H-2 system*

Type strain	H-2 allele	Antigenic specificities [old symbols (letters) shown at top]													
		A	D^b	C	D	E	F	G	H	I	J	K	M	N	P
		1	2	3	4	5	6	7	8	9	10	11	13	14	16
A	a	1	—	3	4	5	6	—	8	—	10	11	13	14	—
B10	b	—	2	—	—	5	6	—	—	—	—	—	—	14	—
D1.C	c	*	—	3	4	(—)	(6)	—	8	—	*	—	13	*	—
DBA/2	d	—	—	3	4	—	6	—	8	—	10	—	13	14	—
ACA	f	*	—	—	—	—	(6)	7	8	9	*	—	—	*	—
HTG	g	—	2	—	—	—	6	(—)	(8)	(—)	*	—	—	14	(—)
HTH	h	1	2	3	—	5	6	(—)	8	(—)	*	11	—	?	(—)
HTI	i	*	—	3	4	5	6	(—)	(—)	(—)	*	—	13	*	(—)
JK/St	j	—	—	—	—	—	6	(7)	—	(—)	—	—	—	—	(—)
C3H	k	1	—	3	—	5	—	—	8	—	—	11	—	—	—
I/St	l	—	—	—	—	—	6	(7)	—	(—)	10	—	—	—	(—)
AKR.M	m	*	—	3	—	5	(6)	—	8	—	*	11	13	*	—
F/St	n	1	—	—	—	5	6	(7)	8	(—)	10	—	—	14	(16)
HTO	o	1	—	3	—	5	—	—	8	—	*	—	—	*	—
P/Sn	p	*	—	3	—	5	6	(7)	(8)	—	**	—	—	*	16
DBA/1	q	—	—	3	—	5	6	—	—	—	—	11	13	—	—
RIII/J	r	*	—	3	—	5	(6)	—	8	—	*	11	—	*	—
A.SW	s	*	—	3	—	5	6	7	—	—	*	—	—	*	—
WB/Re	w	—	2	*	—	*	*	*	—	—	*	—	—	*	*

Data supplied by SHREFFLER to complete the table of SNELL and STIMPFLING

C. The H-2 System in Mice

Among the total number of more than 30 segregating H systems in mice, the H-2 system has a unique position due to the relative strength of the H2-antigenic differences. Skin grafts transplanted between individuals with different H-2 alleles are rejected between the 8th and 15th day and preimmunization produces a vigorous second set reaction. Many tumours (and possibly some normal tissues) are able to overcome the non-H-2 differences yet are rejected regularly when they are opposed by an H-2 barrier. As a rule, antibodies are produced by immunization of recipients with cells carrying different H-2 alleles. The combination of both approaches, i.e., grafting and serological methods, can be used to solve the genetic complexity of the locus. The H-2

(from SNELL *and* STIMPFLING, *1966*; SHREFFLER, *1969)*

Antigenic specificities [old symbols (letters) shown at top]												Inbred strains with probably identical alleles
	S	V	Y	A^1	B^1	C^1	D^1	E^d	D^k	K^b		
18	19	22	25	27	28	29	30	31	32	33	Ss	
—	—	—	25	27	28	29	—	—	—	—	h	AKR.K, B10.A
—	—	22	—	27	28	29	—	—	—	33	h	A.BY, C3H.SW, C57BL/6, C57L, CC57BR, CC57W, D1.LP, LP/J, St/a, 129
—	—	*	*	27	28	29	—	(31)	*	*	h	
—	—	—	—	27	28	29	—	31	—	—	h	BALB/c, C57BL/ks, B10.D2, ST.T6, WH, YBL/Rr, YBR/Wi
—	—	*	—	27	—	—	—	(—)	*	*	h	B10.M, RFM/Un
—	—	22	*	(27)	(28)	(29)	(—)	31	—	—	h	
—	—	—	*	(27)	(28)	(29)	(—)	—	—	—	h	B10.A(2R)
—	—	22	*	*	*	*	(—)	—	—	33	h	B10.A(5R)
—	—	22	?	(—)	(28)	(29)	(—)	(—)	*	*	h	
—	—	—	25	—	—	—	—	—	32	—	l	AKR, B10.BR, CBA, CE, CHI, C5TBR/a, C57BR/cd, C58, D1.ST, MA/J, RF/J, ST/bJ, 101
—	—	22	—	(—)	(—)	(?)	(—)	(—)	*	*	h	N/St(?)
—	—	*	*	27	28	29	30	(—)	*	*	l	
—	—	—	*	(—)	(—)	(—)	(—)	(—)	*	*	h	
—	—	—	*		—	29		31	32	*	h+l	
—	—	*	*	—	—	—	—	(—)	*	*	h	C3H.NB
—	—	—	*	27	28	29	30	(—)	*	*	h	C/St, BUB
18	—	*	25	—	—	—		(—)		*	h	RIII/Wy, LP.RIII
—	19	*	—	—	28	—	—	—	—	—	h	SJL
*	—	*	*	*	*	*	*	—	*	*		WC/Re

are given in parentheses.

system represents the best examined MHS. Other reviews give much more detailed information about this system as well as lists of literature (SNELL, 1948, 1953; SNELL et al., 1964; SNELL and STIMPFLING, 1966; SNELL, 1968; STIMPFLING, 1965; SHREFFLER, 1965, 1967a, b). Consequently, no attempt at an exhaustive review is necessary here.

The H-2 system was discovered by GORER (1937, 1938) when he found that there existed a relation between the distribution of serologically detected erythrocyte antigens and the susceptibility to a transplantable tumor. Later, combining serological, grafting and genetic experiments, the complexity of the H-2 system was elaborated by a number of investigators. Its present status — the H-2 chart — is illustrated in Table 3. The H-2 antigens are determined by a chromosomal segment (region) located in the IXth linkage

group of mice (GORER et al., 1948). An H-2 allele, defined as the respective chromosomal segment, determines a set of H-2 specificities. These were originally indicated by letters, but after the change of the nomenclature (SNELL et al., 1964), they have since been indicated by numbers. Twenty-six H-2 specificities are known (1—33, some numbers being vacant). Nineteen H-2 alleles have been described and their distribution in different inbred mouse strains is given on the right-hand side of the H-2 chart. Actually, the H-2 chart represents a key position for a number of basic aspects in the field of immunogenetics of transplantation.

The unique position of the H-2 system in the mouse is obvious with regard to its relative strength as a histocompatibility barrier (SNELL and STIMPFLING, 1966, Table 5). If random immunization is performed between two inbred strains of mice, H-2 antibodies are formed, whereas non-H-2 antibodies, whether against the defined non-H-2 histocompatibility systems, or erythrocyte blood group systems, are formed less frequently. Only sporadic data on non-H-2 antibodies reacting with leucocytes are available (AMOS et al., 1963; MISHELL et al., 1963; TERASAKI and MCCLELLAND, 1963; REIF and ALLEN, 1964; WINN, 1964, 1965a; CHERRY and SNELL, 1969). While other H systems apparently represent poorer immunogens, erythrocyte blood group differences (possibly non-histocompatibility systems) represent good immunogens but are less polymorphic, with less frequent antibody production by random immunization (for references, see POPP, 1967; STIMPFLING and SNELL, 1968). H-2 represents the only H system in mice which can be easily and systematically used for serological purposes. Anti-H-2 antibodies are detected by red cell agglutination by the dextran method (GORER and MIKULSKA, 1954), PVP method (STIMPFLING, 1961), by leucocyte agglutination (AMOS, 1953; MISHELL et al., 1964; SEVERSON and THOMPSON, 1968), or by the cytotoxic test on lymphoid cells by the dye exclusion method (GORER and O'GORMAN, 1956), or ^{51}Cr release method (GOODMAN, 1961; SANDERSON, 1964; WIGZELL, 1965; BOYLE, 1968). Other methods for detection of H-2 antibodies have been described (MÖLLER, 1961; WINN, 1962, 1964; KLEIN and IVÁNYI, 1963; CEROTTINI and BRUNNER, 1967; DAVIS and SILVERMAN, 1968; GERVAIS, 1968). Anti-H-2 antibodies are produced by different immunization schedules, mainly by the intraperitoneal injection of spleen cells, and/or skin grafting.

Anti-H-2 haemagglutinins develop in female mice after repeated pregnancies with H-2 incompatible males. The immunization is due to the pregnancy itself but there is no evidence of foetal disease or decreased fertility as a result of sensitization. Similarly, females immunized by spleen cell injections do not become sterile or grossly less productive (GOODLIN and HERZENBERG, 1964). Anti-H-2 antibodies are readily transmitted from mother to foetus and suckling offspring (KALISS et al., 1963). The question of why neonatal anlmals are not affected has not been satisfactorily answered (KALISS et al., 1963; KALISS and DAGG, 1964; GOODLIN and HERZENBERG, 1964; RUBINSTEIN and KALISS, 1964).

Immunization with pure erythrocytes does not lead to the production of circulating antibodies nor to a second set reaction. Though this can presumably

be explained by the small quantity of the antigens possessed by the red cells or by their different quality, the difference is somewhat a mystcry. Similarly, the fact that the greatest part of non-H-2 antigens could not be found on erythrocytes, though a few data give evidence of this possibility, remains to be elucidated (HILDEMANN and PINKERTON, 1966; SNELL and STIMPFLING, 1966).

The immunoglobulin character of anti-H-2 antibodies seems to follow the general rule in antibody response: early 19S followed by 7S. While no marked strain differences have been found with regard to the response following primary immunization, there is a marked quantitative difference in the course of hyperimmunization resulting in only minute amounts of 19S and high values of 7S in A.CA and A.SW mice but with an opposite finding in C57BL. (A.SW × C57BL) F_1 hybrids responded in the same way as C57BL, suggesting a dominant genetic factor (WINN, 1965b; ANDERSON et al., 1967). This has not yet been tested on segregating populations.

The antigenic differences in the H-2 system, which represents a strong histocompatibility barrier and leads to the development of circulating antibodies, indicate that the H-2 system occupies a special position among mouse H systems. A number of other facts can be added:

a) the strength of the second set reaction,

b) the strength of the graft-versus-host reaction (splenomegaly, runt disease after injection of allogeneic lymphoid cells),

c) the difficulty of evoking immunological tolerance,

d) the significance for transformation in mixed lymphocyte cultures.

a) The type of second set reaction. While results of individual experiments are mostly dependent on the method of sensitization (number and type of cells, grafts used for sensitization, time interval between sensitization and test skin grafts) when using a standard selected procedure (histological examination of the graft on the 6th day), grafts can be classified morphologically as white grafts (absence of lymphocytic infiltration, epithelial degeneration and necrosis); red grafts (necrosis of graft epithelium, engorgement of graft veins and haemorrhage, accumulation of host inflammatory cells); blue grafts (hyperplasia of graft epithelium and host cell infiltration particularly in perifollicular regions); grafts without any signs of rejection (EICHWALD et al., 1966). EICHWALD et al. (1966)-, proved that only H-2 determined antigens have sufficient strength to cause white grafts without additional antigenic differences. In the absence of an H-2 difference between host and graft, multiple loci are needed to cause the white graft rejection. The degree of variance of genetic aspects of second set skin grafts is illustrated in Fig. 2. While the difference between the observed histological features are continous and merely quantitative with intermediate types, the unique position of H-2 in this grading is evident.

The type of second set reaction is probably the reflection of differences in the dynamics and/or mechanism of acquired sensitivity. MCKHANN (1964b) compared the H-2 and H-3 system from this point of view. In H-2 difference

sensitization appeared rapidly and persisted for a shorter time than in H-3 where sensitization appeared more slowly.

b) The strength of the graft-versus-host (GVH) reaction. The strength of the GVH reaction produced in neonatal animals or F_1 hybrids by injection of allogeneic lymphoid cells, measured either by the splenomegaly assay or

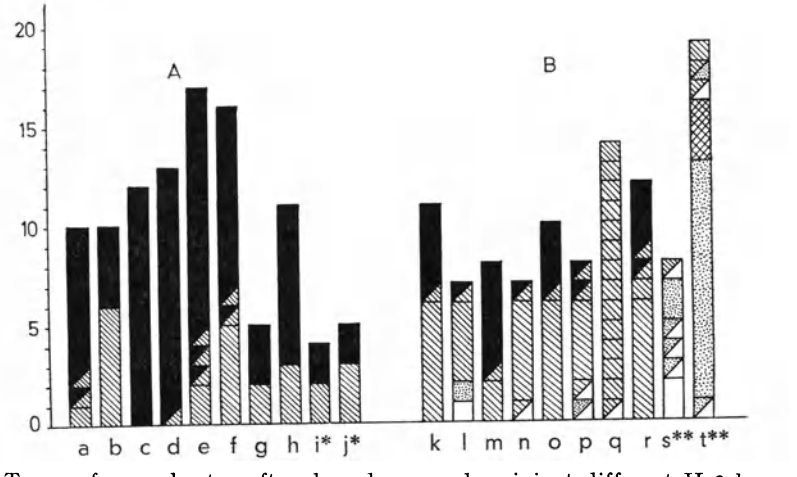

Fig. 2. Types of second-set grafts when donor and recipient differ at H-2 locus (A), or when donor and recipient have same H-2 allele (*B*) (EICHWALD et al., 1966). Solid black: white grafts; diagonal lines: "red" grafts; stippled areas: "blue" grafts; clear areas: no sign of rejection; divided squares: intermediate forms.

	a	b	c	d	e	f	g
Donor	A/J	CBA	C57BL/6	CBA	C57BL/6	BALB/c	CBA
Recipient	CBA	A/J	CBA	C57BL/6	BALB/c	C57BL/6	BALB/c

	h	i*	j*	k	l	m	n
Donor	BALB/c	C57BL/10	B10.D2	B10.D2	BALB/c	DBA/2b	BALB/c
Recipient	CBA	B10.D2	C57BL/10	BALB/c	B10.D2	BALB/c	DBA/2b

	o	p	q	r	s**
Donor	CBA	AKR	C57BL/10	129/SvSn	C57BL/10 (H-1^c)
Recipient	AKR	CBA	129/SvSn	C57BL/10	C57BL/10.129 (5M) (H-1^e)

	t**
Donor	C57BL/10.129 (21M) (H-4^b)
Recipient	C57BL/10 (H-4^a)

* H-2 difference only.

** H-1 or H-4 difference only.

by the production of runt disease, is dependent on the genetic difference (UPHOFF and LAW, 1958, 1959; SIMONSEN, 1962; SANKOWSKI and NOUZA, 1968). No comprehensive analytic study is available but generally the authors agree on this point. While an H-2 difference itself produces a pronounced reaction (NOUZA, 1969), if there is a weak difference only, clinical signs of a GVH reaction are not observed unless presensitized donors of lymphoid cells are used. Non-sensitized cells in a non-H-2 difference produce only a subclinical disease (SANKOWSKI and NOUZA, 1968).

c) The difficulty of evoking immunological tolerance. There is similar general agreement about the role of H-2 in the process of induction of immunological tolerance. It is much more difficult to induce tolerance in neonatal mice across an H-2 barrier than across single or multiple non-H-2 differences (greater or repeated dose of cells required and/or earlier time of injection). The same is true in experiments performed in adults with even more pronounced differences (BILLINGHAM and SILVERS, 1958; BILLINGHAM and BRENT, 1959; MARIANI et al., 1959; SHAPIRO et al., 1961; HAŠEK et al., 1961; MARTINEZ et al., 1962; MEDAWAR, 1963; MCKHANN, 1964c; LENGEROVÁ and MATOUŠEK, 1966; EICHWALD et al., 1968a). Generally, "the ease of toleranceinduction usually appears inversely proportional to the graft rejection rate" (HILGERT, 1967). While there can be little doubt about the validity of such a correlation there is a number of details which require more profound understanding. This is mainly the unique position of H-2 (when compared with other systems regardless of graft rejection time) and the relative strength of different H-2 antigens.

KLEIN (1966) described skin survival times in "weak" (monoantigenic) H-2 differences comparable with those in some non-H-2 combinations. HILGERT (1967) compared the possibility of inducing tolerance in adult mice in "weak" H-2 and "strong" non-H-2 differences. While average skin graft rejection time with only an H-2.2 difference is about 20 days, skin graft rejection in H-1 + H-3 + H-13 difference is about 17 days. Nevertheless, a dose of 8×10^8 spleen cells induced second set rejection of all H-2 incompatible grafts but not for non-H-2 incompatible grafts, where some were permanently tolerated. It could be concluded that in spite of a comparable first set graft rejection time, the H-2 and non-H-2 antigens may turn out to be qualitatively different when tested for sensitization or tolerance induction.

Presumably it is easier to produce tolerance in a weak (monoantigenic) H-2 difference than in a strong (polyantigenic) one. However, this has not been tested conclusively. Perhaps easier tolerance induction will be found only in some cases of weak H-2 differences, as was demonstrated by KLEIN (1966) who observed that there were differences in the speed of first set rejection between different monoantigenic combinations. Second set tolerance and GVH reaction was not related to the number of differences in H-2 (EICHWALD et al., 1968a). Differences and inconsistencies between different experimental designs could be influenced by different donor-*host* combinations. EICHWALD et al. (1968b) observed different rejection times for the same monoantigenic H-2 differences as did KLEIN (1966) after use of different donor-host combinations. Differences in antibody production genetically linked with the H-2 system were described (see this review) and this could influence the outcome of such experiments.

The distinction "weak" and "strong" inside the H-2 system is not well defined and could actually represent a continuous line of increasing differences based probably on the number of differences in the H-2 specificities involved. Alternatively some individual H-2 specificities could represent stronger

"immunogens" for qualitative reasons. In any case, it has been repeatedly pointed out that it is easier to produce tolerance in H-2^a mice to H-2^k cells (A←CBA, difference in H-2.32) than in the reverse combination (CBA←A, difference in H-2, 4, 6, 10, 13, 14, 27, 28, 29) (LENGEROVÁ and MATOUŠEK, 1966). EICHWALD et al. (1968) wrote therefore: "It was was 'extremely fortunate' (BILLINGHAM and BRENT, 1959) that the CBA→A combination which involves a 'weak' H-2 barrier was investigated at an early date". Some aspects of the relative strength of H antigens will be discussed in connection with the human HL-A system.

Enhancement of tumour grafts can be obtained by appropriate pretreatment of the recipients with allogeneic tumour cells or by passive injection of immune sera. This can easily be obtained only if an H-2 difference is involved (KALISS, 1958, 1962).

d) The effect of H-2 on blast transformation in the mixed lymphocyte culture (MLC). It has been demonstrated that an H-2 difference itself is able to produce lymphocyte transformation as well as multiple non-H-2 differences, whereas single non-H-2 differences are unable to effect transformation, at least in a systematically studied comparative approach (DUTTON, 1965, 1966; RYCHLÍKOVÁ and IVÁNYI, 1968). The role of the number of differences in H-2 antigenic specificities similar to those for skin graft survival or induction of tolerance was described by TANAKA et al. (1968) and RYCHLÍKOVÁ and IVÁNYI (1968) but not supported by the findings of HUEMER et al 1968). In any case the unique role of H-2 in the MLC assay seems firmly established. The MLC

Table 4. *Relative concentration in different tissues of the H-2 alloantigens*

Tissue	H-2	
	AMOS et al. (1963)	BASCH and STETSON (1962)[a]
Spleen	4	4
Liver	3	3
Thymus		2
Lung	2	2
Adrenal		2
Gut	2	
Kidney	1	1
Red cell	1	0
Testis	0	
Heart		1/2
Muscle	0	0
Brain	0	0

[a] The values given by BASCH and STETSON (1962) are transformed to a numerical system approximating that used by AMOS et al. (1963). As thus expressed, only the relative concentrations are indicated, with 4 indicating the highest concentration, 0 the lowest (from SNELL and STIMPFLING, 1966).

is considered to be an in vitro immune reaction to H differences (DUTTON, 1966; SMETANA and IVÁNYI, D., 1967; GORDON et al., 1967; WILSON, 1967; SCHWARTZ, 1968).

H-2 antigens are also detectable in a number of other tissues (Table 4). The exact evaluation of these results is not clear from several points of view: the end products of H-2 alleles in cells of organs indicated as — or ± a. are not present at all; b. are present in different form; c. are equally represented even quantitatively, the differences arising only in the course of the experiment (e.g. degradative changes of the antigen might be pronounced in the liver extract, due to the effect of some enzymes or cholic acids, HILGERT and KRIŠTOFOVÁ, 1967); e. the diversity of findings is due to a different representation of individual cell elements in complex organs and thus limits the relative comparison only to the "rough" organ standpoint.

At variance with BARTH and RUSSELL (1964), VOJTÍŠKOVÁ (1969) demonstrated the presence of H-2 antigens on mouse spermatozoa. It was not yet clarified whether this is due to pre- or post-reductional H-2 activity.

Chemistry of the H-2 Antigens

While such questions as the chemical nature of the H-2 end products[4], whether all specificities produced by one allele are the components of one macromolecule[5] and how the genetic fine structure of the locus is related to the chemistry of its products remain unanswered, it seems to be clearly established that the H-2 end products are constituents of the cell membranes (see SNELL and STIMPFLING, 1966). Differences in the distribution of distinct H-2 specificities in the plasma membrane fraction and endoplasmic reticulum were reported by OZER and WALLACH (1967). Soluble preparations of antigens of MHS of relatively low molecular weight seem to be an important step in the clarification of the above mentioned basic questions (KANDUTSCH and STIMPF-

[4] "The chemical nature of these substances remains uncertain. Successive groups of investigators have suggested that the antigenic specificity controlling transplantation depends upon DNA, upon mucoids, or upon lipoproteins, based upon the chemical labilities, gross physical and compositional properties, and enzymatic susceptibilities of their various subcellular extracts. In general, these extracts contain an extremely heterogenous array of components, and the active principle represents but a small portion of the entire mixture. Therefore since the above inferences were based upon observations on complex mixtures, the effects which were noted may well have reflected the nature of the contaminant substances rather than of the active principle" (for references see KAHAN and REISFELD, 1968). KAHAN and REISFELD (1968) described differences in amino acid compositions of the antigens extracted from two histoincompatible lines of guinea pigs, which suggests that H antigens have allotypic specificities related to protein structure.

[5] Several H-2 specificities seem to be present on the same molecule, and in F_1 hybrid mice at least some hybrid molecules exist carrying specificities derived from both parental strains (DAVIES, 1967a, b). Recent evidence is available that different specificities determined by a particular H-2 allele can be separated (SUMMERELL and DAVIES, 1969).

LING, 1963; NATHENSON and DAVIES, 1966a, b; EDIDIN, 1967; DAVIES, 1966, 1967a, b).

It is interesting that analogous methods of extraction, solubilization and fractionation gave identical results in humans (DAVIES, 1967b; DAVIES et al., 1967, 1968; MANN et al., 1968). Physico-chemical characterization of H-2 and HL-A preparations indicates an important homology between the two systems (DAVIES, 1967b; DAVIES et al., 1967b, 1968; MANN et al., 1968). Human and mouse alloantigens solubilized by papain, when applied on a column of "Sephadex G-150", had similar elution patterns demonstrating similar chromatographic properties (MANN et al., 1968).

Genetics of the H-2 Locus

The H-2 specificities detectable on the cellular membranes are end products of H-2 alleles which represent a chromosomal segment in the IXth linkage group of mice. The degree of polymorphism of H-2 is only partially elucidated at this time. While many intensively studied inbred strains had different H-2 alleles when developed from independent genetic origin, there are a few examples indicating that lines without any possible (?) common ancestor have identical H-2 alleles (POPP and AMOS, 1965; SNELL and STIMPFLING, 1966; KLEIN et al., 1967; YEGOROV, 1967a, b). On the other hand, RUBINSTEIN and FERREBEE (1964) when serotyping 47 Swiss-Webster mice of a random bred colony found a high degree of polymorphism; new H-2 alleles and H-2 antigens had to be expected, three mice were negative with all the sera studied. In preliminary studies (IVÁNYI et al., 1969), 38 Wild (W) mice (randomly captured field mice, *Mus musculus*) were serotyped with a number of anti-H-2 sera including reagents against specificities 1, 2, 3, 4, 5, 7, 9, 11, 13, 16, 19, 23, 31. The composition of the products of H-2 alleles could not be resolved conclusively without progeny testing but the following observations could be made. 1. None of the known H-2 alleles could be identified with certainty on the W mice tested. 2. Some of the W mice reacted negatively with all or almost all sera tested so that the presence of all or nearly all of the above listed H-2 specificities could be excluded. 3. A considerable degree of polymorphism for the H-2 antigens could be deduced from the reaction pattern of the sera. 4. Many sera reacted more weakly with the cells of W mice than with the cells of the donor or other inbred strains. 5. The only specificities reacting in comparable strength with those of inbred mice in some of the W mice tested were H-2.3, H-2.4, H-2.5 and the complex of specificities reacting with mixtures of anti-1, 11, 23.

As a word of caution it should be mentioned that the variability observed by RUBINSTEIN and IVÁNYI was based on the serological approach only (haemagglutination by the dextran or PVP method), whereas some of the "variants" could be due only to quantitative influences on the expressivity of the H-2 antigens which could strongly influence the possible interpretations (see page 57—61). Nevertheless, further population studies as one of the keys to the resolution of the complexity of the H-2 system as well as testing of H-2

alleles in further inbred strains seems to be highly desirable. There are few data about minor variants of well defined H-2 alleles (H-$2^{d'}$, H-$2^{k'}$). None of them has been sufficiently studied (SNELL et al., 1953; GORER, 1956; GREEN and KAUFER, 1965; SNELL and STIMPFLING, 1966).

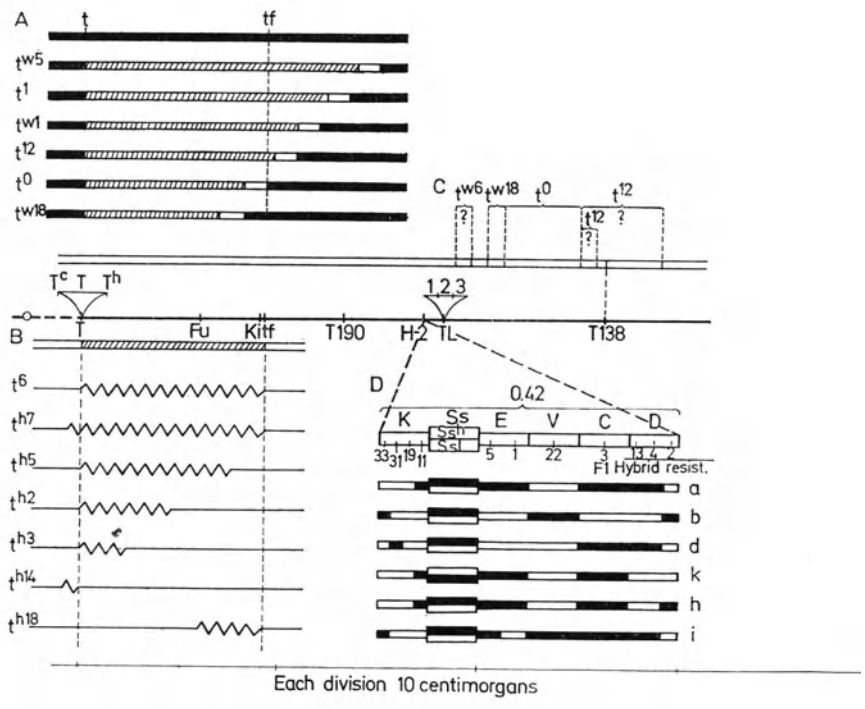

Fig. 3. The IXth linkage group in mice. The linkage map (see middle solid line) was constructed on the basis of data from the works of ALLEN (1955), BOYSE et al. (1965, 1968b, c), BUTLER and MEREDITH (1968), CARTER et al. (1955, 1956), CASPARI and DAVID (1940), CUDKOWICZ (1965), DUNN and CASPARI (1945), DUNN et al. (1962), GORER et al. (1948), GREEN (1967), GREEN and STIMPFLING (1966), LYON (1956, 1959,) LYON and PHILLIPS (1959), LYON et al. (1967, 1968), SEARLE (1966), SHREFFLER (1965, 1967a), SHREFFLER et al. (1966), SNELL and STIMPFLING (1966), ŠRÁM and KLEIN (1967) STIMPFLING (1965), STIMPFLING and RICHARDSON (1965). From left to right: Centromere; alleles of T gene; region of t alleles; Fused; Kinky; tufted; translocation break T190 (9; 13) Ca; H-2 system with the Ss locus inserted between the H-2.K and H-2.E region; F_1 hybrid resistance; TL antigens; translocation break T138(9;2)Ca. A number of minor inconsistencies were discussed in the text, or omitted for the sake of simplification. *A* and *B* diagrammatic representation of the hypothesis concerning the nature of t alleles, from LYON et al. (1964b), and *C* from GEYER-DUSZYNSZKA (1964). *D* diagrammatic representation of the four best studied H-2 alleles (black: specificity present; white: specificity absent)

The set of H-2 specificities determined by an H-2 allele is transmitted as a unit, which could be defined as a complex antigen (see description of the situation in the chicken B system). Nevertheless, crossing over occurs within the H-2 locus (ALLEN, 1955; AMOS et al., 1955) and sufficient data have already

been accumulated for the subdivision of the H-2 locus (chromosomal segment) in to at least five crossover regions. Crossing over between the H-2.11 (H-2.K) and H-2.4 (H-2.D) *ends* is approximately 0.45 % in the heterozygous females and 0.2% in the heterozygous males (STIMPFLING, 1965; STIMPFLING and RICHARDSON, 1965; SHREFFLER, 1965, 1967a, b). The genetic map of the H-2 locus and IXth linkage group are shown in Figs. 3 and 4. Four of the 18 known H-2 alleles given in the H-2 chart were defined as crossovers (i.e., H-2^g from bxd, h from axb, i from axb, o from dxk). H-2^a is probably a crossover derived from dxk crossing over. Actually H-2^a grafts survive permanently on (dxk) F_1 hybrids (the "dk" effect) if the intervention of non-H-2 systems is avoided by using either certain tumours (SNELL, 1953) or skin grafts when working with H-2 congenic lines (KLEIN, 1966).

While the existence of mutations is frequently presumed, a defined H-2 mutation was described only recently by YEGEROV (1967a) in the B10.D2

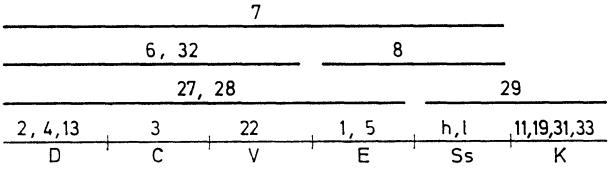

Fig. 4. Recombination map of the H-2 region of the mouse. The numbers on the upper three horizontal lines represent determinants which cannot be assigned to a single subdivision but which lie within the portion of the region encompassed by the line. The different subdivisions are identified by capital letters, as indicated (from SHREFFLER, 1965)

ine (H-2^d). The mutant line B10.504 (H-2^{da}) obtained from the progeny of a male treated by injections of a mutagen (diethylsulphate) does not possess H-2.10 of the H-2^d complex, and obviously possesses some new (changed?) specificity because skin grafts between B10.D2 and B10.504 are mutually rejected within 14—20 days. The strength of the difference was measured also for the ability to evoke the blast transformation in MLC and it was found that the value of transformation between the original and the mutant allele is similar to that of other H-2 differences (RYCHLÍKOVÁ and IVÁNYI, 1968).

Other Genes in the IXth Linkage Group. A Functional Unit or Super Gene?

1. The Ss System

Surprisingly enough a locus designated Ss, which determines a serum variant (high molecular weight 15 S or 19 S β-globulin), is located within H-2 between H-2 regions E and K. Two alleles are known: Ss^h (a high level of serum protein) and Ss^l (a low level). The difference is approximately 20-fold in the concentration detected by the Ouchterlony immunodiffusion method, by the reaction of a rabbit anti-mouse serum globulin reagent. From a number of inbred strains typed, all, except those with H-2k and H-2m (Ss^l), are Ss^h. F_1 hybrids

Table 5. *Differences in the "strength" of various histocompatibility loci as measured by grafts of skin and C57BL/10 transplantable leukaemias made between congenic strain pairs (from* SNELL *and* STIMPFLING, *1966)*

Locus	CR strains	Per cent survival of leukemic allografts in immunized hosts		Median survival (and range), in days, of skin allografts in unimmunized hosts	
		♂♂	♀♀	from C57BL/10	to C57BL/10
H-2	B10.D2	0	0	9	9
H-1	B10.BY	0	0	15 (14—20)	>60 (34—>60)
	B10.129(5M)	0	0	28 (21—36)	>100 (24—>100)
	B10.C3H(40NX)	4	0	17 (15—23)	42 (22—58)
	B10.C(41N)	0	0	25 (21—58)	>60 (26—>60)
	B10.D2(58N)	17	0	33 (18—61)	>60 (27—>60)
H-3 + H-13[a]	B10.LP	2	2	21 (16—27)	35 (28—>78)
H-3[a]	B10.LP-a	10	2	24 (21—28)	38 (24—49)
H-7	B10.C(47N)	2	1	22 (16—58)	44 (21—93)
H-8	B10.D2(57N)	30	16	45 (26—>56)	34 (23—41)
H-4	B10.129(21M)	39	44	127 (43—>261)	29 (29—34)
H-9	B10.C(45N)	67	6	>200 (17—>225)	>225 (24—>225)
H-10	B10.129(9M)	70	61	>75 (21—>250)	>225 (46—>225)
H-11	B10.129(10M)	78	59	124 (24—224)	>200 (19—>250)
	B10.D2(55N)	55	45	123 (22—>200)	138 (30—>175)
Y-linked (♂ to ♀)				26 (16—65)	

[a] SNELL et al. 1967.

have an intermediate level. All of the 53 Wild mice tested were Ss^h (or Ss^{h1}). The system is without known effects on histocompatibility. Thus far the Ss protein is neither antigenically similar nor structurally associated with the H-2 antigen (SHREFFLER and OWEN, 1963; SHREFFLER, 1964, 1965). BERG et al. (1967) described in humans an association between the Lp serum group and the HL-A system.

2. F_1 Hybrid Resistance

In accordance with basic transplantation laws, the parental grafts ought to survive on F_1 hybrids of two inbred lines of mice. Nevertheless, many cases of poor growth of parental tumours and haematopoietic cells (bone marrow, lymphoid cells) have been reported (SNELL, 1958; CUDKOWICZ and STIMPFLING, 1965; GOODMAN and BOSMA, 1967). The results of a study of this phenomenon, called "F_1 hybrid resistance", on a number of F_1 hybrids of inbred

and congenic strains and backcross segregants were "consistent with the supposition that genetic interaction in heterozygotes between alleles at the D region of H-2 modified the expression of parental isoantigen(s) relevant for the fate of the transplanted cells" (CUDKOWICZ and STIMPFLING, 1965). The immunological interpretation consists of the presumption that in the given situation the F_1 hybrid produces an immunological reaction against the parental grafts (CUDKOWICZ, 1961). GOODMAN and BOSMA (1967) and GOODMAN and WHEELER (1968) listed a number of findings which are inconsistent with the above interpretation, and presumed that the poor growth of parental tissues resulted from delay in the initiation of differentiation and cell division. The possible relationship between F_1 hybrid resistance, syngeneic preference and allogeneic inhibition was discussed by CUDKOWICZ (1965) and HELLSTRÖM and HELLSTRÖM (1965). While the mechanism of the observed F_1 hybrid resistance remains unsolved (SANFORD, 1967), it represents a phenomenon restricted to certain H-2 combinations associated or closely linked with the H-2.D region[6]. If the immunological interpretation is supported, the number of "unexpected" findings associated with immunological reactions located genetically on the right side of the T-H-2 functional unit will be extended. It seems pertinent that the phenomenon occurs only in some cases of gene interaction.

3. Position of the Centromere in the IXth Linkage Group

After demonstrating that the centromere in the IInd linkage group is proximate to d and tk (GREEN and STIMPFLING, 1966) and that the sequence is centromere-cw or cw-centromere-d-se-tk (LYON et al., 1968), the observation that in mice with the translocation T (2:9) 138Ca the genes T and d show significant linkage (GREEN, 1967; LYON et al., 1968) indicated that their loci are on opposite sides of the chromosomal break (T proximal, d distal to the break). LYON and PHILLIPS (1959) showed that the 138 break was beyond tf. The order must therefore be centromere-T-tf-T138 break. Since the H-2 locus gives about 7% recombination with tf (GREEN and STIMPFLING, 1966) and the T138 break gives 10—15% (LYON and PHILLIPS, 1959) the order is probably centromere-T-tf-H-2-T139 break, i.e. the IXth linkage group: centromere-T-tf-H-2.K-Ss-H-2.D-Tl (following the presumption of LYON et al., 1968)[7].

Genes on the right side of H-2 (the location of genes for virus susceptibility and antibody production is uncertain):

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[6] Transplanted haemopoietic cells of H-2^b/H-2^b, H-2^a/H-2^a or H-2^d/H-2^d donors grow equally well in isogenic, F_1 hybrid or backcross mice. Haemopoietic cells of different H-2^b/H-2^b donors exert a pure growth on F_1 hybrids or backcross segregants differing in the H-2.D region but not in hybrids differing in the H-2.C and H-2.K regions (CUDKOWICZ and STIMPFLING, 1965).

[7] Studies on H-2 antigenic variants obtained from heterozygous mouse tumours led to the assumption that H-2.D is proximal and H-2.K distal to the centromere. This was based on a hypothesis that a chromosomal event, possibly deletion, is responsible for the occurrence of some of the observed variants (KLEIN, 1961; KLEIN and KLEIN, 1964; OZER et al., 1965).

a) F_1 hybrid resistance (possibly influenced by the H-2.D region),

b) TL locus (thymus leukaemia),

c) Virus susceptibility and resistance,

d) Gene(s) governing the level of antibody response to synthetic polypeptide antigen.

Genses on the left side of H-2:

a) Tufted,

b) Kinky and Fused,

c) T locus and male sterility [the location of male sterility (IVÁNYI et al., 1969) is uncertain].

A nucleolar organizer on the IXth chromosome? (see page 43).

4. TL Antigens

The TL (thymus leukaemia) antigen was determined by an antiserum prepared in C57BL/6 mice immunized with spontaneous or radiation-induced A strain leukaemia. The antiserum reacts positively with the thymus cells of

Table 6. *Reaction of anti-TL sera (characterized by C57BL/6 serum against A strain spontaneous or radiation induced TL + leukaemia*

	TL + strains	TL − strains
Thymus[a]	+	−
Other tissues[b]	−	−
Spontaneous leukaemias or radiation induced	+ (some, i.e. 14/27)	+[c] (some i.e. 70/160)
Production of anti-TL	−	+

[a] Present in thymus of embryos in the same or higher concentration as in adults.
[b] Spleen, liver, erythrocytes, lymph nodes, bone marrow, kidney, brain, lung.
[c] Leukaemias of thymus origin?

A strain (and other TL + strains) and radiation-induced leukaemia of the A strain or C57BL/6 strain. The antiserum does not react with thymus cells of the C57BL/6 strain and with other tissues of either TL + or TL − strains. Thus TL + is a *thymus*-specific antigen in TL + strains and a leukaemia-specific antigen in TL − strains (OLD et al., 1963; BOYSE et al., 1965). The general situation is characterized in Table 6.

The occurrence of TL + leukaemias in TL − strains seems to be a general phenomenon. But not all spontaneous or radiation-induced leukaemias in TL + or TL − strains are TL + (see Table 5). Thus there are two broad classes of leukaemias, regardless of whether the strain of origin is TL + or TL − (OLD et al., 1964; BOYSE et al., 1965).

The quantity of TL + seems to be the same in different TL + thymus cells whereas on F_1 hybrids between TL + and TL − strains the thymocytes contain half the amount of antigen (BOYSE et al., 1964).

Typing of TL+ and H-2 in Bc mice proved a close linkage between the two systems (6 cross-overs from 397 mice, about 1—2% of recombination). One crossover within H-2 placed TL near to the D end of the H-2. A number of further findings confirmed this observation (BOYSE et al., 1964, 1965).

Radiation-induced or spontaneous leukaemias type as TL+ even in TL− mice (C57BL/6). Anti-TL+ immunity in TL− recipients gives no resistance

Table 7. *Definition of TL.1, 2, 3*[a], *by cytotoxic test systems*[b]

Serum	Absorbed	Test cells	Specificity detected
B6[c] (TL−) anti-ASL1 (TL+)		B6(TL+) radiation-induced leukemia	Tl.1 (strong)
B6(TL−) anti-129(TL−)		129(TL−)[d] thymocytes	TL.2 (weak)
(BALB/c × C3H(F1(TL-1) anti-ASL1(TL+)[e]	(B6 × A)F1[f] leukemia cells TL+ (in vivo)	A(TL+) thymocytes	TL.3 (strong)

[a] All Tl.1 strains are also TL.2 and TL.3 (TL.1, 2, 3). Three strains are only TL.2. Consequently three phenotypes are distinguishable: TL−, TL.2, TL.1, 2, 3.

[b] Modified after BOYSE et al. 1968.

[c] C57BL/6.

[d] 3 TL−strains are TL.2+: DBA/2, BALB/c, 129.

[e] Anti-TL.2 absent because BALB/c is TL.2+.

[f] For absorption of anti-TL.1.

to isologous leukaemias even if they have strong cytotoxic antibodies against tumour cells. On the contrary, the TL+ leukaemia cells become TL− when passed into highly immunized mice. The tumour cells regain TL+ antigen when passaged again in nonimmunized TL− mice. The return of the TL+ phenotype is immediate. Consequently the change is not selection of TL− cell variants. This phenomenon of the loss of TL antigen due to the action of antibody to TL has been called antigenic modulation (BOYSE et al., 1963, 1965; SCHLESINGER et al., 1965; OLD et al., 1968).

As regards the mystery in the occurrence of TL+ leukaemias in TL− strains, it is hypothesized that all mouse strains possess the genetic information for synthesis of TL antigen but that its phenotypic expression is determined by another gene. The appearance of TL antigen in the leukaemia cells of TL− strains may then be regarded as a disturbance of the mechanism normally controlling the expression of TL antigen, and the high frequency of this "accident" suggests an intimate connection with leukaemogenesis. Perhaps the genetic locus for TL expression controls some step in the differentiation

of thymus cells. In view of the strong possibility that all leukaemias of the mouse are caused by viruses, TL antigen may be related to an unrecognized virus (BOYSE et al., 1965).

Although both TL and H-2 antigens can be isolated by the same method, soluble TL+ antigen could be separated from soluble H-2 antigens in column chromatography (DAVIES et al., 1967a).

Further serological studies demonstrated the existence of three TL antigens TL.1, 2, 3. As the specificities TL.1 and TL.3 are always together, only three phenotypes are known: TL—, TL.2, TL.1, 2, 3. The method of detection of individual TL specificities and their characterization is presented in Table 7 (BOYSE et al., 1968b).

Antigens of the H-2 system and those of four other genetic systems can be determined on thymocytes: TL, Ly-A, Ly-B, Ø (these are all specific for thymocytes — BOYSE et al., 1968a). While TL and H-2 are closely linked, with the linkage map order H-2.K-H-2.D-TL, the last three segregate independently. BOYSE et al. (1967, 1968a, b, c) investigated the question of how this genetic situation will reflect the mutual quantitative relations of their representation on the cell membrane, i.e., whether the presence of one antigen affects the expressivity of the other and whether these relations — if they really exist — will reflect some genetic realities. Thus BOYSE et al. (1968) studied the question of whether the saturation of receptors for any of the five different antigenic systems (TL1, 2, 3; H-2D, K; Ly-A; Ly-B; Ø) leads to the reduction of absorption capacity for another antibody. They found that this occurs more or less reciprocally for TL-1, 2, 3, H-2.D, Ly-B and Ø respectively for Ø, Ly-A and H-2.K. Presuming that antigens of each of the two groups are neighbours in the space of the cell surface, the result would be in conformity with observed linkage TL+ — H-2 and implies that the genetic sequence is H-2.D:TL.1:TL.2:TL.3. Moreover, the wide "separation" of H-2.D and H-2.K implied that on the heterozygous cells the H-2 genes produced, or specifically modified, more than one macromolecule.

The phenotypic expression of TL antigens was found to reduce the demonstrable amount of certain H-2 antigens to as little as 34% of the quantity demonstrable on TL— thymocytes. In the course of antigenic modulation (change of TL+ to TL— produced by TL antibody) the amount of detectable H-2 antigens on the cell surface increases. The reduction of demonstrable H-2 antigens on the thymocytes of TL+/TL— heterozygotes is half that of TL+/TL+ homozygotes an the reduction affects equally the products of both H-2 alleles (cis and trans in relation to TL+). These findings indicate that the mechanism of H-2 reduction by TL is extrachromosomal. The H-2 specificities affected are those determined by the D end of the H-2 locus, which is adjacent to TL; antigens of the H-2.K end, which is distal to TL, are not depressed. It has been supposed that H-2.K-H-2.D-TL+ antigens occupy adjacent positions on thymocytes reflecting the gene order in the cell surface structure (BOYSE et al., 1967, 1968c).

5. H-2 and Susceptibility or Resistance to Virus-Induced Mouse Leukaemias (Virus S/R)[8]

SNELL (1968) has recently reviewed this problem and therefore only a short survey will be given here.

There are three sources of evidence about a relationship between the H-2 system and virus S/R.

1. Outstanding differences to virus S/R for different viruses in various inbred strains of made it possible to test F_1 hybrids and Bc or F_2 segregating populations. The result in F_1 hybrids does not give evidence for explicit dominance or recessivity. However, in numerous experiments on testing of F_2 or B_c generations it was possible to show that the virus S/R is in linkage with H-2. Some selected examples and references are presented in Table 8.

2. The second source of evidence of the relation between H-2 and virus S/R is given in experiments on H-2 congenic lines. The difference in virus S/R between congenic lines ensues most probably directly from the H-2 locus or from a chromosomal segment closely linked with H-2. Examples and references are given in Table 8.

3. Finally, TENNANT (1967) offered preliminary data about the effect of monospecific anti-H-2 sera on partial neutralization of the leukaemogenic potential of the virus preparation. Further rejection of leukaemic isografts was enhanced in hosts pretreated with virus or allogeneic cells, suggesting the presence of specific (or cross-reacting) histocompatibility antigens in the outer coat of these virus particles.

A number of alternative explanations for the role of the H-2 in virus S/R could be proposed (SNELL, 1968; LILLY, 1968). However, there is a number of internal inconsistencies which do not allow a general view as yet. In F_1 hybrids different allelic combinations behave differently, the heterozygote sometimes resembles the susceptible, sometimes the resistant parent and sometimes is intermediate (see Table 8). For example, the two different substrains of Friend leukaemia virus

a) F-S (passed thraugh ICR mice) revealed dominance,

[8] The view expressed in this review that virus S/R is linked (if not identical with H-2) to a gene to the right from H-2 is obviously forced and does not take sufficient account of experimental results. For the Gross leukaemia virus susceptibility gene one of the alternative explanations was that this gene is located roughly 10 map units away from H-2 (LILLY, 1966a), and for the Friend virus 30 map units (LILLY, 1966b). The virus S/R affecting H-2 associated gene could influence the ability of the host to stage an effective immunological response to the virus as to virus-induced antigens (LILLY, 1968). A gene affecting the level of antibody response (MCDEVITT and TYAN, 1968) seems to be located on the right side or right end of H-2. LILLY (1968) found a slightly better association of virus S/R with H-2 than with T (7% of recoveries in H-$2^{b/a}$ and 81% of non-recoveries in H-$2^{b/b}$, whereas 11% of recoveries in T/+ and only 66% of non-recoveries in +/+). If the locus would be 10—30% of recombinations (making use of the data from experiments with Gross and Friend leukaemia) on the left from H-2, the opposite should be true. Undoubtedly, these data are clearly insufficient to establish this point.

Table 8. *Selected data about viral leukaemogenesis and H-2 locus in inbred and congenic strains of mice*

H-2 allele	Mice strain	Virus	Susceptibility	Comment	References
kk	AKR	Gross-A	100%		LILLY et al. (1964)
kk	C58		100%		LILLY (1966b)
kk	C3H		100%	mean latent period 68 days	
kk	C57BR		100%		
bb	C57BL/6		26%	mean latent period 191 days	
bb	129		very low		
kb	(C3H × C57BL/6)F1		7—10%	susceptibility recessive	
	all together F2		45%		
kk	F2		90%		
kb	F2		25%		
bb	F2		27%		
	all together Bc1		72%		
kk	Bc1		90%		
kb	Bc1		56%		
kb	(AKR × C57BL)F1		very low	susceptibility recessive	
kk	(AKR × C57BL)Bc1		41%		
kb	(AKR × C57BL)Bc1		29%		
kk	C3H	Bittner[a]	100%		
ll	I		low incidence		
kl	(C3H × I)F1		72%	susceptibility dominance	
kl	(C3H × I)Bc1		67%		
ll	(C3H × I)Bc1		45%	non significant difference	
kk	C3H	Friend	+++		LILLY (1966a)
ll	I		—		
kl	(C3H × I)F1		+++, —	susceptible as newborns, resistant as adults	
kk	(C3H × I)Bc1		36%		
kl	(C3H × I)Bc1		14%		
kk	(C3H × I)F2		47%		
kl	(C3H × I)F2		21%		
ll	(C3H × I)F2		27%		

[a] Bittner spontaneous mammary tumours.

Table 8 (continued)

H-2 allele	Mice strain	Virus	Susceptibility	Comment	References
kk	C3H	Friend	+++		LILLY (1967)
	ICR	(F-S)[b]	+++		
dd	DBA/2		+++		
dd	BALB/c		—	susceptible to high doses as adults or repeated doses postnatally	
aa	A		—	susceptible to high doses as adults or repeated doses postnatally	
bb	C57BL/6		—		
db	(DBA/2 × C57BL/6)F1		±	susceptibility recessive	
kk	C3H	Friend[c]	+++		LILLY (1967)
	ICR	(F—B)	+++		
dd	DBA/2		+++		
dd	BALB/c		+++		
bb	C57BL/6		—		
db	(DBA/2 × C57BL/6)F1		+++	susceptibility dominance	
bd	(C57BL × BALB/c)Bc1	Friend	50[d] 7[e]		LILLY (1968)
bb	(C57BL × BALB/c)Bc1	(F—B)	44 81		
dd	(C57BL × BALB/c)F2		79 2		
bd	(C57BL × BALB/c)F2		70 2		
bb	(C57BL × BALB/c)F2		67 44		
dd	BALB/c	(B/T-L)[f]	100%		TENNANT and SNELL (1966)
kk	C3H		4%		
kk	C57BR		9%		
dk	(BALB/cxC3H)F1		92%	susceptibility dominant	
	all together F2		73%	no consistent relationship with H-2 genotypes	
dk	(BALB/cxC57BR)F1		0%	susceptibility recessive	
	all together F2		39%	no consistent relationship with H-2 genotypes	
bb	C57BL/10(B10)		62%	B10 and congenic strains; comparable results	

[b] Friend (F—S) passed through ICR mice.
[c] Friend (F—B) passed through BALB/c mice.
[d] Incidence of splenomegaly.
[e] Recovery from splenomegaly.
[f] BALB/Tennant-leukaemia (B/T-L).

Table 8 (continued)

H-2 allele	Mice strain	Virus	Susceptibility	Comment	Reference
dd	B10.D2		94%		
aa	B10.A		88%		
kk	B10.BR		84%		
dd	BALB/c	B/T-L	100%		TENNANT and SNELL (1968)
bb	C57BL/10 (B10)		39%		
dd	B10.D2		83%		
aa	B10.A		73%		
kk	B10.BR		62%		
ba	(B10 × B10.A)F1		57%		
bd	(B10 × B10.D2)F1		67%		
da	(B10.D2 × B10.A)F1		94%		
aa	A		100%		
bb	A.BY		75%		

The percentage of susceptibility in different experiments cannot be strictly compared due to differences in virus injection schedules and scoring of the effect.

b) F-B (passed through BALB/c mice) revealed recessivity on the *same* (DBA/2 × C57BL/6) F_1 hybrids. This excludes on explanation of relationship on the basis of molecular mimicry since this should be associated with complete dominance of susceptibility (TENNANT and SNELL, 1968). Potent anti-H-2 sera do not produce 100 per cent neutralization of virus particles (TENNANT, 1967). Pretreatment with virus or allogeneic leukaemia cells had different effects in two different susceptible mouse strains (TENNANT, 1967). Finally, the virus dose and age of the recipients strongly influence the outcome of the experiments. This was well demonstrated by LILLY (1968) when he found that the response to Friend leukaemia virus is governed essentially by a single gene with a dominant allele for susceptibility which segregates *independently* with respect to H-2. However, this essential susceptibility is markedly influenced by the host's genotype with respect to H-2 or a gene closely associated with it. In mice with an essentially susceptible genotype, H-2 type influences the threshold virus dose required for the induction of splenomegaly and also the prognosis for recovery (LILLY, 1968). Further, the analysis of LILLY (1968) showed that the influence of H-2 is expressed as a quantitative character which has no effect in the absence of an essentially susceptible genotype. In the experimental design of LILLY (1968) the H-2 associated gene influenced *recovery* from splenomegaly, i.e. *after* cellular infection and virus proliferation had begun. This implies an assumption that a specific immunological response is effectively enhanced in mice of certain H-2 genotypes. Different homozygotes have different degrees of the hypothetical specific immune response ability and heterozygotes belong to different categories producing probably a continuous line. This could be the effect of different results of interactions between the two influencing genes. It is interesting that the t alleles and genes

influencing reproductive performance (linked with H-2) have a similar mode in producing their effects. Nevertheless, a number of alternative hypotheses presuming a more direct involvement of the H-2 phenotype and/or genotype are still tenable:

a) H-genes could interfere with alterations during host-recipient interaction (TENNANT, 1967; BAILEY, 1966);

b) the virus donor-host could influence the outer virus coat (TENNANT, 1967);

c) H loci may be homologous with a part of the antigenic constitution of the outer virus coat (NANDI, 1967; SNELL, 1968).

The experiments of NANDI (1967) indicate that virus-carrying mice (mammary tumour) have the virus coated with H-2 alloantigens of the host. In these experiments BALB/c mice could be infected with red blood cells transmitting mammary tumour virus (R-MTV) only when H-2 compatible allogeneic cells were used. Further, blood from H-2^a donors was noninfective in H-2^d or H-2^k recipients but highly infective in H-2^d/H-2^k heterozygotes which share the complete set of H-2 specificities produced by the H-2^a allele. This resembles the discovery of the "dk" effect by SNELL (1951, 1953) in whose experiments transplantable tumours were rejected due to H-2 differences in incompatible recipients, whereas in the experiments of NANDI (1967) the "incompatible" virus was rejected or neutralized, suggesting that the virus was coated with H-2 antigens. A gene regulating the effectiveness of immunological response linked or identical with H-2 also could interfere with this mechanism.

Well recognized differences in the qualitative and quantitative aspects of the immune response to H-2 antigens in different donor-recipient combinations could illustrate the complexity of the situation depending on rules governing the given H-2 donor-recipient relationship (see also the F_1 hybrid effect in CUDKOWICZ's interpretation, page 25). In other words, even in a pure H-2 congenic situation the virus S/R can vary in combinations, which theoretically should be "identical", the same appears in some experiments with the skin grafts. It was shown that a difference in antigen H-2.32 or H-2.31 leads to markedly different effects (concerning the speed of rejection) in dependence on the donor-recipient combination (KLEIN, 1966; EICHWALD et al., 1968). Analogous reasons could lead to statistically significant differences in virus S/R, for example, in the experiments of TENNANT and SNELL (1968).

6. The H-2 System and Genetic Control of the Antibody Response

There are several data about strain differences in the immune response to different antigens, or to microbial or virus infection. Except for susceptibility or resistance to some virus — produced murine leukaemias, there are no data about a possible linkage with the H-2 or IXth linkage group. MCDEVITT and TYAN (1968) described that "the antibody responses of CBA and C57 mice to a series of multichain synthetic polypeptide antigens are quantitative traits which are under a dominant, determinant-specific type of genetic control"

(see also McDevitt and Sela, 1965, 1967). These antigens are composed of a polylysine backbone with side chains of poly-DL-Alanine terminating in short, random sequences of either tyrosine and glutamic acid (/T, G/-A—L), or histidine and glutamic acid (/H, G/-A—L), or phenylalanine and glutamic acid, (/P, G/-A—L). The multipoly-alanyl-polylysine (A—L) part of these antigens is not antigenic by itself, and the antibody response to (T, G)-A—L is specific for the tyrosine, glutamic acid, and alanine at the end of each side chain. CBA and C3H mice respond well to (H, G)-A—L and poorly to (T, G)-A—L, whereas C57 mice respond poorly to (H, G)-A—L and well to (T, G)-A—L. The quantitative difference between CBA and C57 mice with respect to anti-(T, G)-A—L response is approximately tenfold (McDevitt and Tyan, 1968). While these differences are clearly quantitative, when operationally divided into responders (high responder mice) and nonresponders (low responder mice), it was discovered that the major genetic factor controlling the ability to respond well to (T, G)-A—L and (H, G)-A—L is identical or closely linked with H-2. In other words, the different H-2 alleles were themselves responsible or marked a closely linked gene which in the course of immune response "can discriminate clearly between tyrosine, histidine and phenylalanine in the antigenic determinant" (McDevitt and Sela, 1967).

It is interesting that in the pioneer studies on segregating populations of CBA and C57 mice it has been found that "the differing ability of CBA and C57 mice to make an antibody response to (H, G)-A—L is dominant and in all likelihood due to more than one genetic factor, or to one major genetic factor plus an unknown number of modifiers. This tentative conclusion is based on the observation that in the $F_1 \times C57$ backcross there is not a clear 1:1 Mendelian segregation of C57 and F_1 phenotypes" (McDevitt and Sela, 1967). However, in a modified antibody assay high dilutions of antisera were testable and the difference between CBA and C57 sera became greater and thus more distinct. Mice of congenic strains differing in the chromosomal segment for H-2 only differed also in their ability to produce antibody. While C3H ($H\text{-}2^k$) mice are good responders, C3H.SW ($H\text{-}2^b$) are poor responders to (H, G)-A—L. Results along the same line were obtained in other combinations. There were minor quantitative discrepancies, for example, C3H ($H\text{-}2^k$) and B10.BR ($H\text{-}2^k$) mice produced antibodies with 28% and44% capability of antigen binding to (H, G)-A—L. Experiments on Bc animals serotyped for H-2 showed a close linkage of antibody production with the H-2 type of recipients. In (CBA $\times$ C57) $F_1 \times$ CBA Bc animals there were only 2 "recombinants" out of 38 animals tested. In the (CBA $\times$ C57) $F_1 \times$ C57 Bc animals there was only one exception out of 20 animals tested. Altogether this indicates an approximately 5% recombination between the two traits (McDevitt and Tyan, 1968).

The mechanism underlying the observed strain differences and H-2 linkage for antibody production remains obscure. The genetic control seems to be directly related to the preocess of antibody formation and can be localized in spleen cells (McDevitt and Tyan, 1968). No qualitative correlation could be

found between the ability to respond and the immunoglobulin type of the antibodies produced, or between the immunoglobulin allotype and responder mouse strain, i.e. the ability to respond is not linked to the structural gene coding for the Fc fragments of different mouse immunoglobulin classes (McDEVITT, 1968). The antigens tested and their respective antisera cross-react extensively, though they seem to be unable to cross-stimulate a secondary response (McDEVITT and SELA, 1967). Nevertheless, the CBA and C57 mice differ in their ability to undergo a secondary response (McDEVITT, 1968).

There are three points of reasoning which make one of the alternative hypotheses unlikely, i.e. that the genetic control is determined by shared antigenic determinants between the antigens examined and the animal's own antigens. 1. F_1 hybrids are not uniformly poor responders (as in the virus S/R situation); 2. alloimmune sera do not cross-react with these polypeptide antigens; 3. the ability to produce antibody can by transferred by cells to irradiated recipients.

In connection with the finding of linkage between H-2 and virus S/R, McDEVITT and TYAN (1968) presume that "the IXth linkage group includes a large chromosomal region which is in some way related to antibody formation". It seems that different authors prefer the view that genetic interference with some more general immunologic mechanism is associated with the IXth linkage group or with H-2 directly as the key for understanding the relation of H-2 and virus S/R or the McDevitt phenomenon (LILLY, 1968; SNELL, 1968). Strain differences in the ability to produce predominantly 7S and 19S antibodies have been described (WINN, 1965b; ANDERSON et al., 1967). ANDERSON et al. (1967) pointed out that the quantitative differences between CBA and C57BL mice in antibody response to synthetic polypeptides should be considered as a possible reflection of differences in 7S and 19S antibody production.

7. Genes on the Left Side of H-2

We shall give only a short survey of the genes tf, Ki and Fu, quoted from the list of mutant genes in "Biology of Laboratory Mice" (GREEN, 1966). There are neither data nor presumptions about the possible role of their linkage with H-2.[9] They represent *occasionally* observed mutants in the labora-

[9] The occurrence of three genes in the IXth linkage group, with similar effects on the developmental processes and affecting the tail in heterozygotes is remarkable. A possible relation between T, Fu and Ki has been repeatedly considered. It seems interesting to quote the oldest presumption concerning the genetic situation in the IXth linkage group (DUNN and CASPARI, 1945). "A repeat hypothesis applied to the present case would assume that an original locus $+^T$ may have triplicated to becom $+^T$ $+^{T1}$ $+^{T2}$. One locus may then have mutated to T, another to Ki and a third to Fu." Later, it was thought by DUNN (1956) following the similarities in the effect of three neighbouring genes T, Ki and Fu that (because the region might contain much heterochromatin) any change in this chromosomal segment should result in a disturbance of basic developmental processes. The t alleles whose effect is the most similar to Fu and Ki are t^{w18} (BENNETT and DUNN, 1960) and t^q (MOSER and GLUECKSOHN-WAELSCH, 1967).

tory mice and identical or mutant alleles of the respective loci have not yet been found in populations other than the original one.

"Tf, tufted, recessive, IX. Probably arose spontaneously in a multiple recessive stock. Homozygotes show repeated waves of hair loss and re-growth which begin at the nose and pass posteriorly along the body. Viability and penetrance are good (LYON, 1956).

Fu, fused, semidominant. Arose in stocks at the Bussey Institution prior to 1931. Expression of this mutant shows great variability. Both homozygotes and heterozygotes may have shortened and kinked tails or they may both be normal. Homozygotes tend to be more severely affected than heterozygotes. Expression is not dependent on the residual genotype but offspring of Fu/+ or Fu/Fu mothers are less likely to express the character than offspring of +/+ mothers (REED, 1937). Homozygotes and heterozygotes occasionally show an abnormal behaviour similar to the circling mutants and are deaf (DUNN and CASPARI, 1945). Embryos of homozygotes show some overgrowth and duplication of the posterior part of the neural tube (THEILER and GLUECKSOHN-WAELSCH, 1956).

Fu^{ki}[10], kinky, semidominant. Arose in the stocks of a Florida mouse fancier (CASPARI and DAVID, 1940). Heterozygotes are very similar to fused in abnormalities of the skeleton and may show the same behavioral abnormalities. DUNN and CASPARI (1945) found five probable crossovers between Fu and Fu^{ki} among 505 gametes tested, but DUNN and GLUECKSOHN-WAELSCH (1954) found none in 971 and concluded that the previous study was in error and that kinky is an allele of Fu. Unlike Fu, kinky homozygotes are inviable. They show tissue hyperplasia and twinning at 7 days and die between 8 and 10 days of embryonic life (GLUECKSOHN-SCHONHEIMER, 1949)." (From GREEN, 1966.)

8. The T Locus

The T locus is located about 15 crossover units proximal to the H-2 system in the IXth linkage group (GORER et al., 1948; SNELL, 1952; ALLEN, 1955). There is a number of reasons for the hypothesis that the linkage between the T and H-2 systems represents a functional unit: super-gene (SNELL, 1968). The T locus thus represents one of the key positions for understanding the biologic role of the H-2 system. It seems justifiable to recognize the H-2 system or a chromosomal segment including H-2 as a major gene interfering with a number of immunological processes, i.e. histocompatibility, thymus leukaemia, virus susceptibility, antibody production, F_1 hybrid resistance. The genetic fine structure of the T locus regulates the effectiveness of these processes. While a number of internal problems of these complex genetic systems are unsolved, it is evident that the respective H-2 gene frequencies in nature mouse populations are basically influenced by alleles of the T locus.

[10] In the Linkage Map of the Mouse compiled by BUTLER and MEREDITH (1968) in Mouse News Letter the loci Ki and Fu are separated by about 5 crossover units.

It is impossible to give details about this complex system in this review. The findings were summarized by DUNN (1964) and BENNETT (1964). The basic data about the system are summarized in Fig. 5. The following points will be briefly discussed:

a) The dominant T alleles;

b) The recessive t alleles; 1. T/t interaction; 2. Lethality of t alleles; 3. Abnormal segregation ratio of t alleles; 4. Crossover suppression by t alleles;

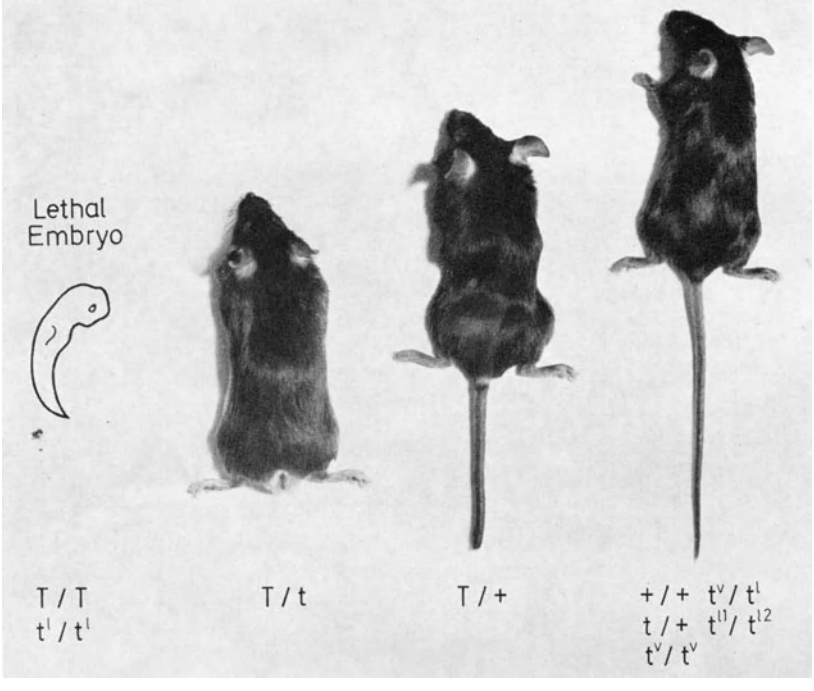

Fig. 5. The T system in mice. t^v viable t alleles; t^l lethal t alleles; t^{l1}/t^{l2} different lethal t alleles

5. Reproductive performance and t alleles; 6. Polymorphism of t alleles in Mus musculus; 7. Cytological and biochemical findings;

c) Genetics of the T locus.

a) The Dominant T Alleles

The T locus represents a complex genetic system occupying a chromosomal segment of uncertain length between the centromere and gene tf (tufted). The first mutant gene was discovered by DOBROVOLSKAIA-ZAWADSKAIA (1927) in the Institute Curie, designated T, the short-tail gene or Brachyury. This was a dominant mutation, which when heterozygous (T/+) produced a short tail and when homozygous (T/T) was lethal for 10-day embryos. Identical or similar mutations at this locus are not frequent and were detected only 2—3 times [CARTER and PHILLIPS, 1950; KUMINEK, 1959, 1960; LYON, 1959 (T^h);

SEARLE, 1966 (T^c)]. While it is generally assumed that the T alleles have a normal transmission ratio, DUNN and RYNERSON (1967) and DUNN and BENNETT (1968) reported a "low ratio" (Lr) T allele (transmitted from males to about 14% of the offspring, from females to 50%). The Lr factor is located about 5 crossover units from T and closely linked with tufted.

b) The Recessive t Alleles

1. T/t interaction. The greatest interest in the T locus is focused upon a series of recessive t alleles. They were discovered when T/+ (Brachy) mice were outcrossed to some laboratory or randomly captured Wild mice and when tailless (T/t) mice were observed in their progeny (DOBROVOLSKAIA-ZAWADSKAIA and KOBOZIEFF, 1927, 1932; DOBROVOLSKAIA-ZAWADSKAIA et al., 1934). The T-modifying factors, which behaved as "alleles" of T, were designated as t; T/t are tailless, t/t are lethal or viable with normal tail, and t/+ are viable and with normal tail. The different t alleles are maintained in stocks combined with T, the lethals in balanced lethal lines, with the following breeding system:

	T/t	×	T/t	
T/T		T/t		T/t
lethal		tailless		lethal

With a frequency of about 0.2% viable normal tail animals are observed in balanced lethal lines. This is the result of occurrence of a variant t allele (compounds of different t alleles, i.e. t/t', are not lethal) which occurred probably by rearrangement in the chromosomal segment bearing the t allele. Some of these variant t alleles do not result in tailless phenotype in T/t' mice (t^{h18}, t^{h13}: LYON and MEREDITH, 1964a; t^{h7} and t^{h14}: LYON and MEREDITH, 1964b) and limit thus the absolute validity of the original definition of t alleles.

2. Lethality of t. Many t alleles (mainly those derived from wild populations) are lethal in the homozygous state (t^1/t^1) while t^{11}/t^{12} heterozygotes for different t alleles are viable. The lethal t alleles act at different stages of embryonic development. The earliest acting t allele (t^{12}) (DUNN 1956) acts at the stage of 4 days morula when t^{12}/t^{12} embryos degenerate without undergoing any transition into blastocysts. The latest acting lethal t allele (t^{w1}) has a very extended lethal period, some embryos dying as early as the 9th day of gestation while others survive until birth (BENNETT et al., 1959a, b).

3. Abnormal transmission ratio of t alleles. Relatively few genes show apparent abnormalities from classical Mendelian segregation ratios. Evidently the t alleles are the most widespread ones of this type in mammals (CHESLEY and DUNN, 1936; DUNN, 1953, 1960). While heterozygous females (t/+) mated with +/+ males give birth to about 50% of t/+ and 50% +/+ progeny, an extremely high transmission ratio of t alleles was observed when heterozygous males (t/+) were mated with +/+ females. Except in a few cases, all lethal t alleles have abnormally high transmission ratios, average values recorded by PETRAS (1967) and DUNN (1957a, 1960) were 0.89—0.95. Viable t alleles may be transmitted in high ratios when found in Wild popula-

tions, the pooled value recorded by PETRAS (1967) for 6 t^v alleles was 0.79, but more frequently the value is found to be normal or low (20—40 %) when derived from laboratory stocks.

The observed abnormal transmission ratio has probably many causes or interacting factors (BRADEN and WEILER, 1964; BRADEN, 1958; YANAGISAWA et al., 1961; DUNN, 1943, 1960). While it is mostly presumed that the effect of t alleles is realized before ejaculation, the mechanism and cause of the segregation distortion is not known and several hypotheses have been proposed (DUNN and SUCKLING, 1956; DUNN and GLUECKSOHN-SCHOENHEIMER, 1939; DUNN, 1967; BRADEN, 1958).

4. Suppression of crossing-over in the IXth linkage group by t alleles. (DUNN and CASPARI, 1945). Lethal t alleles almost regularly, viable t alleles sometimes suppress recombination for a distance of at least ten crossover units (DUNN, 1964) in the IXth linkage group, e.g. t^6 was found to suppress recombination in the interval between T and tf from 8.3 to 1.2 (LYON and MEREDITH, 1959, 1964a, b, c; DUNN et al., 1962; DUNN, 1956). The effect of t alleles on recombination frequency is obviously connected with the fine genetic structure of the T locus and its mechanism is not fully understood. It was alternatively presumed that t alleles, which suppress recombination, may be the properties of inversions (DUNN, 1956), deficiencies (lethal when homozygous, DUNN et al., 1962) or functional changes on No. 9 chromosome (LYON and MEREDITH, 1964a, b, c). In the absence of conclusive cytological evidence the alternative arguments remain hypothetical (see also the section on "Genetics of the T locus").

5. Male sterility and t alleles (DUNN, 1937). As in abnormal transmission ratios the complete or partial sterility connected with t alleles is restricted to males homozygous for a viable t allele (t^v/t^v) or heterozygous for viable, lethal or a combination of viable and lethal t alleles (t^{v1}/t^{v2}, t^v/t^l, t^{l1}/t^{l2}). Females of the same phenotype are normally fertile. No effect of t alleles on male fertility in t/+ heterozygotes was described. It should be pointed out that the sterility or quasi-sterility (less than 20 % or normal fertility) is apparently due to interaction of these alleles rather than to a direct effect of single alleles. Complete or quasi-sterility was observed in certain combinations of viable and lethal t alleles, while homozygotes for the same viable t alleles or compounds of the same lethal but different viable alleles appeared not to affect male fertility at all (DUNN and GLUECKSOHN-WAELSCH, 1951; DUNN and BENNETT, 1969).

The mechanism of sterility or reduced fertility produced by the t alleles is not fully understood. Litters from males with affected fertility are less frequent and smaller in size. On the other hand, even in sterile males, spermatogenesis is not impaired, the number of sperms ejaculated is not markedly reduced, the proportion of abnormal sperm cells is not greatly increased; sperm number and morphology seem to be almost normal. Affected males were found to have reduced sperm mobility resulting in low numbers at the site where fertilization should occur, but some spermatozoa were found at

the site of fertilization even in completely sterile males (BENNETT and DUNN, 1967; GLUECKSOHN-WAELSCH et al., 1950; DUNN, 1950, 1952; RAJASEKARASETTY, 1951; BRADEN and GLUECKSOHN-WAELSCH, 1958; DUNN and BENNETT, 1969). However, the finding of normal spermatogenesis in sterile males was not supported in all works. BRYSON (1944) observed an extensive degeneration of testes in t^0/t^1 sterile males. BRYSON (1944) and RAJASEKARASETTY (1951) found a high number of abnormal sperm cells. Recently JOHNSTON (1968) confirmed the findings of high morphological abnormalities and significant reduction in numbers and sperm mobility in sterile t^{w2}/t^{w2} males. Significantly lower testes weights of sterile males also suggested disturbed spermatogenesis. In a work to be published (DUNN and BENNETT, 1969) sterile males homozygous or compound for semi-lethal alleles from wild populations which have no spermatozoa in their ejaculates will be described.

The problem of impaired reproductive performance linked with H-2 was extended by experiments in which randomly captured wild males were mated with females of inbred strains C57Bl/10 (hereafter B10), A, C3H (which are all +/+ by definition, because when mated with T/+ they have no tailless progeny) (IVÁNYI and DÉMANT, 1968; IVÁNYI et al., 1968). All types of hybrid females and (C3H × W) F_1 hybrid males were normally fertile. (B10 × W) F_1 and (A × W) F_1 hybrid males produced by a number of Wild males were completely sterile. The male sterility was accompanied by reduced testes weight and an almost complete arrest of spermatogenesis, at the stage of primary spermatocytes (i.e. a premeiotic stop). The presence of "typical" t alleles was not a prerequisite because this type of sterility was observed in t/+ and also +/+ hybrid males. When different types of segregating hybrids were serotyped for H-2 antigens it was found the a genetic factor associated with H-2 and thus located in the IXth linkage group is responsible for the observed sterility; the presumed gene was provisionally designated W^{st}. The involvement of the genetic sites of the dominant T gene and H-2 alleles seemed to be unlikely. It was hypothetically presumed that t alleles without the T-modifying effect could be responsible for this type of male sterility (IVÁNYI et al., 1969 a, b).

The impairment of spermatogenesis was the result of *interaction* of genes located on both IXth chromosomes of the hybrid males involved. In different combinations variations in reproductive performance presented a continuous line from clear-cut extrems of complete arrest of spermatogenesis to only slight differences in male fertility. The difficulty of recognizing these differences is underlined by the fact that litter size was grossly identical but litters were less frequent, resulting in a lower number of offspring per mating unit from different types of hybrid males. The consequences of these findings in population genetics aspects in mice cannot basically differ from those discussed in connection with t alleles, nevertheless they also allow the assumption of more general aspects based on the observed high polymorphism of the respective alleles and variations due to the effect of their different compounds (IVÁNYI et al., 1969b). The hypothesis is put forward that reproductive performance underlined by *quantitation* of spermatogenesis, though doubtless a polygenic

trait, is in *each individual* affected by at least one major gene localized in the IXth linkage group and thus linked with the MHS.

6. Polymorphism of t alleles in Mus musculus. For the aspects of this review it is of primary importance to emphasize that *different t alleles are very frequent and almost consistently present in natural populations of Wild mice.* The presence of t^w alleles was confirmed in almost all out of more than 30 different wild populations from widely different centers in the USA (DUNN and MORGAN, 1953; DUNN, 1955; DUNN and BENNETT, 1960; DUNN et al., 1960; PETRAS, 1965, 1967) and in small trapped examples in Japan, Britain, Australia and Czechoslovakia (TUTIKAWA, 1954; DUNN, 1964; IVÁNYI et al., 1969a, b).

The greatest part of Wild mice bearing t alleles is heterozygous, the frequency of t/+ genotype is frequently 40—50% (DUNN, 1956, 1957a, b; DUNN et al., 1960). In an extended study of PETRAS (1967) the overall gene frequency for t was found to be 0.16, with variations from 0.0 to 0.50 in different buildings of a rural area. Similarly, wide differences were found in the same trapping place from year to year [e.g. 0.0 (1959), 0.50 (1960), 0.20 (1961), 0.17 (1964) in a chicken coop]. Mice live in relatively small closed units and periodically undergo substantial changes in the effective number of the population size due to various external influences. Nevertheless, the distribution of t alleles is being intensively directed by the specific properties of the T locus (lethality, abnormal transmission ratio, male sterility) (ANDERSON et al., 1964; BENNETT et al., 1967; DUNN and BENNETT, 1967). All this indicates that the T locus represents the most intensively studied example in mammals for population studies, similar to those performed in Drosophila (SANDLER et al., 1959; HARTL et al., 1967; WATANABE, 1967). Several model situations based on data from feral and confined populations were studied by computer simulation and compared with actual situation (PROUT, 1953; BRUCK, 1957; LEWONTIN and DUNN, 1960; DUNN and LEVENE, 1961; PETRAS, 1967; LEWONTIN, 1968).

Generally lethal t alleles are more frequent in Wild populations than viable alleles. Alleles found in different populations, frequently widely separated, fall into groups of identical lethals. This can be basically explained by two possibilities: a) common origin, dispersion by human factors; b) repeated independent occurrence of the same mutation (DUNN, 1955; BENNETT and DUNN, 1958, 1964; DUNN et al., 1960; BENNETT et al., 1959b; PETRAS, 1967).

7. Cytogenetic and biochemical abnormalities associated with t alleles. While a number of findings and assumptions indicate that the T locus could represent a grossly abnormal chromosomal segment, actual findings along this line are scarce and inconclusive (JAFFE, 1952; BENNETT, 1965; BRYSON, 1944). The findings of GEYER-DUSZYNSZKA (1964) indicate that alleles t^0, t^{w6} and t^{w18} are interstitial deficiencies involving different chromosomal regions, t^{12} is either a big terminal deficiency or a small interstitial one inducing constant asynapsis in adjacent terminal segments of the bivalent. The conclusions drawn by GEYER-DUSZYNSZKA (1964) were "(i) that the T locus occupies the main part of the long arm of the chromosome of linkage group IX, (ii) that the t alleles are much more scattered along the chromosome than was previously

supposed and (iii) that they are not unilocal neither with T nor with each other". A diagrammatic presentation of these findings is included in Fig. 3. These findings should basically alter the genetic concepts of the locus. Nevertheless, these cytologic observations have not yet been confirmed and the "cytogenetic evidence on the structure of this chromosome region is at present indecisive" (DUNN, 1964).

SMITH (1956) found in t^{12}/t^{12} lethal embryos (the earliest acting lethal t allele) abnormalities in the shape of the nucleoli in the morulae. The normally appearing sharp increase in the cytoplasmic concentration of RNA was absent at this stage. This finding may be due to t^{12} interference with RNA synthesis, presuming the presence of a nucleolar organizer on the IXth chromosome (BENNETT, 1965). KLEIN and RAŠKA (1968) found a low saturation level of DNA (about 30%) from $t^{12}/+$ heterozygotes with ribosomal RNA from +/+ mice. It was concluded that $t^{12}/+$ mice have a deficiency at the locus for RNA synthesis, which supports the possibility that this locus represents one part of the nucleolar organizer region in mice.

c) Genetics of the T Locus

The designated T locus seems to be an extremely complex genetic system. In the absence of conclusive cytological and biochemical data, the occurrence of variant (mutant) t alleles, the frequency of this event and the different features of t alleles, serve as a basis for different hypotheses (DUNN and GLUECKSON-SCHONHEIMER, 1953, 1950; DUNN, 1954, 1956, 1964; LYON and PHILLIPS, 1959; DUNN et al., 1962; BENNETT, 1964; LYON and MEREDITH, 1964a, b, c).

While "mutants" from lethal to viable t alleles were repeatedly observed, no example of the reverse, or $+ \rightarrow t$ mutation was reported. Nevertheless, the occurrence of new t alleles in balanced lethal lines was observed in the relatively high frequency of about 1/500 mice born in balanced lethal lines. The high frequency of occurrence of these variants indicates that they are not mutations (which are in the range of 8×10^{-6} per locus per gamete in the mice, SCHLAGER and DICKIE, 1967). The new t alleles have different properties than the t allele from which they were derived, i.e. they have lost some part of the original complex property. In all mutants either the t-lethal factor or the t-T modifying factor was present. This led to the view that the two respective factors are determined by the two extreme parts of the respective chromosomal region (see Fig. 3). The two extreme variants did not reconstitute the original "complete" (t^6) allele when placed on the same chromosome thus providing the basis for the assumption of the existence of a middle piece responsible for male sterility, crossover suppression and abnormal transmission ratio. Alternatively, the whole length of the abnormal region is dependent (LYON and MEREDITH, 1964a, b, c).

Two types of variants deserve special attention. t^{h18} and t^{h13} lost the T-modifying factor (short tailed T/t mice); t^{h7} and t^{h14} gained an opposite T-modifying effect (normal tailed T/t mice) (LYON and MEREDITH, 1964b).

Three possibilities were considered to explain the occurrence of these variants:

i. mutation in the classical sense of the term;

ii. changes in the homologous locus of the partner chromosome induced by t alleles;

iii. crossing-over in a chromosomal region representing the T locus.

All the above findings indicated that t alleles occupy a considerable part of the length of the IXth chromosome (from T to Fu-Ki-tf). Different interpretations about their nature are possible[11], mainly:

i. t alleles are series of spatially separated inversions and/or series of deficient, deleted or duplicated sections, i.e. structural differences (DUNN et al., 1962);

ii. t alleles are series of functionally changed abnormal regions of different length causing inhibition of effective pairing (LYON and MEREDITH, 1964a, b, c);

iii. t alleles are deficiencies or deletions scattered along the chromosome (GEYER-DUSZINSZKA, 1964).

The concept of the T locus and its possible role as a part of the T—H-2 supergene (SNELL, 1968) is not yet fully understood. In conclusion, from the point of view followed in this review the extreme polymorphism of the T system should be emphasized. This could not be studied in such detail without the interaction of T/t resulting in tailless animals. If analogous systems *linked* with the MHS exist in other species, but are not detectable by similar visible effects, its definition would be much more difficult. Nevertheless, the B system of chickens displays remarkable analogies. Preliminary findings in rats indicate the possible existence of a similar linkage group. In humans an analogous situation would be even more difficult to tackle due to selection against "t" alleles. Some preliminary findings indicate a similar situation (see page 62).

The T system is the only known example of lethal and male sterile alleles in mammals with a remarkable polymorphism in natural populations maintained by a complicated balanced system, in which an abnormally high transmission ratio contributes not only to the maintenance but also to progressive dispersion of the deleterious gene (DUNN, 1964). The hypothesis is put forward that forced heterozygosity, in a neighbouring chromosomal segment regulating a number of basic immunological processes, could represent the performing part of a certain evolutionary effort.

[11] STIMPFLING and RICHARDSON (1965) when discussing H-2 recombinations and the problem of T locus wrote: "Similarly, the notion that the H-2 locus may be constituted of two or more contiguous gene duplications, as opposed to its being a pseudoallelic system, provides a plausible explanation of its origin and complex character. However, the lack of critical evidence makes a detailed consideration of this view premature." (See also footnote on page 36.) A similar view was applied to the human HL-A system (DAUSSET et al., 1967c). This is probably only a reflection of a somewhat general idea to hold this hypothesis for phenotypic expressions of the "asymmetric" features in complex loci. Even in allotypic differences, where more exact biochemical information is available, analogous theories obtained a more concrete basis (SMITHIES et al., 1962; SMITHIES, 1964).

D. HL-A, the Major Histocompatibility Systems in Humans

1. Indroduction

Data about skin graft survival could give an insight into the general situation on histocompatibility in humans. There are no conclusive data about permanently surviving grafts transplanted between unrelated individuals; none out of a few hundred grafts transplanted by different teams survived longer than a few dozen days, while the overwhelming majority were rejected by the 12th day after grafting. Only 16% of the grafts transplanted from children to fathers survived longer than 15 days, only two out of 62 grafts survived longer than 20 days but not a single graft survived longer than 90 days (DAUSSET et al., 1969a, b). The mean rejection time found by CEPPELLINI et al. (1965) in sibs was 14.7 days. When skin grafts were transplanted between sibs recognized as HL-A identical and non-identical, survival averaged 26 days (15—36) and 12 days (11—13) respectively (AMOS et al., 1967b; BACH et al., 1967; AMOS and BACH, 1968). There are no data available which would contradict the assumption that in humans a similar number of segregating H-loci as in other species exhibits polymorphism. It is evident that in such a situation combined with other "technical" difficulties the identification of the MHS could be extremely difficult. A short survey of procedures leading to its discovery will be given here, since it serves as a suitable illustration of difficulties resulting from the complexity of the situation.

It was suggested by DAUSSET (1954) that leucocyte agglutinins are alloimmune[12] rather than autoimmune antibodies and could serve for serotyping of H antigens in humans. These were obtained from patients after multiple transfusion (DAUSSET, 1958) and later mainly from multiparous women (PAYNE and ROLFS, 1958; VAN ROOD et al., 1958, 1959).

The first leucocyte antigen detected by alloimmune human sera was described by DAUSSET (1958) and designated MAC. However, as late as 1965 (DAUSSET et al., 1965a) it became clear that MAC belonged to the human MHS. During this time, difficulties with available serological techniques (leucoagglutination, plateletcomplement fixation, lymphocytotoxic test and others) and the practical impossibility of obtaining two identically reacting sera from large batteries of randomly chosen reagents led VAN ROOD (1962) and VAN ROOD and VAN LEEUWEN (1963) to introduce computer techniques in this field. On the basis of the chi-square values, a computer program helped to select sera with highly similar reaction patterns. A group of positively associated but not completely identical sera (as in antigen MAC) served for the definition of leucocyte antigens following the reasoning that an individual possesses a certain antigen if his leucocytes react positively with all or almost all sera of the respective group of similar sera. The reaction pattern of the

[12] Human leucocyte antigens detected by rabbit heteroimmune agglutinating sera were defined by REJHOLEC et al. (1954) and called Z and V, with 0.55 and 0.65 frequency in the Prague population. Z and V antigens exerted a significant negative coefficient of correlation. They were not followed further and it is not known whether they belong to the HL-A system.

individuals reacting with some of the sera defining a given specificity was considered to be either the result of extra antibodies and was classified as negative, or of ANAP (i.e. cells negative in the agglutination test with certain

Table 9. *New HL-A nomenclature and previous designation (Bull. World Health Org., 1968)*

New HL-A nomenclature[b]	AMOS	BATCHELOR	CEPPELLINI	DAUSSET	KISSMEYER-NIELSEN
HL-A1	19	1	To-8	11	LA1
HL-A2, or HL-AMac	1	5	To-9	1 or Mac	LA2
HL-A3	4	—	To-10	12	LA3
HL-A4					
HL-A5	45	25	To-5	5	—
HL-A6					
HL-A7	2	—	To-20	10	—
HL-A8	41	2	To-7	8	—

New HL-A nomenclature[b]	PAYNE/ BODMER	VAN ROOD	SHULMAN	TERASAKI	WALFORD
HL-A1	LA1	LA1	—	1	Lc-1
HL-A2, or HL-AMac	LA2	8a	PIGrLy^{B1}	2	Lc-2
HL-A3	LA3	LA3	Hill	8	Lc-3
HL-A4					
HL-A5	—	Da5	—	6	—
HL-A6					
HL-A7	4d	7c	—	5	Lc-8
HL-A8	7d	7d	—	11	Lc-7

[a] A dash (—) indicates that no symbol has been allocated within the nomenclature concerned.

[b] HL-A4 will be reserved for one of the higher frequency 4[a] factors and HL-A6 for 4[b]. Before assigning these specificities an exchange of sera among collaborating laboratories will be necessary.

sera but positive when tested by absorption: agglutination-negative- absorption-positive) and classified as positive. The discovery of the first pair of antigens with a contrasting distribution in the population (4a, 4b) evoked a number of further studies.

A basically similar approach in the years 1964—1967 helped to define a number of leucocyte antigens in different laboratories (see Table 9 and Histocompatibility Testing Conferences and Workshops 1964, 1965, 1967) designated, for example, by VAN ROOD et al. (1967b) 4a, 4b; 5a, 5b; 6a, 6b; 7a, 7b, 7c, 7d; 8a; 9a. This nomenclature approach followed the finding that pairs or groups of the described antigens had an alternative distribution in the population with a significant negative coefficient of correlation indicating the

possibility that the respective pairs of antigens could be determined by alternative genes of an "allelic" system. Family studies supported this possibility (VAN ROOD and VAN LEEUWEN, 1965; VAN ROOD et al., 1965). After serotyping 130 individuals, DAUSSET et al. (1965a) examined in greater detail all mutual coefficients of correlation between 50 alloimmune sera subdivided into 10 groups of similarly reacting sera which served for the definition of 10 antigens. On the basis of significant positive and negative correlations[13] between the respective sera and defined antigens, they first concluded that the 10 antigens described belong to one complex genetic system (proposed to be called Hu-1); the antigens were denoted by numbers 1—10 (later extended up to 17). The authors thus followed ROSENFIELD et al. (1962) and SNELL et al. (1964) in designating specificities of complex alloantigenic systems by "genetically neutral" numbers. Serological workshops and exchange of sera demonstrated that some of these antigens had been recognized previously or were found later by a number of different laboratories. The conclusion deduced from population data about one complex system (Hu-1) was confirmed by family studies of DAUSSET et al. (1967a, b, c) and CEPPELLINI et al. (1967) and by an extensive study at the Turin Histocompatibility Workshop (CEPPELLINI, 1967). Following the recommendation of the WHO Nomenclature Committee, the MHS in humans is called HL-A (AMOS, 1968). While different laboratories used different designations for HL-A antigens, the WHO Nomenclature Committee (1968) recently recommended an international designation of 6 best defined HL-A antigens (Table 9, Fig. 6).

At present the "number" of HL-A specificities recognized in different centers differs widely, mainly due to the different approaches (definitions and methods of statistical analysis). At the last Histocompatibility Workshop, AMOS et al. (1967a) reported that at least 50 antigenic specificities could be recognized, BATCHELOR and SANDERSON (1967) recognized 26 different antigenic specificities, DAUSSET et al. (1967c), VAN ROOD et al. (1967), CEPPELLINI et al. (1967), PAYNE et al. (1967a) worked with 7—14 HL-A antigens and MICKEY et al. (1967a) recognized 5 antigen groups. The above numbers are not at all comparable due to basic differences in the definitions of the terms used (AMOS, 1967). TERASAKI's team examined the greatest number of alloimmune sera (495 mentioned by MICKEY et al., 1967a). What would perhaps

[13] The coefficient of correlation $r = \sqrt{\frac{x^2}{N}}$ was introduced in the field of human leucocyte typing by FEINGOLD (1966) and used extensively mainly in the works of DAUSSET et al. (1965a, 1966, 1967e). The values of r vary from -1.0 to $+1.0$. A positive correlation indicates a similar distribution pattern for two sera or antigenic specificities in the human population. A negative correlation indicates an alternative distribution and is frequently considered to be an indication for the existence of alternative genes. DÉMANT et al. (1966) computed the values of correlation coefficients of antigens controlled by allelic genes in a population at equilibrium. ANDERSEN et al. (1963) published similar data for the chi-square values. For application to human leucocyte typing see also PAYNE et al. (1964), BODMER and PAYNE (1965), DÉMANT and IVÁNYI (1967a, b), DÉMANT et al. (1967), MICKEY et al. (1967a).

seem to be a useless play with symbols reflects in fact the genetic complexity of the HL-A system (DAUSSET et al., 1965a, IVÁNYI and DAUSSET, 1966). "The ambiguity is perhaps in the nature of the problem at hand and in this case it is appropriate that the ambiguity is not masked" (MICKEY et al., 1967a).

At the Torino Histocompatibility workshop some of the HL-A antigens were defined by identical or almost identical sera, at least on the limited number

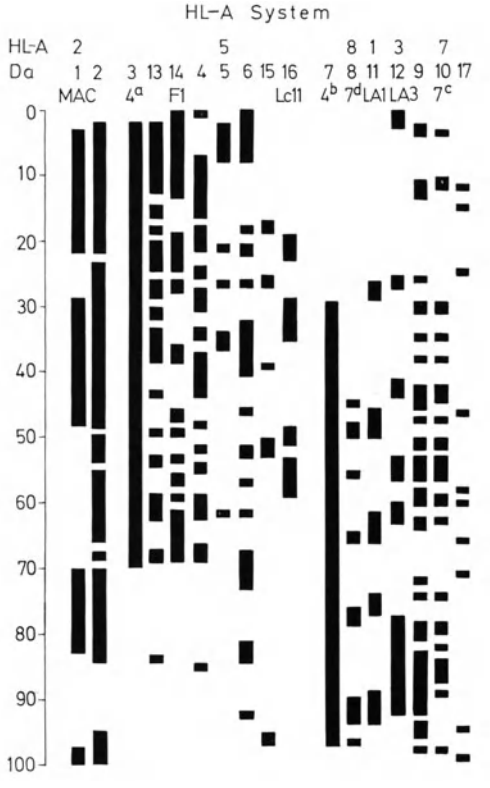

Fig. 6. The MHS in humans, the HL-A system. Distribution of 17 HL-A antigens in the French (Parisian) population. Nomenclature (see also Table 9): first line: international designation of six HL-A antigens; second line: DAUSSET's nomenclature; third line: original designation (DAUSSET, 1969)

of cells tested (Fig. 9). This could serve as a basis for the first attempt to give an international designation to at least some HL-A antigens (WHO Nomenclature Committee 1968, Table 9).

At present, for the actual presentation of the HL-A system, data from one leading laboratory must first be selected, even though there is a basically similar situation in different centers. In Fig. 6 the present situation of the HL-A system in the laboratory of DAUSSET is given. Six defined HL-A antigens obtained an internationally accepted designation. Antigens 3 and 7 are "identical" with 4a and 4b antigens originally described by VAN ROOD. Both these antigens form such complex entities in the populations hitherto studied

that the absence of reproducible identical monospecific sera made it impossible for the Nomenclature Committee to give them a definitive designation (Fig. 6, Table 9)[14].

A number of non/HL-A leucocytes and/or platelet antigens were defined; a list of them is given in Table 11. There are no data about their possible role in histocompatibility and they are obviously members of substantially less polymorphic genetic systems.

2. HL-A Antigens and Histocompatibility

After a number of preliminary findings (summarized by VAN ROOD, 1966), DAUSSET et al. (1965b) and VAN ROOD et al. (1965) could demonstrate that HL-A is a system with a number of strong H antigens. Individuals preimmunized by HL-A incompatible cells exerted an accelerated or white graft rejection of incompatible test skin grafts, while "compatible" transplants were rejected by a normal first set reaction. Later it was found that skin grafts in sib combinations survive significantly longer in HL-A identical than in non-identical combinations (AMOS et al., 1967b, BACH et al., 1967; BACH and AMOS, 1967; AMOS and BACH, 1968) and that skin graft survival in child-parent combinations is influenced by the number of HL-A differences (DAUSSET et al., 1969a, b). Kidney grafts have a significantly longer survival time and a better clinical course (fewer rejection episodes, better kidney function) in HL-A matched donor-recipient pairs than in mismatched pairs. This was demonstrated as early as 1964 in a retrospective study of patients grafted with kidneys from related donors (TERASAKI et al., 1965, 1967a; VREDEVOE et al., 1965), and confirmed by a number of teams (VAN ROOD et al., 1967a; PAYNE et al., 1967a, b; DAUSSET et al., 1967d, e; DORMONT et al., 1967). The significance of HL-A matching for clinical transplantation in unrelated donor-recipient combinations was recently also found by MORRIS et al. (1968a), TERASAKI et al. (1968) and PATEL et al. (1968). Acute rejection of kidney grafts in patients with antilymphocyte antibodies, probably anti-HL-A, was reported by TERASAKI et al. (1967), KISSMEYER-NIELSEN et al. (1967) and MORRIS et al. (1968b).

It should be pointed out that, due to the complexity of the situation from the clinical point of view and the inevitable interference of a number of weak H systems, clinical donor selection remains an approach offering only a statistically higher probability of success for the patient which cannot be individualized. Though further refinement of HL-A typing can be expected, there is little, if any, hope that it will be possible to select "completely" compatible pairs for all H systems. Nevertheless, donor selection can reach a highly effective operational stage (see discussion about the relative strength of HL-A an-

[14] Confusions could arise if we want to discuss in this review the *complexity* of the HL-A system as we should have to refer to a number of specificities not yet recognized by international designation and designated only by ad hoc terms. We prefer for the purpose of this review to follow the terms used in one laboratory selected as an example; a comparison with the WHO HL-A nomenclature is given in Table 9 and Figs. 6, 7, 8.

tigens) which, combined with available or improved immunosuppression, could signify a basic change in the clinical situation. It was proposed that the final outcome should be a broad panel of prospective recipients typed for HL-A (and ABO[15] antigens) and the donor's kidney should be grafted, after matching, to the relatively best recipient. Computer and storage facilities could enable laboratories to effectively apply this possibility even for such a large geographical area as Europe (VAN ROOD, 1967).

Different approaches were proposed for clinical donor selection (see RUSSELL et al., 1966), mainly the third party test (RAPPAPORT et al., 1960; MATHÉ et al., 1961; WILSON et al., 1963; MATSUKURA et al., 1963; IVÁNYI, 1965a), the normal lymphocyte transfer test (BRENT and MEDAWAR, 1963; GRAY and RUSSELL, 1964; GRAY, 1966) and the mixed lymphocyte culture test. The experiments of DAUSSET et al. (1965b) and VAN ROOD et al. (1965) demonstrated that the outcome of the third party test is essentially dependent on the HL-A antigens of the respective donor and recipient. The same was found by VAN ROOD et al. (1965) for the normal lymphocyte transfer test.

The mixed lymphocyte culture test (MLC) deserves special attention for its contribution to the genetics of HL-A and because it represents a "histocompatibility reaction in vitro". BAIN et al. (1963) and HIRSCHHORN et al. (1963) demonstrated that an activation of lymphocytes occurs in short-term cultures of human blood lymphocytes from two unrelated individuals. This can be measured morphologically by counting the percentage of blast cells (after 4—6 days of incubation), by measuring the level of incorporation of labelled precursors into RNA or DNA (after 2—3 days of incubation) (HIRSCHHORN, 1968; BACH, 1968), or according to changes in the nucleoli of small lympocytes after staining with buffered toluidine blue (on the 2nd—3rd day) (SMETANA and IVÁNYI, D., 1967; IVÁNYI, D., et al., 1968). It was found that genetic differences between the two cell populations are responsible for the observed lymphocyte transformation. MLC between uniovular twins were negative, between related individuals negative, slightly positive or positive, and between unrelated individuals almost always positive (BAIN et al., 1964; CHALMERS et al., 1966). When performing 132 MLC tests in unrelated individuals, RYCHLÍKOVÁ et al. (1967) found that values of blast transformation produce a continuous line, down to about 10% of "low grade values". DE WITT (1965) found a correlation between MLC values and leucocyte typing with undefined sera in human and chimpanzee. DAUSSET et al. (1965a) and IVÁNYI, D., et al. (1967) demon-

[15] The ABO blood group system is also a human H system. Organ grafts are promptly rejected by an acute haemorrhagic reaction if the donor is A or B incompatible, while O and AB individuals are "universal" donors and recipients respectively. Preimmunization with AB substance produces a white graft rejection (DAUSSET and RAPPAPORT, 1966). Strong circulating antibodies and their effect on graft vessels is probably responsible for this observation. Owing to a number of internal differences between ABO and HL-A system, the former is not considered to be the MHS in humans (following the given definition, see this review) and represents possibly a situation unique to humans (for details see CEPPELLINI et al., 1966; DAUSSET et al., 1967c; CEPPELLINI, 1968; DAUSSET and RAPPAPORT, 1968).

strated that the value of transformation in MLC in unrelated individuals is dependent on differences in HL-A antigens. BACH and VOYNOW (1966) found by one-way MLC studies in siblings that about 25 % of MLC between randomly chosen siblings are negative. Serotyping confirmed that they represent HL-A identical siblings (BACH, 1967; BACH and AMOS, 1967; BACH et al., 1967; AMOS and BACH, 1968). A quantitative assay applied to pairs of siblings known to be identical or differing in one or two HL-A alleles made possible the demonstration of a higher transformation in the latter (BACH et al., 1967; ALBERTINI and BACH, 1968). The MLC test thus represents a unique possibility for distinguishing identical, semi-identical and non-identical siblings in situations where the parents cannot be serotyped and genotyping of siblings is therefore frequently not possible[16] (ALBERTINI and BACH, 1968).

Further clinical aspects of HL-A typing. The role of anti-HL-A antibodies in febrile transfusion reactions and neonatal neutropenias and thrombocytopenias was described by several authors (DAUSSET et al., 1957; PAYNE, 1957; PAYNE and ROLFS, 1958, 1960; SHULMAN et al., 1962; JENSEN, 1963; PEARSON et al., 1964; SHULMAN et al., 1964; SVEJGAARD et al., 1967; COLOMBANI et al., 1968; HRONKOVÁ and IVAŠKOVÁ, 1968).

HL-A antibodies were found more frequently in women with an anomalous course of pregnancy (premature delivery, stillbirth, etc.) (JENSEN, 1962; IVAŠKOVÁ et al., 1967). To explain the high percentage of antileukocyte antibodies in women with a pathological course of pregnancy, two possibilities may be considered: a) antibodies occurring in certain genetic situations have an aetiological relation to some form of pathological pregnancy, b) antileukocyte antibodies occur as a subsequent non-harmful side-effect (a more frequent and easier immunizing stimulation of the mother by the foetus) in this state. The fact that even women with strong antileukocyte antibodies can have a normal pregnancy suggests that we should preferentially consider the latter possibility.

Choriocarcinoma is a surviving allograft in women. HL-A typing made it possible to study the relationship between histocompatibility and choriocarcinoma (CHO). Essentially, two possibilities can be considered: a) the CHO occurs when there is a small antigenic difference between mother and foetus. In this case, tolerance to grafts may develop. b) The CHO occurs when a relatively large antigenic difference between mother and foetus is involved and/or is related to a certain antigen (or antigens) of the HL-A system. In this case, the humoral immune reaction against the graft might produce enhacement of tumour growth. Antileukocyte antibodies are frequently observed in patients with CHO (MATHE et al., 1964; MOGENSEN and KISSMEYER, 1968; IVAŠKOVÁ et al., 1968). Preliminary findings of ROBINSON et al. (1967) and MOGENSEN and KISSMEYER-NIELSEN (1968) indicated that the survival of CHO presupposes a high degree of histocompatibility between the foetus and the mother.

[16] At a very polymorphic locus such as HL-A (see data on gene frequencies) both parents will usually be heterozygous for different alleles and four groups of siblings can be presumed: a/b × c/d: ac, ad, bc, bd. 1/4 of siblings are identical, 2/4 semi-identical and 1/4 non-identical for the respective HL-A alleles.

IVAŠKOVÁ et al. (1968) found an excess of HL-A.MAC+ husbands and HL-A. MAC— patients (CHO, mole hydatidiform, and mole proliferans) indicating that HL-A incompatibility is more frequent in these couples. The same group of hus bands and patients was examined also with a battery of unclassified cytotoxic sera and it was found that the patients reacted with a significantly lower number of sera. The hypothesis was advanced according to which 1. the probable condition for the occurrence of CHO is the presence of HL-A alleles producing relatively few antigenic specificities; 2. in this situation, the type of immune reaction to the husband's HL-A antigens may in some cases be capable of enhancing the growth and perhaps causing malignancy of the allograft; 3. incompatibility for antigen MAC, or its alternative antigens, might play a decisive role in this situation (IVAŠKOVÁ et al., 1968). MOGENSEN and KISS-MEYER-NIELSEN (1969) when evaluating a greater number of patients arrived at the conclusion that HL-A compatibility is more frequent in patients with a generalized form of the disease while incompatibility is found in more localized forms.

MATHÉ et al. (1967) and BACH et al. (1968) presented preliminary data indicating that in the course of bone marrow transplantation the graft-versus-host reaction was reduced or eliminated in an HL-A compatible situation.

The presence of HL-A antigens was demonstrated on different tissues (VAN ROOD, 1962; BRUNING et al., 1964; ENGELFRIET et al., 1966; IVAŠKOVÁ, 1967; MELIEF, 1968; BERAH et al., 1969). The relative concentration on different tissues is similar to that of H-2 in mice (BERAH et al., 1969). The emerging difference is the absence (?) of HL-A antigens on erythrocytes (for discussion see ROSENFIELD et al., 1967; HRONKOVÁ et al., 1968). The presence of HL-A antigens on reticulocytes was demonstrated (HARRIS and ZERVAS, 1969).

3. Genetics of HL-A

There is general agreement that antigens of the HL-A system are probably determined by a chromosomal region; each HL-A allele is responsible for the production of a set of HL-A specificities. There are preliminary data about possible crossing-over between some specificities (AMOS et al., 1967a; SINGAL et al., 1968; WARD et al., 1969).

Population and family studies demonstrated a number of unexpected findings.

1. Alloimmune sera reacting with human leukocytes (and other nucleated cells and platelets but not with erythrocytes), collected randomly from immunized persons or multiparous women, contain antibodies more than 90% of which react with HL-A antigenic specificities. No precise data can be gathered concerning this point because most sera are polyvalent and thus are not fully resolvable, nevertheless data reported by VAN ROOD et al. (1965), TERASAKI et al. (1966), VREDEVOE et al. (1966) and MICKEY et al. (1967a, b) are in agreement with the possibility that, as in other species, the immunogenicity of the MHS (HL-A) is much greater than of other (weak) H systems.

2. The HL-A system is extremely polymorphic; the frequency of individual HL-A alleles is possibly no higher than 5—7% and frequently lower than 1%, as demonstrated by a list of the 47 most frequent alleles (Table 10) which corresponds well with those deduced from a study of 25 families (DAUSSET et al., 1967c). The recently described HL-A antigens Da 15, 16, 17 (Fig. 6) are not included in Table 10 and will certainly lead to an increase in number and decrease in frequency of HL-A alleles. GEPPELLINI (1967) presented a similar conclusion at the Turin Workshop: "When the 44 chromosomes (corresponding to the 22 parents) have been deduced and compared, no two identical combinations have been found: thus 44 different 'alleles' have been observed out of 44 analyzed. This is an extraordinary case of genetic polymorphism of a degree almost unknown in higher organism." Consequently, the HL-A systems represents the most polymorphic human genetic system; 17 antigens could be defined on a sample of 200 French individuals. From this point of view, the Rh system is much less polymorphic.

The results obtained with the MLC test, which in humans depends basically on HL-A differences, are also indicative of a high degree of polymorphism, because unrelated individuals almost always produce positive mixtures and only HL-A identical sibs give negative results (see BACH, 1968, and page 50).

The consequences of the presumed existence of 50 or 26 HL-A antigenic specificities for gene frequencies have not yet been evaluated. As a word of caution it should be emphasized that the definitions of antigens (antigenic specificities), and thus also of the respective gene frequencies are operational. A higher frequency of identical HL-A alleles is expected by VAN ROOD (1968). As will be discussed below, the genetic situation underlying the phenotypically observed complexity of the HL-A (and other MHS) is not fully understood. Some restrictions (interference of the synergic effect of weak H systems) can also be applied to the genetic interpretation of the MLC test.

3. When groups of similarly reacting sera defining some HL-A antigens were tested simultaneously on different populations (Caucasian, Japanese, Malian) (DAUSSET et al., 1965a, 1967a), it was found that not only is there a difference in the frequency of the respective antigens but also the individual typing sera of the same group behave differently, some reacting at a higher and some at a lower frequency (Fig. 9). This was interpreted as due to the complexity of the individual HL-A antigens, consisting of different antigenic factors in different populations (IVÁNYI and DAUSSET 1966). Similar results were obtained by BRAIN and HAMMOND (1968) in three race groups examined in South Africa. RUBINSTEIN et al. (1967) found differences in frequencies of HL-A antigens in Mapuchi Indians and pointed out that some cells were not agglutinated by any of the antisera used.

4. According to the distribution of HL-A antigens in the Caucasian population, these are clustered in three series of positively associated antigens, i.e. groups of antigens which are more frequently found together (Fig. 7a, b, c). Some of the antigens are "inclusion", this is the situation in which, at least

Table 10. *The 47 most frequent alleles of the HL-A system (From* DAUSSET *et al., 1967c)*

Allele									Frequency	Allele							Frequency
1	2	3	13						0.006	2	1	7				6	0.009
1	2	3	13	4					0.012	2	1	7					0.040
1	2	3	13	4		14			0.017	2	1	7			10		0.017
1	2	3	13		5	14	6		0.030	2	1	7	8				0.031
1		3	13		5				0.009	2	1	7	8	9	10		0.012
1	2	3		4					0.053		11	7					0.025
1	2	3					6		0.013		11	7			10		0.012
1	2	3				14	6		0.036		11	7	8				0.039
	2	3	13	4					0.042		11	7	8	9			0.021
	2	3	13	4		14			0.077		12	7					0.030
	2	3	13		5	14	6		0.029		12	7			10		0.018
	2	3	13			14			0.010		12	7		9			0.038
	2	3	13		5	14			0.004		12	7		9	10		0.051
	2	3		4					0.015			7		9			0.053
	2	3			5		6		0.006			7		9	10		0.011
	2	3				14			0.022			7					0.015
	2	3							0.029	2	1			9			0.016
	2			4					0.011	2	1			9	10		0.030
	2					14			0.002	2	1				10		0.005
	2						6		0.009		12			9		6	0.012
	2			4		14			0.005		12					6	0.033
	2	3		4		14			0.005					9		6	0.008
	2	3						11	0.011					9			0.006
	2	3		4				11	0.015								

in the population sample studied, one antigen (with a lower frequency) is present *only* when a second one (with a higher frequency) is also present. The genetic mechanism underlying this kind of distribution of HL-A antigens is not understood and is indicative of the selection of some alleles or of preferential fertilization. It can be hypothesized that this is based on a chromosomal

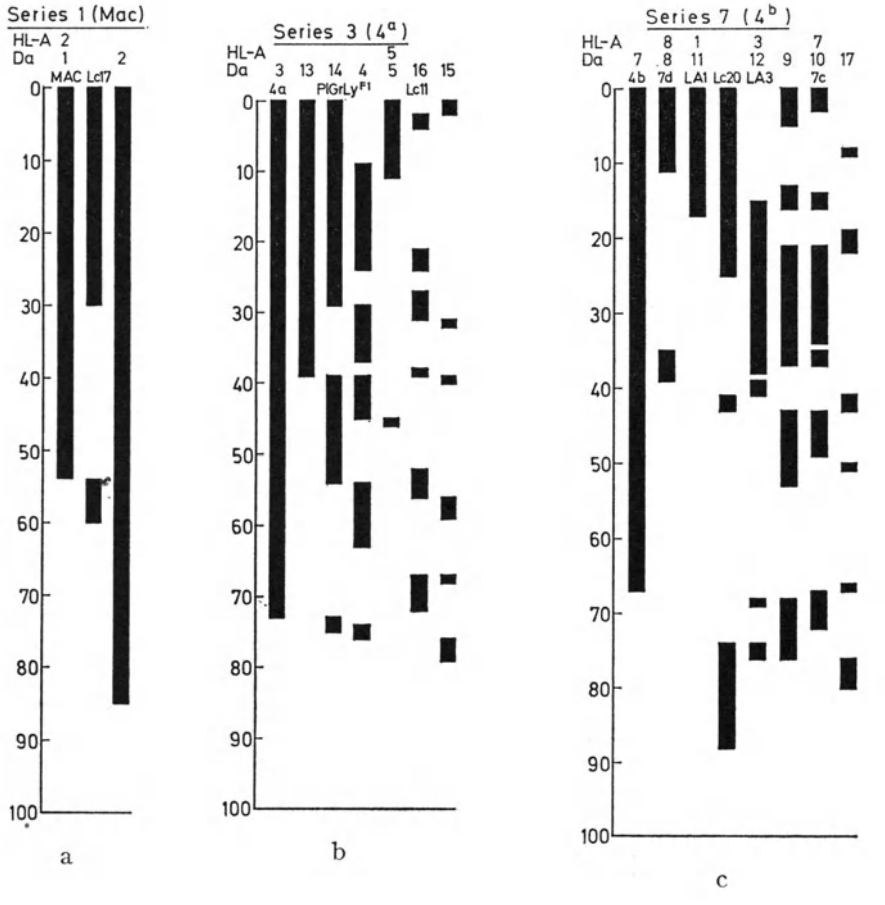

Fig. 7a—c. Distribution of HL-A antigens in the population. The three groups of positively associated HL-A antigens (DAUSSET, 1969)

region derived by duplications, with subsequent mutations of the duplicated part. The duplicated part could be predisposed to deficiencies, or alternatively some factors could prevent crossing over in the original part (proximal to the recombination suppressor factor) but allow recombination of the duplicated part (distal to the recombination suppressor factor) (schematically $A \rightarrow A'$, $AA' \rightarrow A''$, $AA'A'' \rightarrow A'''$, $AA'A''A''' \rightarrow$). This could result in a situation in which only A, AA', AA'', AA'A'' or AA'A''' alleles are present or frequent in the population, while A' or A'A'' and A''A''' are rare or absent. A number of similar speculations are possible (DAUSSET et al., 1967c; CEPPELLINI et al.,

1967; KISSMEYER-NIELSEN et al., 1968); the one presented is favoured by the author only for the hypothetically possible interference of a "T" system in humans.

5. There are two series of HL-A antigens which behave as though determined by two closely linked genes with a number of alternative sites for each of them (Fig. 8a, b). The antigens included in these two series have mutual negative associations in the population, no individual possessing more than two

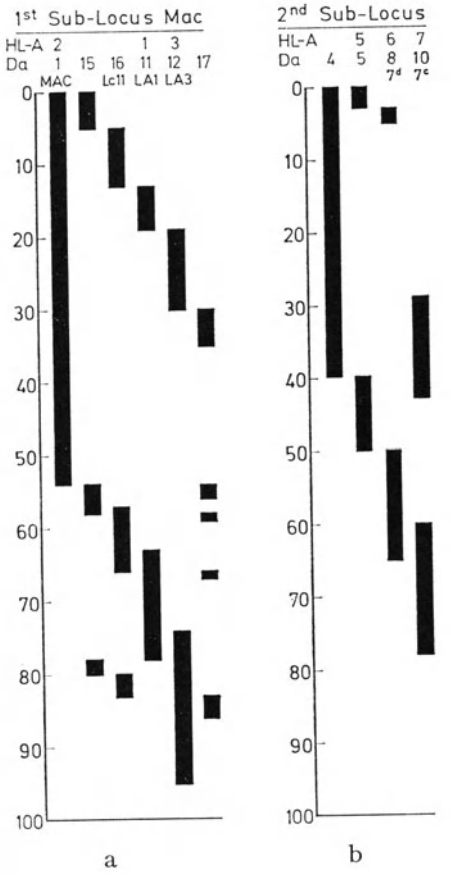

Fig. 8a and b. Distribution of HL-A antigens in the population. The two series of negatively associated HL-A antigens (DAUSSET, 1969)

antigens of each series in his phenotype, and family studies show that each allele determines only one antigen for each of the two series. This led to the hypothesis (DAUSSET et al., 1969b; SINGAL et al., 1968; KISSMEYER-NIELSEN et al., 1968) that the HL-A chromosomal region consists of at least two subregions (sub-loci) possibly separable by crossing over and with a number of alternative sites determining the HL-A antigens of the two series. A third sublocus was tentatively suggested by SINGAL et al. (1968). For the present the alternative explanation remains that the situation observed is the result

of certain gene frequencies in the population studied and could change sharply when other human populations are tested.

4. The Complexity of the HL-A System

There are no genetic or biochemical data which could allow any realistic assumptions concerning the relationship between HL-A phenotypes (as determined from the reaction pattern of a battery of typing sera) and the genetic fine structure of the corresponding chromosomal segment. The existence of a subdivisable chromosomal region is signalized by the description of possible recombinants and HL-A subloci. Similarly, as in the H-2 system and other MHS, the products of different HL-A alleles are "*asymmetric*", i.e. some produce more specificities than the others. Consequently, different genetic hypotheses are possible:

a) HL-A alleles are series of functionally changed regions which produce amorphous parts;

b) HL-A alleles are series of structurally changed regions (deficient or duplicated);

c) HL-A alleles are symmetric with a one-to-one relationship between HL-A antigens and genetic sites although this is "masked" by the existence of not yet described specificities and by the interference of rather "peripheral" factors (cross-reactions, quantitative differences in expressivity, etc.);

d) HL-A alleles are symmetric for a number of subloci, each of which can produce a different number of HL-A antigens (see also the H-2 linkage map). The question arises why different subloci of HL-A (or other MHS) produce a possibly different number of specificities and the hypotheses listed above (a, b, c) should be applied at the level of subloci.

While operationally effective typing (applicable effectively to clinical situations) is now possible, the serological complexity of the HL-A system, from the "peripheral" point of view, emerges from a number of features.

1. Of the basic serological techniques used — leukoagglutination (LA), lymphocytotoxicity (CY), platelet-C'-fixation (PCF) — only the last one has nearly perfect reproducibility, while the reproducibility of the first two is about 90—95 % (VAN ROOD, 1968). More details and discussion about the serological techniques used are given by WALFORD (1960), DAUSSET and RAPPAPORT (1968) and mainly in the Proceedings of the Histocompatibility Workshops (1964, 1965, 1967). The different techniques represent possibly different sensitive approaches, the PCF being the least sensitive and the CY the most sensitive (VAN ROOD et al., 1966). Individual sera exert a different frequency of positive reactions on a panel of cell samples when tested by different techniques. Frequently sera highly polyvalent in LA and/or CY are monospecific in PCF or do not react at all. A relatively pure anti-3 (4a) serum in CY reacts as a monospecific serum anti-5 (COLOMBANI, 1969). Many sera react only in LA or CY; CPF is never positive if LA and CY are negative.

2. It is extremely difficult to find two identically reacting sera which fulfil the requirements: a) operationally[17] monospecific, b) tested on a large panel of cells derived from different populations, c) identical results with different techniques.

In 1967 (a) after testing more than 400 randomly selected sera MICKEY et al. stated: "The failure to find at least one pair of identical sera would then suggest that there are a large number of serum types."

Comparison of selected sera at the Leiden and Turin Histocompatibility Testing Workshops (1965, 1967) indicated the identity of some sera when tested on a relatively low number of cells (Fig. 9). Nevertheless, some sera

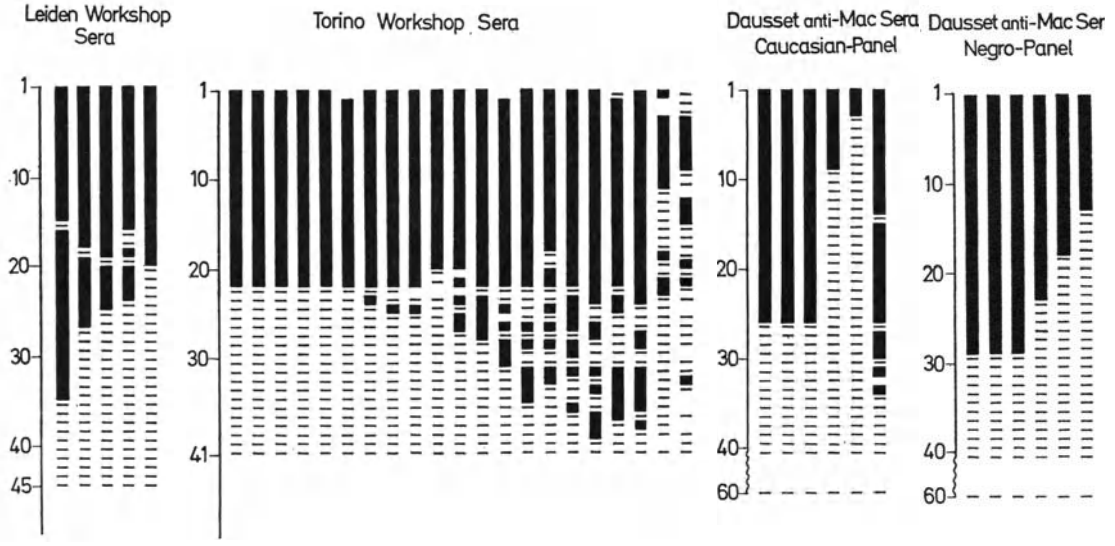

Fig. 9. Complexity of HL-A antigens. Anti-HL-A.MAC sera from the Leiden and Torino Histocompatibility Workshops (BRUNING et al., 1965; CURTONI et al., 1967) and from DAUSSET et al. (1967c)

exhibited a few "extrareactions", others were "shorter", i.e. the specificity detected by them was included in the well-defined antigen. Both these findings are of primary importance.

i. Different sera gave different extrareactions (even though these are more or less restricted to a certain part of the cells tested while others remained "completely" negative). There are no data about the possibility of conclusively subdividing such sera into two parts, i.e. the "main" antibody and the "extra" antibody.

[17] Operational monospecificity is defined by at least 30—60 absorption experiments. Nevertheless, if there are two antibodies in the examined serum, and both react with specificities which are associated in more than 90—95%, there are considered as a unit for the given population (WALFORD and TROUP, 1967; WALFORD et al., 1967). Effectively monospecific sera are those which were tested by absorption with a number of cells such that more absorptions cannot effectively strengthen the statistical value of previous experiments (KLOUDA et al., 1969).

ii. The sera with a lower reaction frequency signalize the existence of further frequently associated specificities. This was considered by the WHO Nomenclature Committee which recommended accepting a coefficient of correlation $r \geqq 90$ for the definition of a second associated specificity. The criterion is evidently operational and could lead in its theoretical consequences to an "indefinite" subdivision of any antigen if a large number of sera could be tested on a sufficient number of cell samples.

3. Some monospecific anti-HL-A.MAC sera reacted almost identically with other anti-MAC sera which were shown to possess several antibodies (DAUSSET et al., 1965a, 1966). A similar situation was studied by computer simulation of complex immunogenetic systems (DÉMANT et al., 1967; DÉMANT and IVÁNYI, 1967a, b).

4. Cross-reacting HL-A antibodies or antigens were described by DAUSSET et al. (1969b) and SVEJGAARD and KISSMEYER-NIELSEN (1968)[18].

5. The synergic effect of anti-HL-A antibodies in the CY test was described (IVAŠKOVÁ et al., 1969). Mixtures of two polyvalent or two monospecific sera (reacting with the same HL-A antigens) exhibit a higher titre and percentage of killed cells than the individual sera.

6. Monospecific sera react at different titres with different positive cells, indicating quantitative or qualitative differences in the antigenic specificities.

7. Monospecific sera change their mutual correlations when tested on different populations (DAUSSET et al., 1965a, IVÁNYI and DAUSSET, 1966; DAUSSET et al., 1967c; BRIAN and HAMMOND, 1968).

8. HL-A antigens seem to differ in their immunogenicity (DAUSSET et al., 1965a, 1967c; VAN ROOD, 1968).

The similarly complex features exerted by the chicken B system were discussed on page 8. There are apparently analogous findings concerning the H-2 system. Preliminary data from testing H-2 antigens in non-inbred or field mice (see page 22) are along the same line, but more data are highly desirable. The synergic effect of H-2 sera was described by MÖLLER and MÖLLER (1962). Strain differences in the production of 19S and 7S alloantibodies were described by ANDERSON et al. (1967). Cross-reactions between some H-2 antigens were presumed (GORER, 1961; RUBINSTEIN and FERREBEE, 1964), and quantitative differences in individual specificities produced by different

[18] The phenomenon is not fully clarified and can be illustrated by quotations: "A new phenomenon consists in the fact that an apparent cross-reaction exists between antigens 1 and 15. A serum containing both anti-1 and anti-15 cannot be broken into its constituent parts by cross-absorption. Nevertheless, an anti-15 reacts much stronger with antigen 15 than with antigen 1 and upon appropriate dilution will react only with the former" (DAUSSET et al., 1969b). "Analysis of the families, however, provided two distinct antigens, LA2 and Ba, and that the corresponding genes always segregate in repulsion. Thus the antibodies in sera Ba and Du can be considered cross-reactive, and as KABAT stressed, it does not seem desirable to define new antigens by such antibodies. Accordingly, we prefer to regard cross-reactive antibodies as dispecific rather than monospecific" (SVEJGAARD and KISSMEYER-NIELSEN, 1968).

H-2 alleles were observed. Some specificities are difficult to detect by the "routine" haemagglutination techniques and were described by the cytotoxic test (possibly the analogy of the effectiveness of different serological techniques for HL-A). The underlying genetic mechanism is unclear. SHREFFLER et al. (1966) described how a recombinant between H-2^d and H-2^k (both H-2.3) resulted in a new H-2^0 allele producing an "H-2.3" specificity with a different reaction pattern. Further, H-2^0 mice produced an antibody which was tentatively defined as anti-3. From different alternative explanations, including the possibility that H-2.3 is determined by different sites in different alleles

Table 11. *Non-HL-A leucocyte and/or thrombocyte alloantigens (From* DAUSSET *and* RAPPAPORT, 1968, *and* VAN ROOD *et al., 1967)*

System	Antigens	Population frequency
Zw	Zw^a	98.9
	Zw^b	26.2
Ko	Ko^a	15.2
	Ko^b	99.4
Pl^E	Pl^{E1}	99.1
	Pl^{E2}	5.0
L_y^D	L_y^{D1}	36.0
NA	NA-1	81.0
NB	NB-1	99.0
9^a	9^a	58.1
5	5^a	37.2
	5^b	97.1

[a] Possibly Linked with HL-A.

(a situation which could arise if H-2 alleles are "series of duplications, rearrangements or other chromosomal aberrations"), it was concluded that the anomaly could be the result "either of unequal crossing-over or of alteration of one antigenic specificity as a result of recombination" (SHREFFLER et al., 1966). Serological studies with several antisera revealed the existence of *complexes of specificities* associated with the D and K end of H-2 and their quantitative and qualitative variation in products of different alleles (SNELL and DÉMANT, 1969).

There is no realistic basis for discussing the genetic basis of this complexity. No doubt there must be a mutual interference among the points listed above. At the operational level, a hypothesis was put forward by DAUSSET et al. (1965a) and IVÁNYI and DAUSSET (1966) which assumed that defined HL-A antigens are in fact complexes of antigenic factors frequently associated in the population, i.e. an HL-A antigen is composed of the ABCDEFGH. . . . antigenic factors. From the reaction pattern of groups of positively associated sera it was presumed that antigens 1, 3 and 7 are composed of a minimum of 5, 6 and 6

antigenic factors respectively (DAUSSET et al., 1965a). The different combinations of factors, the different immunogenicity of some combinations and the synergic action of the respective antibodies can underlie a great part of the observed complexity of the serological findings. Their actual resolution is impracticable; the hypothesis represents a resolution on an operational level, defining an antigen by a set of positively associated sera. While this approach could formally clarify a number of observations, other mechanisms can also interfere, resulting in quantitative differences in the expressivity of different specificities. These are possibly: intra- and inter-allelic interactions, dosage effect, interference of other genes, different sensitivity of different specificities to the serological technique used, differences in molecular classes of antibodies, inhibition of binding of antibodies to adjacent antigenic determinants, anti-complementary effects of individual sera. It can be concluded that the frequently observed variants of HL-A antigens are qualitatively and quantitatively different and that the participation of both components depends, inter alia, on the test system applied.

5. The Relative Strength of HL-A Antigens

While the relative strength of different H systems has been extensively elucidated (see pages 6 and 16 and Table 5), there are only scanty data about the strength of individual antigens of a given H system (KLEIN, 1966). The problem was recently reviewed from different points of view (LENGEROVÁ and MATOUŠEK, 1966; ELANDT-JOHNSON, 1968; MCKHANN, 1968; STIMPFLING, 1968). Essentially two possibilities can be considered:

a) qualitative differences between antigens of different immunogenicity;

b) differences in the complexity of individual antigens, the more complex antigens representing the stronger immunogen.

The features of the distribution of HL-A antigens allow an extension of the second possibility, if a *synergic effect* between different antigens can be presumed. The synergic effect of antigens of different H systems was described (see page 7) and the findings of KLEIN (1966) and RYCHLÍKOVÁ and IVÁNYI (1969) also agree in part with this possibility. The results of DAUSSET et al. (1969b) indicate that more HL-A incompatibilities are involved when skin grafts from children to fathers are rapidly rejected. It is obvious from the distribution pattern of HL-A antigens (Figs. 6, 7a, b, c) that in a HL-A.Da3 + donor — HL-A.Da3 — recipient combination there is a significantly higher probability of a *simultaneous difference* in more HL-A antigens (i.e. in those positively associated with 3) than in a combination where both donor and recipient are 3+. The same is true of other groups of positively associated antigens. It was argued by DAUSSET et al. (1965b) that HL-A antigens MAC, $3(4^a)$ and $7(4^b)$ are the "main" antigens of the system. In other words the existence of groups of positively associated antigens determines the existence of more or less numerous histocompatibility differences. Applying the above evaluated operational concept to the relative strength of individual HL-A antigens

and their importance for clinical transplantation, it can be suggested that the relative strength of individual HL-A antigens is determined by their *mutual associations* in a given population. This hypothesis is based on the presumed synergic effect of different HL-A specificities.

6. Data Indicative of an Analogous Linkage Group in Humans and Mice

There are no definite data in humans concerning the existence of a linkage group analogous to the IXth linkage group in mice. Possible indications are listed below.

a) Is HL-A Associated with Reproductive Performance?

1. Data from the kidney transplant register (GLEASON and MURRAY, 1967) and skin graft experiments (CEPPELLINI et al., 1966, 1968) indicate a relationship between the P blood group system and histocompatibility (no similar indications were found for other blood group loci, except ABO). HRONKOVÁ et al. (1968) found that antigen P_1 and HL-A.MAC are not randomly distributed in the Czech population ($r = -30$, $P < 0.01$); this was confirmed by DAUSSET (1968). Family studies gave no evidence of close linkage between the two systems

Table 12. *Segregation of HL-A antigens in backcross matings;* $\chi^2 > 3.84$ *(*$P \leq 0.05$*, 1 d.f.). In the first column the international and Torino nomenclature is compared (From* CEPPELLINI *et al., 1967)*

HL-A antigens		Total of reciprocal backcrosses (+/− × −/−)			
		No. of matings	Children +/−(a)	−/−	x^2
HL-A"4?"	2	6	15	8	2.17
HL-A"4?"	3	11	26	24	0.10
HL-A"4?"	4	12	35	21	3.52
HL-A.5	5	11	31	21	1.94
HL-A"6?"	6	10	21	29	1.30
HL-A.8	7	10	35	16	7.10
HL-A.1	8	15	41	28	2.46
HL-A.2	9	18	42	37	0.33
HL-A.3	10	14	31	34	0.15
	11	18	44	34	1.29
	12	14	23	37	3.26
Total			344	289	

(DAUSSET et al., 1967c, KISSMEYER-NIELSEN et al., 1968), and the nature of the P-HL-A association is not clarified. REED (1968) tested the possible role of blood group systems in reproductive performance, following the view that a difference between husbands and gravidae in frequencies of phenotypes within a given blood group system could reflect selection. This possibility was

tested for seven blood group systems (ABO, MNSs, Rh, Kell, Duffy, Lutheran and P). None of the comparisons was significant, except for the P system, in Caucasians where χ^2 (1 df) was 10.43 ($P < 0.005$). This was an excess of P_1 gravidae over P_1 husbands accompanied by an increase in the frequency of the husband $-\times$ gravidae $+$ mating over the reciprocal one. The yearly heterogeneity in the P result for husbands and the relatively poor reproducibility of the P grouping made this conclusion uncertain (REED, 1968). The findings of HRONKOVÁ and of REED are preliminary, but taken together they indicate that the human MHS is associated with gene(s) which interfere with reproductive performance.

2. Data about an excess of 4a4b and 6a6b heterozygotes are repeatedly found in the papers of VAN ROOD et al. (1965, 1966, 1967) and were confirmed by CEPPELLINI et al. (1967). The significance of this finding remains obscure and more data are required about the frequency of $--$ and possibly $++$ alleles. Nevertheless, these data will perhaps rank as the first on the action of selective fertilization in the HL-A system. CEPPELLINI et al. (1967) reported a general tendency to heterozygous excess in intercrosses and reciprocal backcross matings. This was significant for 3 antigens (TO4, 7, 12) in backcross matings where the mother was positive for the factor but remained significant only for TO7 (and almost significant for TO4) when reciprocal matings were pooled (Table 12). There were no indications of a possible immunological elimination of certain individuals.

b) Is HL-A Linked with a Serum Group?

BERG et al. (1967) described the influence of a serum group system (Lp) on skin graft survival. Preliminary data indicated a nonrandom distribution of Lp serum groups and HL-A antigens.

c) Is HL-A Associated with Human Leukaemia?

KOURILSKY et al. (1967) found no correlation between HL-A antigens and human leukaemia. AMIEL (1967) reported that patients with lymphogranuloma are more frequently HL-A.5 than would be expected by random distribution[19].

There is not a single definitive finding about the linkage of HL-A with genes analogous to those present in the IXth linkage group of mice. Nevertheless, there are a number of indications for this possibility. As was pointed out by SNELL (1968), the different type of "T"-H locus interaction could be extremely complicated, but whatever the true explanation is, "a forced heterozygosity at a strong H locus lends evidence to the assumption that the heterozygote-retaining effect of T on H-2 is not an accidental one".

[19] It was suggested by GREEN et al. (1960) that lymphogranuloma could be a maternal to foetal lymphocyte chimaera. An association between malignant trophoblastic disease and HL-A antigens was described (see page 51). While the authors assume the involvement of immunologic factors, the interference of a virus in the process of malignization cannot be excluded.

E. Histocompatibility Antiges in Chimpanzee, Rhesus Monkey, Rabbit, Dog, Cattle, and Pig

There are substantially less data about H antigens in other species but it has already been possible to undertake the first important steps which will be further developed in a not too distant future. The identification of HL-A antigens in the chimpanzee deserves special attention. In this review only brief data about other species will be given.

1. Chimpanzee

Cross-reactions between human and chimpanzee alloimmune antileukocyte antibodies and serotyping with defined anti-HL-A sera demonstrated that the chimpanzee possesses some of the HL-A antigens (SHULMAN et al., 1964, 1965; SHULMAN, 1964; METZGAR et al., 1965; METZGAR and ZMIJEWSKI, 1966; BALNER, 1966, 1968; BALNER et al., 1967a, b).

The complex nature of HL-A in chimpanzee parallels the findings in humans. A relatively high number of HL-A antigens was detectable in a small number of animals. The reaction pattern of sera exhibits groups of similar but not identical sera.

In the previous chapter it was shown that the HL-A system seemed to be composed of at least two closely linked subloci. Preliminary findings indicate that antigens produced only by one of these subloci are present in chimpanzees (i.e. antigens linked with the system 4 of VAN ROOD (Figs. 1, 2 and 3). This finding, if confirmed, could give rise to some hypothesis about the evolution of the HL-A locus in humans (BALNER et al., 1967b; BALNER, 1968).

2. Rhesus Monkey

Rhesus monkey alloimmune sera produced by random immunization when tested on a panel of Rhesus leukocytes exerted complex reaction patterns similar to those described in humans (Fig. 10). By the use of computer analysis and later selected donor-recipient immunizations and/or absorptions, relatively pure typing sera were obtained which helped to define 5 alloantigens. It can be assumed that they represent the first step in the recognition of the MHS in Rhesus monkeys (BALNER and DERSJANT, 1965; BALNER et al., 1965a, b, 1966, 1967c; BALNER, 1968). It was actually found that some of them are strong H antigens (BALNER et al., 1965b; BALNER, 1968) and the respective identity of host and donor mitigated the graft-versus-host reaction after bone marrow transplantation (BALNER et al., 1967c). There are preliminary data that Rhesus H antigens (1a and 1b) cross-react with the human and chimpanzee HL-A system when tested by absorption experiments. The same could be found in Speciosa monkeys (Macacca speciosa) but not in Baboons (Papio Cynecephalus) (BALNER, 1968).

3. Rabbit

Preliminary data of COHEN et al. (1964) about the role of the Hg blood group system in histocompatibility were at variance with a number of findings

of IVÁNYI (1962, 1964, 1965b, 1966a). FABIAN et al. (1963) described long-term skin survival in spite of these blood group differences.

Recently DÉMANT (1966, 1968) and BLACK (1967) used an approach similar to that described for humans. A number of randomly produced alloimmune sera were tested by the cytotoxic test on a panel of rabbit cells. Groups of

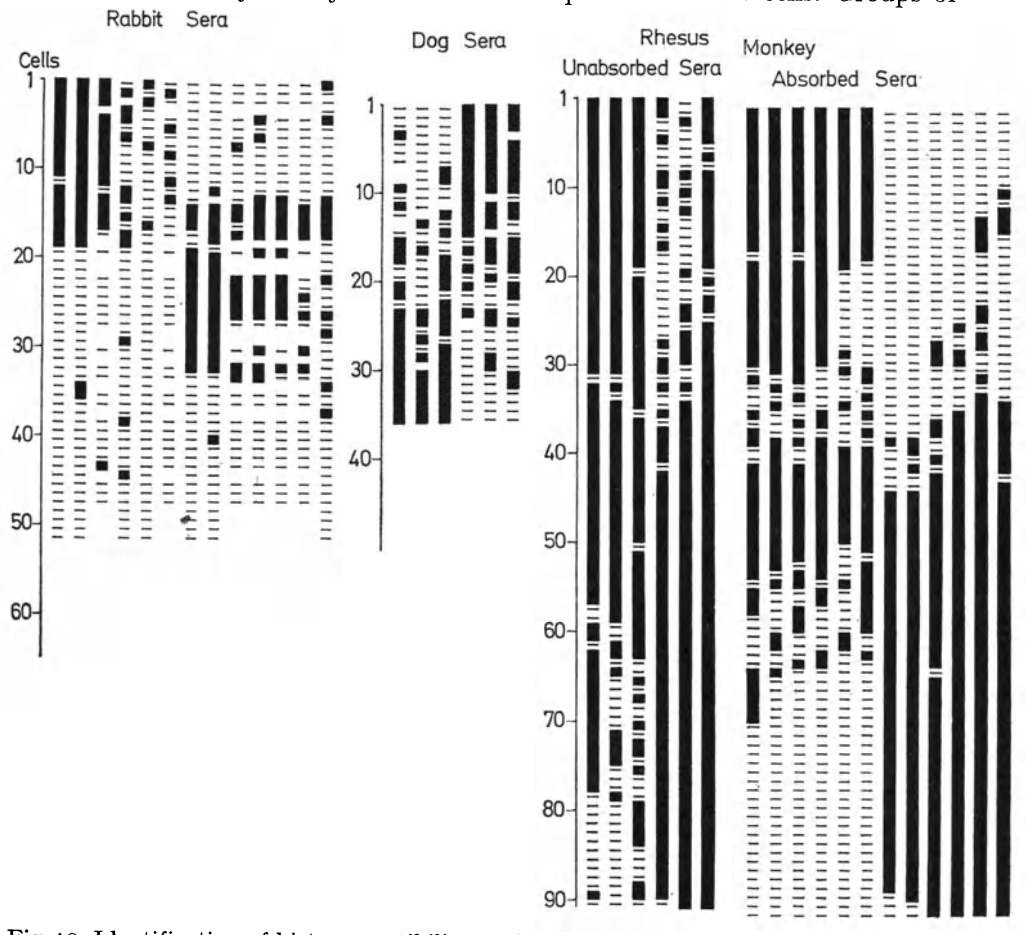

Fig. 10. Identification of histocompatibility antigens in rabbit, dog, and Rhesus monkey (From BLACK, 1967; CLETON et al., 1967; and BALNER et al., 1967c)

similarly reacting sera were found and 7 (DÉMANT) and 3 (BLACK) antigens were determined (Fig. 10). The distribution pattern of the former indicated the existence of a MHS. BLACK (1967) reported that skin graft survival was correlated with compatibility for the described specificities. DÉMANT (1966, 1968) found longer survival time of maternal versus paternal skin grafts in newborn rabbits. Compatibility for the defined specificities correlated with prolonged survival of maternal skin grafts (transplacental tolerance produced in the absence of strong H difference) while accelerated rejection was observed in incompatible combinations (immunity against strong H antigens present in the mother and absent in the newborn).

4. Dog

The same approach as in humans led to the definition of 2 alloantigens in dogs. 24 antileukocyte sera were produced by random immunization by intradermal injections of leukocytes and skin grafts. The sera were tested on a panel of cells, analysed for mutual correlation coefficients and sera with similar reaction patterns served to define the first two dog leukocyte alloantigens (Fig. 10). Their distribution indicates the existence of one major leukocyte antigen system in the dog. Skin graft survival was correlated with compatibility for the detected antigenic specificities (CLETON et al., 1967). EPSTEIN et al. (1968) used four alloimmune cytotoxic typing sera for donor-recipient sibling pair selection in experiments with bone marrow grafting. None of the mismatched recipients lived longer than 14 days; all of the matched recipients lived beyond 40 days. Similar results were obtained by STORB et al. (1968) with unrelated dogs.

5. Cattle and Pig

There are no conclusive data about H antigen identification in these species; BOROVSKÁ and DÉMANT (1967) tested alloimmune sera against different cattle blood group systems by means of the cytotoxic test. A correlation between the reaction pattern of the anti-erythrocyte antibodies and that tested by the cytotoxic test was found only for the S system. HÁLA (1967b) tested alloimmune sera against different pig blood group systems by absorption test on lymphoid cells. Only antibodies against the E blood group system could be absorbed by lymph node cells. Both these findings give preliminary indications that the S or E blood group systems are the MHS for the given species; both are the most complex systems in these species[20].

IV. A Hypothesis and Summary

MHS as a super-gene involving two parts: one major gene for reproductive performance and one for immunological processes

A large number of Histocompatibility systems was found in all species sufficiently studied. The estimates of a "number" of the respective H loci are limited by the experimental approaches. They depend also on general definitions. SHREFFLER (1965) pointed out that it is rather a question of definition whether the TL locus should be considered as a part of the H-2 chromosomal region. Does the H-2 system represent one or two (or more) systems, considering a 0.04 recombination value between its two end points and the insertion of the Ss locus in the H-2 region? H-1 and H-4 located in linkage group I are separated by about 24 crossover units (SNELL and STEVENS, 1961); H-3 and H-13 in linkage group V are separated by about 15 crossover units and the

[20] More antigenic specificities and alleles were described for the bovine B blood group system than for the S system. Nevertheless, considering the analytic data obtained from limited populations, the presence of "compound" antigens and "cross-reacting" antibodies, the S system resembles rather the serological characterization of a MHS (see GROSCLAUDE, 1965).

existence of a third H system is presumed in the same linage group (SNELL et al., 1967). With these limitations in mind it appears to be an operational dictum: there are at least 30 segregating H systems in the species studied.

It seems highly probable that in every species one H system or one group of closely linked loci occupies a special position for which the designation Major Histocompatibility System is proposed. There are insufficient genetic and biochemical data to elucidate the genetic fine structure of the former and its relationship to the antigenic specificities. The definition of the MHS is operational; the framework is given below.

The historical approach which served for the discovery of the first MHS (H-2) remains the simplest and thus the leading one. If transplants are performed on a segregating population, preferentially consisting of F_2 or Bc individuals from two inbred mouse strains, the best correlation (if not the only one) of graft survival with H antigens can be found for the MHS. Consequently, the MHS is the main or strong histocompatibility sytem for a given species. While incompatibility in MHS antigens leads to the shortest possible survival time, the survival time of "compatible" transplants is determined by the synergic action of weak loci. If a general statement is made and histocompatibility is defined as being determined genetically by a *polygene*, the MHS represents the "major gene" and the weak systems the "minor" or "multiple genes".

Characterization of the MHS

1. The MHS is the main (strong) H system for any studied species.

2. Random immunization leads to antibody production against the MHS in the majority of donor-recipient combinations; antibodies against weak systems are feeble, less frequent or not at all detectable.

3. A second-set reaction to the MHS frequently leads to a "white graft" type rejection.

4. The graft-versus-host reaction is more pronounced against the MHS than against weak systems.

5. The MHS is solely or basically responsible for lymphocyte transformation in vitro in mixed lymphocyte cultures.

6. When grafting organs or tissues characterized as "less sensitive" or "particular" (ovary, kidney, tooth germ), these are rejected when opposed to a MHS barrier but survive in spite of weak H differences.

7. Immunologic tolerance and immunosuppression is more easily obtained in the absence of an MHS antigen difference.

8. The MHS is extremely complex when defined by serotyping large numbers of individuals by a number of randomly selected alloimmune sera.

9. The MHS is extremely polymorphic; it represents one of the most polymorphic genetic systems for any studied species.

The MHS is produced by a chromosomal segment which can be subdivided by recombination. Individual MHS alleles produce a number of serologically

defined antigenic specificities; the products of different alleles appear to be asymmetric.

10. There are preliminary data about homologies in the chemical characterization of MHS in different species.

11. The MHS is linked with a chromosomal region which represents a major gene for reproductive performance. Both systems form a functional unit (super-gene, SNELL, 1968).

The chromosomal region for the T locus in mice is suggested as representing an abnormal chromosomal segment formed by a series of duplications and deficiencies and/or functional changes. Abnormal DNA/RNA hybridization indicates that it could be part of a nucleolar organizer region.

The effect of different alleles differs grossly in different heterozygotes. Consequently, their effect in a "population" is highly variable due to the *interaction* of different alleles. This is the main difficulty when elaborating the respective systems.

The T-H-2 system was described in detail in mice. Indications of a similar situation in other species are as follows.

a) Association of the chicken MHS (B) with
   i. persistence of heterozygosity in highly inbred chicken lines;
   ii. such features as embryonic mortality, hatchability, juvenile viability etc.

b) Indications of abnormal segregation in the rat MHS (H-1, Ag-B) due to prezygotic selection.

c) i. Indications of abnormal segregation in the human MHS (HL-A).
   ii. Non-random distribution of HL-A and P blood group system antigens. The latter was indicated as being involved in abnormal reproductive performance.
   iii. Non-random distribution of HL-A and Lp serum types. The latter may be analogous to the mouse Ss serum group linked with H-2.

None of the above listed findings are conclusive. They are presented together in an effort to assess data indicative of linkage of MHS with reproductive performance in different species. Therefore they represent only the network of a hypothesis.

12. The role of the functional unit T-H-2 (SNELL, 1968) might be to ascertain the biological role of the MHS in mice (SNELL, 1968). This role has not yet been clarified but is possibly reflected by the forced maintenance of the extreme degree of the MHS polymorphism.

The role of H-2 and/or genes closely linked with H-2 for *histocompatibility*, *tumorigenesis*, *virus susceptibility* and *antibody production* indicates the role which MHS systems could play in the evolutionary and biological life of the species as a genetic system involved in a number of basic immunological processes. There are no similar data (except histocompatibility) in species other than mice.

Summary. Data are presented that support the hypothesis that in different species a "super-gene" exists which consists of two parts. One represents a

major gene for reproductive performance and the other a major gene for a number of basic immunological processes. The simultaneous appearance of both in one linkage group appears not to be due to chance.

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Applied Aspects of Microbial Genetics

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With 22 Figures

Contents

For a long time microbiologists held the opinion, that they could solve all of the problems connected with research on microorganisms without the support of any allied sciences, including genetics. The main thesis of such a point of view was that microorganisms are not subject to mutational change but that variation in microorganisms is based on direct adaptation to the environment independent of spontaneous hereditary changes yet connected in some way with the genetic apparatus of the cell.

The first breach in this concept was made by mycologists whose splendid investigations on *Neurospora* not only confirmed the universal character of genetic laws, but served as a basis for the proclamation of a very important scientific principle, i.e. "one gene — one enzyme", which has proved substantially true even until now. Thus microbiologists were forced to make concessions to the fungi, while ardently defending Lamarckian principles in bacteriology. The citadel of Lamarckism was defended for a long time until a number of fundamental studies dispersed the army of Lamarckists, leaving only isolated soldiers, who were still beating off the great number of facts that disproved the false concepts of pure microbiological principles; the occasional rebel followed such a path either because of lack of information or because he was fanatically devoted to erroneous ideas bequeathed to him.

The foundations of modern microbial genetics were formulated on the basis of four discoveries. The first was the discovery of genetic transformation. The history of that discovery was rather paradoxical. Since through it the bacterium, stubbornly defended from the "aggression" of genetics, became the very object that proclaimed the main principle of modern genetics, namely the recognition that the cell's genetic apparatus is DNA in nature. Of great importance too, was the second discovery, that the concept of mutational variation is also applicable to microorganisms. This was an experimental

rebuttal of the existence of continuous variation in bacteria or of the inheritance of so-called acquired characteristics. The work of DELBRÜCK and LURIA (1943), NEWCOMBE (1949) and LEDERBERG (1952) has left no doubt that variation in microorganisms follows the regularities inherent in highly-organized living beings. The third discovery which had an impact on the development of modern genetics was the discovery of a specific sexual process in bacteria, so-called conjugation, on the basis of which hybridization or, more exactly, genetic analysis developed. The resolving capacity of this analysis far surpassed the genetic analysis of *Drosophila*. The fourth discovery was connected with the establishment of the genetic interrelations between phages and the bacterial cell (so-called genetic transduction) and, further, with the development of phage genetics where recombination methods greatly increased the resolving capacity of genetic analysis and brought it to the molecular level. All four discoveries have played a great role in the development of modern genetics, now also called "molecular genetics".

Parallel with its decisive role in the development of modern genetics, microbial genetics has been uniquely important in the development of a microbiological industry. It is possible to say even more than that: microbial genetics, or more specifically applied microbial genetics, has played the decisive role in the formation of the microbiological industry.

The development of microbial genetics occurred at the same time as the development of the microbiological industry, and this was no coincidence. The discovery of biochemical mutations in *Neurospora* was followed by the discovery of antibiotics, products of microbiological synthesis. Subsequently, it was shown that the powerful apparatus of microbial synthesis can produce vitamins (e.g. B_{12} and riboflavin), enzymes (e.g. pronase, keratinase), amino acids (e.g. glutamic acid, tryptophan, lysine etc.) and growth factors (e.g. gibberellin).

Finally, the microbiologists discovered the capacity of microorganisms to participate in various stages of chemical syntheses, e.g. microbial transformation of steroids, synthesis of polymers (dextran) and participation in an intermediate stage of vitamin C synthesis (oxidation of sorbose into sorbitol). All the discoveries of industrial microbiology coincided in time with the fundamental discoveries in microbial genetics mentioned above. These discoveries created a need for selection of strains with appropriate productive capacities and this was the meeting point between the demands of practice and the achievements of modern genetics. The practical application of microbial genetics was most efficacious in the fermentation industry. The major practical applications of genetics in this new industry have been 1. the use of induced mutations, particularly those leading to a genetic block in the pathways of biosynthesis, and 2. hybridization. It should be noted that these genetic methods had already been used before to some extent in the selection of microorganisms. But whereas the use of mutagen-induced variant has been one of the main methods of selection for almost all producing microorganims (and principally for those producing antibiotics) the method for obtaining

biochemical mutations influencing mechanisms of biosynthesis (lysine, inosine, glutamic acid and other compounds), and the method of hybridization so well elaborated in *Actinomycetes*, *Aspergilli* and yeasts, have not yet found sufficiently wide application in the selection of bacteria.

Induced Mutagenesis in the Selection of Microorganisms

Selection by the use of mutagens has become the most intensive field for the practical application of genetics in microbiology, particularly in the selection of antibiotic-producing microorganisms. A large amount of literature on the selection of penicillin-producing strains dates back to 1948—1958. Most of these publications, however, contain no information on any regularities in the mutational pattern of *Penicillium chrysogenum* with regard to its capacity to produce penicillin. In most cases communications dealt with mutants obtained by the use of ultraviolet light, X-rays, nitrogen mustards, ethylenimine and other physical and chemical agents.

The most important data were obtained in investigations on induced variation of *P. chrysogenum* by DULANEY (1953), by BACKUS and STAUFFER (1955) and by ALIKHANIAN and co-workers (1958). The studies of STAUFFER and BACKUS have been discussed by us in detail in a number of reviews and there is no need to discuss them once more. We should like to point out only that these investigators demonstrated the effectiveness of selecting variants of penicillin-producing strains from populations derived from conidia treated with mutagens such as nitrogen mustard or UV-light. The authors further demonstrated that a substantial rise in productivity could be achieved only as a result of stepwise selection. By accumulating very small, sometimes hardly noticeable changes in productivity at each selection step, indicative of polygenic control, the authors managed to triple the initial strain activity.

DULANEY (1953) obtained similar results with *Streptomyces griseus*, the producer of streptomycin. But before starting such selection, DULANEY assumed (and he was quite right) that a shift in the activity of a strain may be limited by its sensitivity to increased concentrations of streptomycin in the medium. To avoid such limitations DULANEY selected on media with gradually increasing streptomycin concentrations, a variant that was resistant to a concentration of 600 units of streptomycin per ml. Following this DULANEY brought the strain's activity from 250 to 2,000 units per ml., using UV-light and X-rays at 8 different stages of selection.

ALIKHANIAN et al. (1959) using mutagenic agents confirmed the effectiveness of selection in the case of penicillin and streptomycin-producing microorganisms. In addition ALIKHANIAN et al. (1959) showed a very strong effect of mutagens in the selection of actinomycetes producing oleandomycin, erythromycin, oxytetracycline and chlortetracycline. Similar effectiveness of mutation and selection was shown with *Fusarium moniliforme* producing gibberellic acid.

A detailed listing of all cases in which the use of mutagenic agents resulted in a positive effect on microbial selection is not the subject of this report. It will be more interesting to discuss some peculiarities of induced mutagenesis in microorganisms, with special regard to quantitative characteristics, particularly the capacity for antibiotic production.

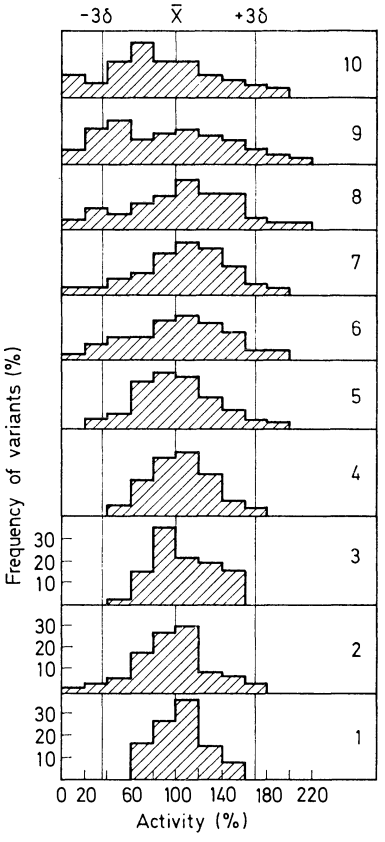


Fig. 1. Distribution of antibiotic production of variants of *Actinomyces erythreus* strain A-5 after different kinds of treatment. *1* Control; *2* 1500 erg/mm²; *3* 1:100 DES 30′; *4* 1:100 DES 60′; *5* 1:100 DES 90′; *6* 1:100 DES 30′ + 150 erg/mm²; *7* 1:100 DES 60′ + 1500 erg/mm²; *8* 10 Krad; *9* 20 Krad; *10* 30 Krad

The literature contains a large amount of material, allowing conclusions to be drawn about relationships between induced variation in quantitative characteristics and the effects of mutagens. Of these, the following have been worked out best:

1. Comparative effects of various mutagens.
2. Role of different doses of mutagens.
3. Correlation between morphological variation and variation in antibiotic production.
4. Role of the genotype in variation in antibiotic production.

5. Peculiarities of variation in highly active and less active strains.
6. Effect of "major mutations" in induced variation.

Comparative studies on the effect of various mutagens have been carried out with some actinomycetes with particular reference to the frequency of occurrence of plus variants. In the oleandomycin-producing actinomycete, *Actinomyces antibioticus*, the effects of ethylenimine, diethylsulfate (DES),

Table 1
Frequency of plus variants induced by different mutagens in Actinomyces antibioticus

Mutagen	Dose	Survival %	Number of variants checked	Frequency of plus variants
None (control)	—	100	282	1.8 ± 0.25
Ethyleneimine	0.1%, 15 min	0.65 ± 0.25	247	6.5 ± 1.4
Fast neutrons	10^4 rad	0.045 ± 0.012	252	9.9 ± 1.8
Diethysulfate (DES)	0.2%, 60 min	0.0005 ± 0.00004	253	6.7 ± 1.5
UV-rays	1500 erg-mm^2	0.00047 ± 0.0002	248	5.6 ± 1.4
X-rays	100 kr	0.00005 ± 0.00001	253	6.7 ± 1.5

Table 2. *Frequency of plus and minus variants of Act. erythreus strain A-5 depending on treatment*

Treatment	Number of variants checked	Plus variants		Minus variants	
		number	%	number	%
None (control)	250				
1500 erg/mm^2 + UV	250	4	1.60 ± 0.80	10	4.0 ± 1.24
DES, 30′	249	0	0.0	0	0.0
DES, 60	250	4	1.60 ± 0.80	0	0.0
DES, 90	260	7	2.69 ± 1.00	8	3.09 ± 1.08
DES, 30 + 1500 erg/mm^2	252	8	3.17 ± 1.1	18	7.14 ± 1.62
DES, 60′ + 1500 erg/mm^2	253	4	1.58 ± 0.63	5	1.98 ± 0.88
10 Krad fast neutrons	255	13	5.09 ± 1.38	39	15.29 ± 2.26
20 Krad fast neutrons	251	13	5.18 ± 1.34	44	17.52 ± 2.40
30 Krad fast neutrons	257	6	2.32 ± 0.94	30	11.69 ± 2.01

fast neutrons, UV-light and X-rays were studied with regard to the frequency of occurrence of plus variants. The results of these experiments are presented in Table 1. These data show that: 1. The frequency of plus variants induced by all of the mutagens is higher than their frequency in untreated cultures; 2. a comparison of UV-light, diethylsulfate and fast neutrons showed that the

last are the most effective (see Fig. 1 and Table 2); 3. a comparison of diethylsulfate and the hydrochloride of the ethyl ester of 5,2-chloroethyl-DL-cysteine revealed a peculiar specificity of their action on the variation of some quantitative features (vitamin production). While inducing considerable variation in plus variants, β-chlorethyl-DL-cysteine-ethyl-ester-HCl does not increase the extent of norma variation of minus variants (Table 3).

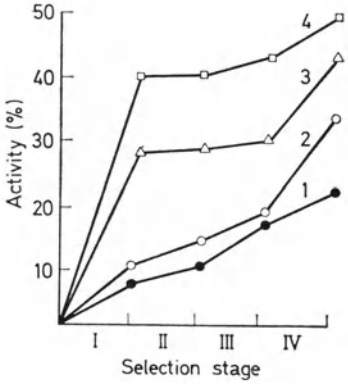


Fig. 2. Activity increase in four lines of *Act. antibioticus* during four stages of selection. *1* without mutagen, *2* ethylenimine, *3* fast neutrons, *4* X-rays

Table 3. *Number of plus and minus variants in Act. olivaceus (producing vitamin B_{12}) after mutagen treatment*

Treatment	Number of variants checked	Plus variants		Minus variants	
		number	%	number	%
None (control)	263	0.0	0.0	0.0	0.0
UV	293	21	7.2 ± 1.5	0.0	0.0
DES	176	2	1.1 ± 0.2	4	2.2 ± 1.0
Ethylenemine	301	24	8.0 ± 1.6	11	3.7 ± 1.1
Sarcolysine [t-Di-(2-chlorethyl) aminothymilamine]	287	26	9.1 ± 1.7	3	1.1 ± 0.1
Ethyl ester of 5.2-chloroethyl-DL-cysteine	326	24	7.4 ± 1.4	12	3.7 ± 1.0
N-Formyl-DL-sarcolysine	282	40	14.2 ± 2.1	0.0	0.0
Embychine (N-methyl-N.di-2-chlorethylemine)	288	13	4.5 ± 1.2	5	1.71 ± 0.03

With regard to the very complicated apparatus of genetic control of quantitative features, it should be noted that the comparative study of induced variation gives only a relative picture of the effectiveness of a mutagen. The only approach which may bring us nearer to the solution of

this problem may be experiments in parallel many-stage selection of the capacity for antibiotic production in one and the same initial strain, using several mutagens. Fig. 2 illustrates the results of one such experiment, in which the effects of selection in strain 32 of *Act. antibioticus* under the action

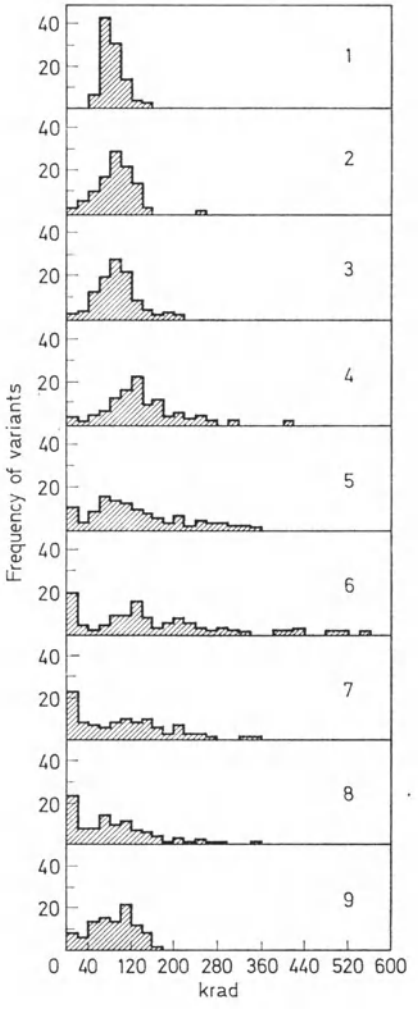


Fig. 3. Range of variation in antibiotic production of *Act. subtropicus* after different X-ray doses. *1* control, *2* 20 Krad-Krad, *3* 40 Krad, *4* 80 Krad, *5* 160 Krad, *6* 320 Krad, *7* 480 Krad, *8* 560 Krad, *9* 640 Krad

of fast neutrons, X-rays and ethylenimine were studied. As is evident from these data, fast neutrons and X-rays led to a higher rate of selection during four stages of the selection process.

There is much material in the literature on the effect of different doses of mutagen on variation in plus and minus variants. In most experiments with X-rays, UV, gamma radiation, fast neutrons, ethylenimine, diethylsulfate,

dimethylsulfate and a large number of 2-chloroalkyl compounds, as well as with combinations of ethylenimine and UV, it has been shown that the majority of variants with increased production capacity occur within the range of moderate doses. This may be illustrated by an example obtained in experiments with *Act. subtropicus* treated by X-rays (Fig. 3). A very important peculiarity was found on comparing the variation in plus and minus variants in strains of different origin. With "wild" strains, i.e. strains not previously subjected to active selection, the potential for selection of plus variants is much higher than for minus variants. The opposite picture is observed in highly active strains, isolated as a result of multiple-step selection with the aid of mutagens. In such strains the frequency of minus

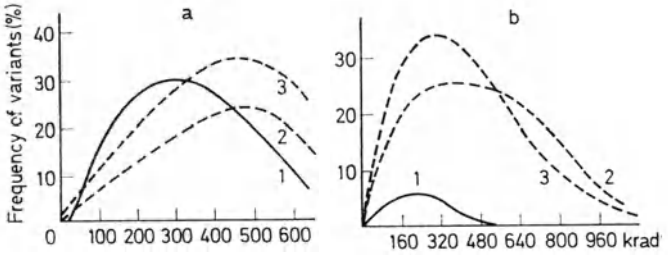


Fig. 4a and b. Frequency of plus and minus variants as a function of the X-ray dose using strain S N 39 (a) and N 788 (b) of *Act. subtropicus*. *1* Plus variants, *2* minus variants, *3* morphological variants

variants exceeds that of plus variants. In both cases the maximum of plus variants is observed with moderate doses, while the frequency of minus variants increases with increasing mutagen dose, forming a peak at the dose inducing the maximum frequency of morphological and other mutants (Fig. 4).

The material just presented may serve as a basis for the following conclusions:

1. Mutagens increase the variation in quantitative features and create heterogeneous populations of actinomycetes, favourable for the selection of active variants.

2. The majority of mutagens are most effective within the range of moderate doses.

3. For each species of actinomycetes, and even for different strains within one and the same species, different mutagens may be differently effective and this means that one can not generalize about comparative characteristics of mutagen effectiveness.

Highly active strains seem to have been saturated by different physiological mutations and the decrease in this low rate of yield of plus variants greatly resembles the decrease in frequency of mutations after a certain dose of mutagenic factor has been applied. This suggests a selective death of types carrying more than an optimal load of mutations. Probably the decreasing selection tempo in highly-active forms is a consequence of the repeated

artificial or natural exposure to mutagenic agents and is connected with the decrease in frequency of plus variants, typical of highly-active strains.

There is some material in the literature on means for overcoming such limits in highly selected and therefore highly active strains and on the peculiarities characteristic of all highly active strains. MOROZOVA and ALIKHANIAN (1966) reported that some of the mutagens tested on a highly active

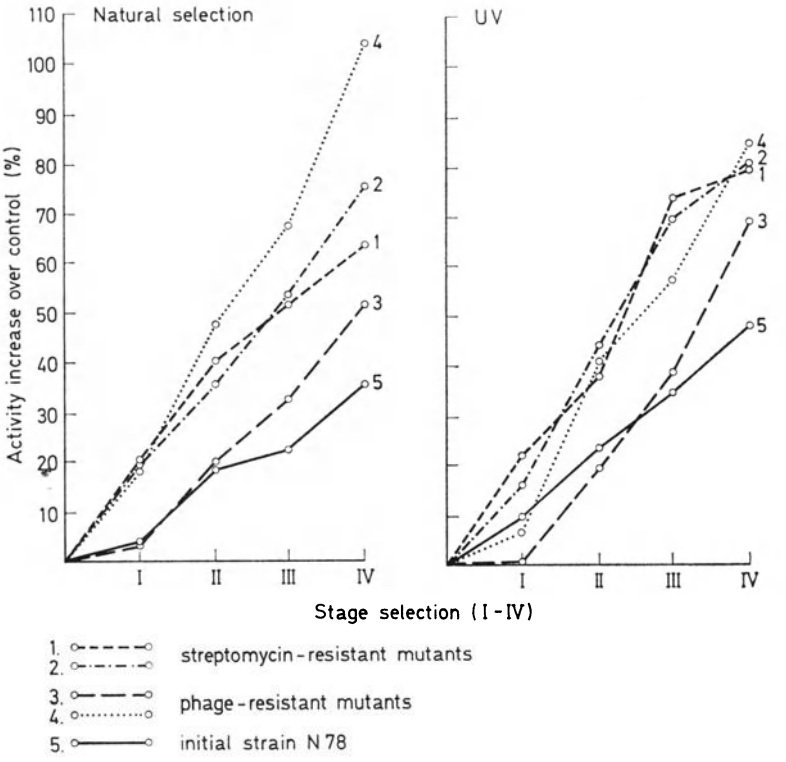


Fig. 5. Activity increase of *Act. streptomycini* strain 78 and strains selected without mutagenic factor and after UV treatment. *1* and *2* Streptomycin-resistant mutants, *3* and *4* phage-resistant mutants, *5* inital strain

streptomycin-producing strain of *Act. streptomycini* did not produce any increase in the production of the antibiotic by any direct change in synthetic mechanisms, but did produce it by four mutations having no apparent direct relation to streptomycin production. Two mutations to phage resistance were obtained by selection among the colonies of the initial strain 78, grown on medium with phage; then, two streptomycin-resistant mutations were obtained by selection on media with increased content of streptomycin (about 10,000 un/ml). All four mutants showed a 25 % increase in streptomycin production over the initial strain (MOROZOVA, 1966).

How can this fact be explained? The authors supposed that the increased activity of phage-resistant mutants could be the consequence of a mutagenic effect of phage, and a derepression of the specific gene controlling strepto-

mycin synthesis in streptomycin-resistant mutants. Subsequently, all four strains were treated, in a four-stage selection process, with UV; controls received no mutagen treatment. As shown in Fig. 5, the increase of activity for all four mutants outstrips that of the initial non-mutant strain.

A study of variation in antibiotic production showed that in a wide range of strains, whose activity level had been much improved as a result of long multiple-stage selection with the use of mutagens, the coefficient of variation falls sharply. From Table 4 it can be seen that the coefficient of variation of the first strain group of *Act. antibioticus* falls gradually and reaches a level

Table 4. *Variability of antibiotic production in natural population of oleandomycin-producing strains*

Designation of isolate and type of treatment	Total number of variants	σ	CV
N 39 without mutagen	202	22.18	24.9 ± 1.8
N 2 ethylenemine	195	21.8	22.9 ± 1.1
N 889 ethylenemine + UV	100	20.2	21.3 ± 1.5
N 1790 without mutagen	154	17.3	17.8 ± 1.0
N 5001 diethylsulfate	113	"great" mutation	
N 5442 without mutagen	150	25.2	32.1 ± 0.8
N 6274 without mutagen	153	17.7	21.8 ± 1.2
N 6852 diethylsulfate	151	13.8	14.2 ± 0.8
N 32 without mutagen	282	12.9	13.7 ± 0.5
N 280 X-ray	195	15.8	16.3 ± 0.82
N 416		14.8	14.3 ± 0.99

where selection effectivity is practically nil. "Plus" variants occurred infrequently and the tempo of selection declined. The "big" mutation at step (5001) was obtained at the fourth selection stage under the influence of diethylsulfate, when the variation coefficient fell sharply and selection did not give any effect.

It was impossible to calculate the coefficient of variation for this mutation because its range did not obey the rules of normal distribution: of 113 colonies obtained in the progeny of the strain, 45 colonies were scored zero, i.e. they were practically without antibiotic-producing capability. The mutant strain differed from the initial strain by its intensive pigment production, the diameter of colonies was one-half, it was characterized by a short cycle of development, by the absence of spore production and by a number of other new characteristics. In colony 5442, selected from the mutant population 5001, the coefficient of variation, which had increased sharply in the natural yield, increased sharply again (Table 4). Such a shift in the coefficient of variation as a result of a large mutation was described in another species, *Act. erythreus*. Once a definite threshold has been reached, the stabilization of the production trend can be damaged by a radical reconstruction of the genotype.

Some cases of wide morphological variation among the colonies grown from spores treated by mutagens and among more active strains have been described in the literature. Two types of colonies (namely morphologically changed and unchanged) of *Act. subtropicus*, obtained following X-irradiation, were distributed in three groups according to activity: zero variants, minus variants and plus variants. As shown in Fig. 6, the zero and minus forms prevailed in the group of morphologically changed forms, whereas plus variants composed the greater part of unchanged forms.

Examining the picture described, we can come to the following conclusions:

1. Drastic changes in morphological characteristics very often are associated with the entire loss or with a significant loss of activity.

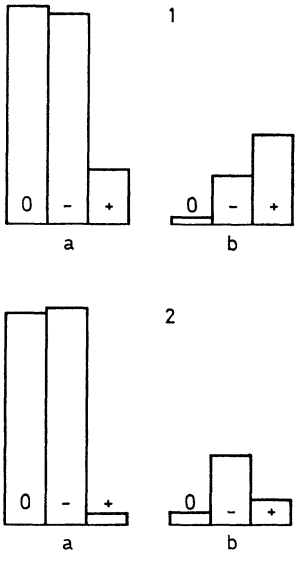


Fig. 6a and b. Frequency of active and inactive variants of *Act. subtropicus* among morphologically changed (a) and unchanged (b) forms. *1* strain N 39, *2* strain N 738

2. Drastic changes in activity are very often associated with morphological changes in colony type.

In concluding this chapter we must indicate that, on one side the peculiarities of quantitative variation, and on the other side the absence of data on the mechanism of biosynthesis of antibiotics, particularly the lost factors, greatly influence the character of the investigation of induced mutagenesis in its practical application to microorganisms and bear the mark of empiricism. Therefore, the development of investigations in microbial genetics for the benefit of the microbial industry must make available methods of genetic analysis which will facilitate the study of quantitative features of induced mutagenesis. However, in spite of existing limitations, the most widely used and valuable method of developing highly active strains from producers of different metabolites is the process of selection from colonies grown from spores and cells treated by mutagens.

Hybridization in the Selection of Microorganisms

While the investigation of the genetic apparatus of *Escherichia coli*, *Bacillus subtilis* and *Salmonella typhimurium* is far advanced, these microorganisms unfortunately are so far of no practical use for applied purposes, and therefore the knowledge of their genetic apparatus can be used only indirectly. In contrast, such economically important forms as *Micrococcus glutamicus* or actinomycetes are only now being subjected to intense genetic investigations thanks to studies by SERMONTI, HOPWOOD, SPADA-SERMONTI, MINDLIN and ALIKHANIAN.

The phenomenon of genetic recombination in actinomycetes was described by SERMONTI and SPADA-SERMONTI (1955), as well as by ALIKHANIAN and MINDLIN (1956), but the mechanism of this phenomenon remained obscure until recently. Although genetic recombination in actinomycetes was previously considered to be similar to parasexual processes in fungi, it may now be considered certain that in its principal features the genetic mechanism of recombination in actinomycetes resembles very closely that of bacteria.

The use of genetic recombination in selection offers the opportunity of using hybridization methods for the breeding of strains with new and valuable properties. The use of recombination also enables one to use the methods of genetic analysis for the elucidation of pathways of antibiotic biosynthesis and a study of the genetic control of antibiotic production.

The phenomenon of genetic recombination was recognized in a number of antibiotic-producing representatives of the genus Streptomyces[1] i.e. *Actinomyces rimosus*, *Act. erythreus* and *Act. streptomycini* but there is only one strain, *Streptomyces coelicolor* A3 (2), on which genetic studies were carried out regularly for many years by HOPWOOD and SERMONTI (1962—1966). Hence *Str. coelicolor* has become a classical subject for genetic studies, like *E. coli* and *S. typhimurium* among gram-negative bacteria.

Genetic recombinants in actinomycetes are usually obtained by joint cultivation of two strains, differing from each other in some genetic features, on a complete medium with subsequent transfer of spores from the mixed culture to a minimal medium. Recombinant forms, having new combinations of characteristics of the parent strains, can grow on the minimal medium, while the parent forms cannot. Thus the procedure for obtaining genetic recombinations in actinomycetes consists of several stages and does not differ substantially from the methods used for bacteria and fungi.

In experiments with *Act. rimosus*, ALIKHANIAN and MINDLIN (1959) have shown that the ratio of spores (or mycelium fragments) of the two strains in mixed cultures is the factor determining the yield of recombinants. It is possible that the cause of this phenomenon is connected with the difference in growth rates of the two strains and their unequal capability for sporulation.

[1] The taxonomists have not yet reached agreement on how to name the ray fungi. We shall follow the names used by the authors of the works mentioned.

Some colonies growing on selective media are heterogenous and contain a mixture of several recombinant types. When identified, they may be erroneously classified as prototrophs, since they can grow on different diagnostic media. Therefore, it is recommended that the supposed prototrophic forms be re-identified on a minimal medium, and that obviously heterogenous colonies be ignored in the analysis of yields from crosses.

Some of the heterogenous colonies growing on selective media can be the result of a mechanical mixture of several genotypes, involving the development of a colony from several spores (or mycelium fragments) that adhered together. Other heterogenous colonies or heteroclones, as they are usually called, arise as the result of a substantially different mechanism. SERMONTI and colleagues (1960) showed that heteroclones arise from spores that outwardly resemble usually haploid recombinants. In contradistinction to the latter, however, they are diploid (ore more exactly, partially diploid) in their genotype, and all possible types of haploid recombinants can be found in their progeny. This property of heteroclones enables one to differentiate them easily from haploid recombinants by transferring them to a selective medium of identical composition. If spores are transferred, colonies of haploid recombinants grow up on this medium, while heteroclones do not, since in the course of sporulation they form haploid recombinant spores, characterized by different combinations of the parental genetic factoes.

To obtain heteroclones, two strains of *Str. coelicolor* A3 (2) are usually used, each carrying one of two closely linked genetic markers. As a selective medium, one employes a medium free of the growth factors required by the parental types. Under such conditions, heteroclones with the genotype a^+b^-/ a^-b^+, as well as haploid recombinants a^+ b^+, will have a selective advantage over the parent strains but, due to the close linkage of the markers, the frequency of occurrence of haploid recombinants will be insignificant as compared with that of heteroclones. Besides the closely linked markers, the participants in the cross are marked with other genetic factors, which can be used as non-selected markers in the course of assay of heteroclones.

On the basis of the data obtained by analysis of the segregation of genetic factors in the progeny of heteroclones, a method of genetic analysis of actinomycetes was worked out. This method has been used alongside conventional methods, based on the determination of the relative frequency of occurrence of various types of haploid recombinants in a mixed culture. HOPWOOD and SERMONTI (1962) worked out two methods for genetic analysis of *Str. coelicolor* A3 (2), one of which was based on an analysis of heteroclones.

The genetic analysis of haploid recombinants (a selective method of genetic analysis) consists in the use of selective media for the growth of such recombinants. This feature considerably limits the number of recoverable genotypes in the progeny. For example, if a four-factor cross yields theoretically 16 possible types of progeny (both parent types included), the use of selective media results in the recovery of fewer (9) genotypes, because all genotypes

with a contribution of parent markers are automatically eliminated (Table 5). On the other hand, only four types of recombinant colonies can grow on each selective medium, which makes it necessary to use all possible selective media for the selection of recombinants from each cross. To determine the frequency of recombinants between corresponding markers, the mean of two complementary genotypes must be calculated. Thus, the recombination frequency in a four-factor cross is determined on the basis of the data obtained from

Table 5. *The genotype of recombinants, selected on four types of selective media, in a four-factor cross ABcd × abCD of Streptomyces coelicolor* (HOPWOOD and SERMONTI, 1962)

Index	Medium			
	BD	AD	BC	BD
Selected allele	BD	BC	AD	AC
Recombinant Genotype	ABCD ABcD ABcD aBcD	ABCD aBCD ABCd aBCd	ABCD AbCD ABcD AbcD	ABCD AbCD ABCd AbCd

quantitative analysis of seven different recombinant types. The relative frequency of recombinants of various genotypes is determined by recording the respective figures in a special column where the frequency of each recombinant type is shown as a percentage of the total number of recombinants.

The method of selective genetic analysis of *Str. coelicolor* A3 (2) has permitted the identification of two groups of unlinked genetic loci showing different degrees of linkage within each group.

It has already been mentioned that the heteroclones of *Str. coelicolor*, when reproduced by spores, undergo segregation with formation of different recombinant types. We must mention that in addition to the recombinant types in the progeny of heteroclones, both initial parent types also segregate. Thus, when one cultivates heteroclone spores on a complete medium and subsequently determined the genotype of the colonies grown from them, one can find all possible progeny classes expected in a given cross (for example, eight classes in three-factor and sixteen in four-factor crosses). The principal peculiarity of genetic analysis in heteroclones is the possibility of determining the magnitude of the linkage between genetic loci in absolute units. However, a direct determination of the magnitude of linkage between loci has not proved possible in every case. The cause of this difficulty in the genetic analysis of heteroclones is that complementary recombinant classes arise in the progeny of heteroclones in extremely unequal proportions. The correlation between parental classes also appears to be unequal in the progeny. HOPWOOD and SERMONTI accordingly, suggested that heteroclone nuclei have an incomplete diploid character. According to this hypothesis, heteroclones arise from incomplete heterozygotes in which one or two terminal chromosome segments in each

linkage group are absent, i.e. each chromosome carries one or two terminal defects. On the basis of this hypothesis HOPWOOD and SERMONTI developed models of segregation in heteroclones, describing different cases of asymmetric segregation (HOPWOOD and SERMONTI, 1962; HOPWOOD, 1965a). The results of the genetic analysis of heteroclones fully confirmed the conclusions made on the basis of selective analysis concerning the presence of two linkage groups and the sequence of loci within each of these groups. Good agreement

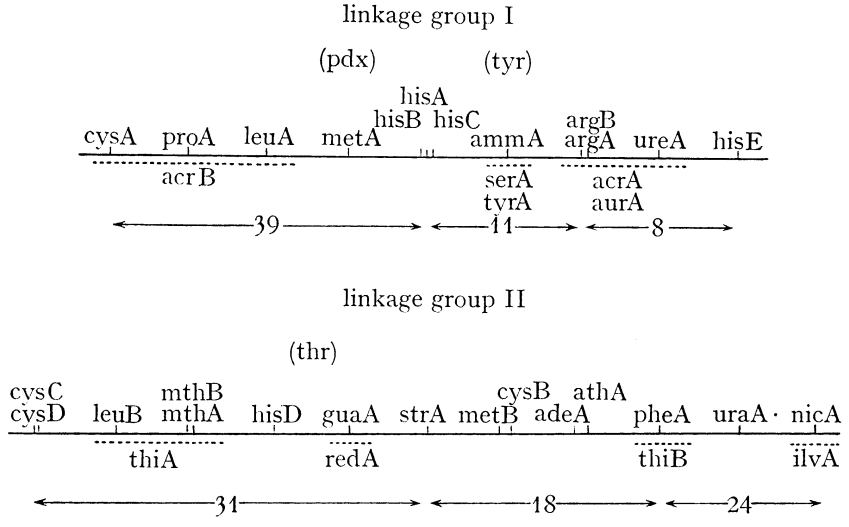


Fig. 7. Genetic map of *Str. coelicolor* showing two linkage groups. *acr* resistance to acriflavin, requirement for: *ade* purines; *amm* NH_4 or glutamic, or aspartic acid; *arg* arginine; *ath* purines and thymine; *aur* arginine and uracil; *cys* cysteine; *gua* guanine; *his* histidine; *ilv* isoleucine and valine; *leu* leucine; *met* methionine; *mth* methionine and threonine; *nic* nicotinamid; *pdx* pyridoxine; *phe* phenylalanine; *pro* proline; *ser* serine and *glycine; thi* thymine; *thr* threonine; *tyr* tyrosine; *ura* uracil; *red* the formation of red pigment; *str* resistance to streptomycin; *ure* urease deficiency (HOPWOOD, 1965)

has also been obtained as a result of determining the distance between neighbouring loci by the two methods.

The genetic map of linkage in *Str. coelicolor* A3 (2), on the basis of data obtained up to 1965, is presented in Fig. 7. In it HOPWOOD (1965a) has shown the distribution of 39 genetic loci in two linkage groups. The presence of two linkage groups in *Str. coelicolor* implied that the genetic system of actinomycetes differs substantially from that of bacteria. More recent investigations, however, have shown that the conclusion regarding two linkage groups in *Str. coelicolor* is not final. New data obtained by HOPWOOD (HOPWOOD, 1965b) favour the concept of one linkage group in *Str. coelicolor*.

The circular linkage map of *Str. coelicolor* plotted by HOPWOOD (1966b) gives the clearest picture of the distribution of various genetic loci. Aside from noting the position of biochemical deficiency markers, this map illustrates the

positions of various temperature-sensitive mutants not connected with known genetic loci; temperature-sensitive mutations are characterized by their inability to grow on complete medium at 38°. As is evident from Fig. 8, the most characteristic peculiarity of the genetic map of *Str. coelicolor* is an irregular distribution of genetic loci concentrated mainly in two regions, corresponding to the two linkage groups described before. The other two map

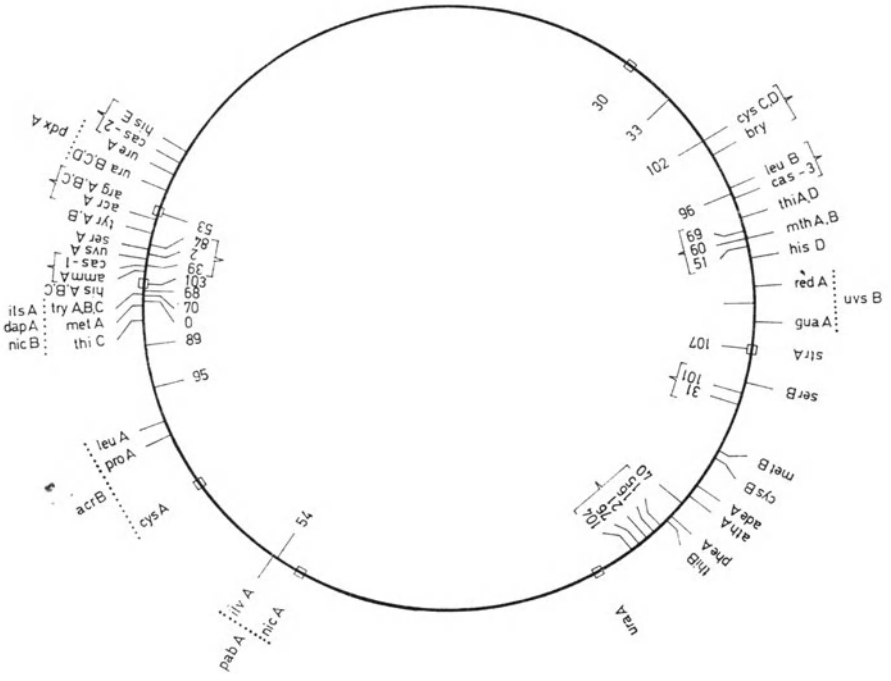


Fig. 8. Circular linkage map of *Str. coelicolcolor* A 3 (2): The numbers on the inside of the circle indicate the loci of temperature-sensitive mutations; designations outside the circle give the loci of biochemical mutations, mutations resistant to some agents and some other mutations (HOPWOOD, 1966b)

regions are practically empty. Consequently these two groups of loci appear to be quite distant from each other, a fact that accounts for the earlier conclusion regarding their location in separate linkage groups.

The model of the *Str. coelicolor* linkage map just presented cannot be considered as final. For example, the question of the regions empty of genetic material is not yet clear. Do they really exist? Is it possible to define them as non-described genetic factors, or are they artefacts resulting from an unusually high frequency of crossing-overs? There also are still no data on the structure of the *Str. coelicolor* chromosome, and the circular linkage map does not at all imply that the chromosome is necessarily circular.

Essential features of resemblance in the genetic systems of bacteria and actinomycetes were also revealed as a result of studying other peculiarities of genetic behavior in both groups of microorganisms. All *Str. coelicolor* strains

used in the majority of genetic investigations of that organism originate from one and the same strain, A3 (2). This fact probably explains why it proved impossible for a long time to find *Str. coelicolor* strains similar to the F^+ and F^- strains of *E. coli*, genetic recombinants arising in any combination on crossing different strains. However, as a result of more thorough studies, SERMONTI and CASCIANO (1963) have found that some strains of *Str. coelicolor* do not form genetic recombinants in crosses between each other, though they possess such a capability when crossed with other strains. The group of strains which are sterile in crosses with each other was named R^-, and strains fertile both in crosses with each other and with strains of group R^- were named R^+. Thus, recombinants occurred in crosses $R^+ \times R^+$ and $R^+ \times R^-$ but were absent in crosses R^- and R^-.

A characteristic peculiarity of the genetic recombination process in actinomycetes, as was shown above, is the formation of incomplete zygotes (merozygotes). This peculiarity is also inherent in the genetic recombination occurring in bacterial conjugation. The presence of merozygotes in actinomycetes may best be proved by the analysis of genetic segregation in heteroclones. Such an analysis shows that only some of the genetic markers of both parents are present in the heteroclone nucleus in the diploid state. On the other hand, an analysis of a large number of heteroclones from one cross leads to the conclusion that the diploid sections of the genome in the heteroclone nucleus are different in different heteroclones, just as the merozygotes of *E. coli* are different in their structure.

SERMONTI et al. (1966) have worked out a new method of obtaining heteroclones which permits their isolation directly from the substrate mycelium of a mixed culture immediately after their emergence on a complete medium. This is done by the use of cellophane discs applied to the surface of a complete medium which sustains the growth of both strains. After one day the cellophane discs are transferred to a minimal medium (or a minimal medium with certain additions) on which only recombinant forms can grow. Two important observations were made with the use of the cellophane method. When all the markers of one parent (streptomycin-resistant) were present in the heteroclone progeny, only some of the markers of the other parent (streptomycin-sensitive) were found in it. In other words, the initial strains made unequal contributions to the formation of the recombinant forms.

The second important conlusion resulting from the study of the dynamics of heteroclone emergence was that the formation of a partially diploid nucleus is time-dependent; in this case the length of the genetic fragment of the streptomycin-sensitive strain was the greater the longer was the period of interaction between the two parental strains.

Thus, one of the most important peculiarities of the genetic recombination process in bacteria, namely, the partial and gradual transfer of the genetic material from the donor to the recipient, has proved to occur in actinomycetes as well.

Studies on the location of genetic loci in the *Str. coelicolor* linkage map have shown that in some cases loci controlling related biochemical functions are closely linked to one another.

On the other hand, one may probably say even now that the genetic system of actinomycetes (and of *Str. coelicolor*, in particular) is different in some features from that of bacteria. One of the important differences is the duration of the merozygotic stage. In contrast to *E. coli*, the merozygote stage in *Str. coelicolor* lasts for a long period of time, covering many nuclear

Table 6. *List of Actinomycetes species showing heterokaryosis and genetic recombination*

Type of *Actinomycetes*	Hetero-karyosis	Genetic re-combination	References
Streptomyces coelicolor	—	+	SERMONTI and SPADA-SERMONTI, 1955; HOPWOOD, 1957
S. fradiae	+	+	BRAENDLE and SZYBALSKI, 1957
S. rimosus	+	+	ALIKHANIAN and MINDLIN, 1957
S. griseoflavus	+	+	SAITO, 1958
S. griseus	+	+	BRADLEY, and LEDERBERG, 1956
S. scabies	+	+	BRAENDLE and SZYBALSKI, 1959
S. aureofacieus	+	+	GREGORY, 1959
S. spheroides	+	—	ALIKHANIAN and BORISOVA, 1961
S. cyanes	+	—	BRAENDLE and SZYBALSKI, 1959
S. albus	+	—	BRADLEY and LEDERBERG, 1956
S. venezuelae	+	—	BRAENDLE and SZYBALSKI, 1959
S. erythreus		+	LI-CHUAN-LO, 1962
S. antibioticus	+	+	VLADIMIROV, 1966

divisions. Besides that, a phenomenon of post-zygote elimination was described in *Str. coelicolor*. Studies of the so-called heteroclones of the second and third order, arising sometimes from the progeny of the first and second order heteroclones respectively, have shown that in a number of cases the length of the stage of the merozygote is shorter than its initial length. In other words, in the process of reproduction of a partially diploid heteroclone nucleus, the loss of a fragment of the genetic material is observed in one of the parents: this is revealed by the absence in the heteroclone progeny of the second and third order of some genetic factors originally present in the heteroclone progeny of the first order.

The long-time preservation of the diploid state in heteroclones is the cause of another genetic peculiarity of actinomycetes, namely, the possibility of direct complementary analysis in heterozygotes.

The use of selective methods in isolating presumptive recombinants has shown that the phenomenon of genetic recombination is widely spread among different representatives of the *Streptomyces* (*Actinomyces*) genus (see Table 6).

Although attempts to obtain interspecific hybrids in actinomycetes have usually yielded negative results, there are some exceptions to this rule.

POLSINELLI and BERETTA (1966) have described a case of interspecific hybridization between *Str. Aureofaciens* and *Str. rimosus*, two species producing tetracycline antibiotics (chlortetracycline and oxytetracycline). A cross of multiple auxotroph strains of these organisms yielded, in some combinations, stable prototroph and auxotroph recombinants.

MINDLIN et al. (1962) studied the recombination process in *Act. rimosus* with the use of the cellophane method and analyzed some stages of that process. On crossing biochemical mutants, differing in their sensitivity to streptomycin, they found that the streptomycin-resistant prototroph recombinants (supposedly haploids) appear at later stages than the streptomycin-sensitive prototroph recombinants (supposedly diploids).

A peculiarity that is probably characteristic of actinomycetes is the formation of an incomplete zygote (merozygote) at the first stages of genetic recombination. Indeed, in crosses between multiple biochemical mutants of *Act. fradiae* and *Act. criseoflavus*, as well as strains of *Str. coelicolor*, other than strain A3 (2), it is possible to find only a part of the theoretically expected recombinant genotypes. In particular, BRAENDLE and SZYBALSKI (1959) have shown that, when crossing strains marked with streptomycin-sensitivity, as a rule only one of the alleles of the parent strain is found on examining the haploid recombinants. Cases of segregating auxotrophs in which the progeny of prototroph recombinants carried biochemical deficiency factors of only one of the parents, were described by ALIKHANIAN et al. (1959) and ALIKHANIAN and BORISOVA (1961) in *Act. rimosus* and *Act. aureofaciens*. These cases also testify in favor of the concept of formation of merozygotes in the process of genetic recombination in actinomycetes.

Thus, the data obtained as a result of studying genetic recombinants in various species of actinomycetes correlate with those obtained from genetic studies of *Str. coelicolor*. The choice of *Str. coelicolor* as an object for genetic investigations of actinomycetes has proved to be fortunate in many respects, except for one, namely, that *Str. coelicolor* is not an antibiotic-producing microorganism. That is why antibiotic production, a feature possessed by many actinomycetes, was not used in the genetic analysis of this organism.

On the other hand *Act. rimosus*, which produces oxytetracycline, has been very poorly studied genetically. It is evident, however, that a knowledge of the genetics of antibiotic producers is a necessary prerequisite for the successful use of hybridization in the selection of antibiotic-producing microorganisms.

Studies of the genetic control of oxytetracycline synthesis were aimed at solution of the following tasks:

1. to obtain mutations controlling antibiotic production which might be used as genetic markers;

2. to map genetic factors controlling antibiotic production, and to try to determine their number.

For this purpose it was necessary to have mutants with impaired antibiotic synthesis at different stages of biosynthesis, i.e. inactive mutants. Such

mutants were obtained in great numbers by ALIKHANIAN et al. (1961) from active strains of *Act. rimosus* with the aid of various mutagenic agents. While the initial strain synthesized about 3,000 μg/ml of oxytetracycline, the amount produced by inactive mutants was about 20 to 70 μg/ml and some produced only traces of the antibiotic. With some combinations of inactive mutants, the producion of considerable quantities of the antibiotic was observed when

Table 7. *Antibiotic production by inactive mutants of Act. rimosus cultivated separately and together*

Strain	Antibiotic activity mg/ml	Mycelium dry weight mg-%	Strain	Antibiotic activity mg/ml	Mycelium dry weight mg-%
Initial	3046	1041	white + black		
White mutant			T1 + T572	1030	1075
T1	0.5	1095	T2 + T572	790	1033
T2	0.2	435	T11 + T572	830	1002
T11	0.4	1160	T12 + T572	1000	1083
T12	0.9	1360	T16 + T572	600	990
T16	0.8	1012	T956 + T572	720	1088
T256	0.3	932	T999 + T572	760	930
T999	0.5	1042	white + white		
Black mutant	20.0	872	T2 + T956	5.0	860
			T2 + T999	4.0	956
			T12 + T956	1.0	924
			T12 + T999	3.0	926
			T16 + T956	4.0	656
			T16 + T999	4.0	905

they were cultivated together on a fermentation medium. Some remarkable results were achieved by combinations of "white" and "black" mutants, so named after the colour of their colonies (see Table 7). Although producing no antibiotics when cultivated alone, these mutants cultivated together, synthesized about 1,000 μg of oxytetracycline per ml, while all other possible combinations of white mutants remained inactive. The mechanism of synthesis of intermediate metabolites leading to oxytetracycline synthesis was revealed by transplanting the mycelium of one mutant on to the fermentation medium of the other. The "black" mutant mycelium synthesized oxytetracycline in the presence of metabolites produced by the "white" mutants.

On the basis of experiments similar to those described above, all inactive mutants were divided into several classes. Mutants of the same class did not produce the antibiotic when cultivated together, while mutants of different classes were complementary in the course of antibiotic synthesis under such conditions. A chromatographic analysis of the culture broth of the mutants confirmed the correctness of their phenotypic classification by showing that the mutants of the same class produce the same oxytetracycline precursors (MINDLIN et al.,1968).

Representatives of each class of mutants were then used for genetic analysis.

An essential feature of the method of crossing inactive mutants was that only one component of a cross was marked by biochemical deficiency and resistance to streptomycin, while the other component was streptomycin-sensitive. Prototroph recombinants were selected in the progeny. MINDLIN

Table 8. *Genetic analysis of inactive mutants* (MINDLIN et al., *1964*)

	ser$^+$	str$^+$	otc3	otc11$^+$
Cross of black mutants (572) Cross of white mutants (12)				
	ser	str	otc3$^+$	otc

Total number of recombinants	ser$^+$ str otc3$^+$ otc11		ser$^+$ str otc3 otc11$^+$		ser$^+$ str otc3$^+$ otc11	
ser$^+$ str	number	%	number	%	number	%
490	454	92.6±1.2	3	0.61±0.35	33	6.74±0.99

Table 9. *Crosses of inactive mutants from different classes with the white mutant* (MINDLIN et al., 1964)

Cross	Total number of recombinants ser$^+$ str otc 11$^+$	Non-actives among the total number of recombinants otc11$^+$otc1 (2.3.4.5)	
		number	%
572 otc3 × 12 otc11	339	16	4.7±1.1
638 otc3 × 12 otc11	281	13	4.6±1.2
384 otc4 × 12 otc11	371	49	18.0±1.3
739 otc1 × 12 otc11	367	367	100
241 otc2 × 12 otc11	389	389	100
35 otc5 × 12 otc11	238	238	100

et al. (1964) reported that when white (12) and black (572) mutants were crossed (see Table 8), the prototrophs resistant to streptomycin could be divided, as was to be expected into classes forming three types of colonies: 1. inactive white, 2. inactive black, 3. active black. (Recombinants of a fourth type, carrying both mutant factors for antibiotic production could not be differentiated from recombinants of the first type.) Recombinants of the second type emerged at the lowest frequency, a fact that testified to the close linkage between the antibiotic-production factor in the black mutant (otc-3) and the streptomycin locus (crossing-over between them occurred very rarely).

In crosses of other inactive mutants, the relative frequency of occurrence of the second and third type recombinants was taken into account (see

Table 9). The data thus obtained have shown that there exist at least two groups of genetic loci which control antibiotic production. The first group includes loci otc. 3 and otc. 4; the second — otc. 1, otc. 2, otc. 5 and otc. 11 (Fig. 9). In crosses of the second-group, mutants between each other (for example, otc. 1 × otc. 11). inactive recombinants did not occur, a fact that testifies to the close linkage of the respective loci. Active recombinants appeared in crosses between mutants of these two groups.

Let us consider the practical, i.e. selectional, aspect of using recombinations in actinomycetes and fungi on the one hand, and the use of hybridization and

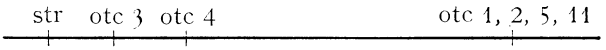


Fig. 9. Location of genetic loci controlling oxytetracycline biosynthesis in *Act. rimosus* (MINDLIN et al., 1964)

recombination for the study of the genetics governing the formation of practically important metabolites on the other hand.

Table 10. *Activity of penicillin-producing diploids obtained from Penicillium chrysogenum mutant Wis 47-1564* (CAGLIOTI and SERMONTI, 1956)

Diploid	Genotype in regard to penicillin product. charact.	Activity after 96 hours of growth (un/ml)	Diploid	Genotype in regard to penicillin product. charact.	Activity after 96 hours of growth (un/ml)
Wis 47-1564	+	441	DXV 124/63	P/P_3	0
D III 26/29	+/+	361	DXI 124/121	P/P_4	0
DXII 46/65	$+/P_2$	385	DV 165/63	P_2/P_3	0
D IV 51/63	$+/P_3$	238	DIX 122/63	P_2/P_3	0
DVII 46/63	$+/P_3$	236	DX 65/121	P_2/P_4	0
DVIII 124/65	P/P_2	0	DXIII 63/121	P_3/P_4	0

Hybridization has been employed for the isolation of desired microbial strains for the last 10 years only and the practical results in this field are as yet still rather poor. The first attempt to study the genetics of penicillin production by diploid strains of *P. chrysogenum* was made by CAGLIOTI and SERMONTI in 1956. Four biochemical mutants (P) producing no penicillin were isolated from strain Wis 47-1564 of the Wisconsin series, which produced about 250 units penicillin per ml. A number of diploids was obtained by crossing these four mutants pairwise. In addition, two of the mutants were crossed with active variants, and a diploid between two active mutants of Wis 47-1564 was obtained (see Table 10). On the basis of the resulting data the authors concluded that the capability of producing penicillin is determined by a specific gene, whose different alleles (P_1, P_2, P_3, and P_4) are the result of interdependent mutations. This is confirmed by the fact that diploids obtained from P-mutants in all possible combinations did not produce penicillin. If the P-muta-

tions block the penicillin production of different genes, their combination in a heterozygote should result in restoration of the capacity to synthesize penicillin. The diploids obtained between the allele P_2 and the newly obtained inactive mutants (P_5, P_6, P_7 and P_8) were inactive.

On the other hand, crossing of the allele P_2 with three mutants carrying the allele P_9 gave three active diploids (Table 11); e.g. a mixed culture of strains $96P_9$ and $99P_2$ showed a production capacity average between the capacities of the initial strains (Fig. 10).

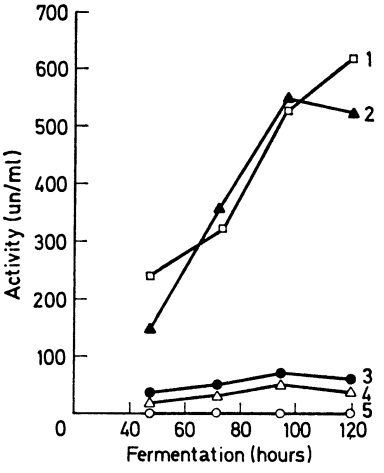


Fig. 10. Activity of penicillin formation of the *P. chrysogenum* strain Wis 47-1564 (*1*), of mutants 99 pr (*5*) and 96 pr (*3*), of mixed culture of these mutants (*4*) and of diploid 65 Pi/96 pd (*2*)

These data allow us to conclude that the eight P alleles (1—8) are alleles of one and the same locus, whereas the allele P_9, differing from the others by in complete suppression of penicillin production, belongs to another locus, since combining it with any of the other eight P alleles restores the capability to synthesize the antibiotic.

Table 11. *Penicillin production by mutants of P. chrysogenum strain Wis 47 1564* (SERMONTI, 1956)

Diploid	Genotype of diploids	Activity after 120 hours of fermentation u/ml	Diploid	Genotype of diploids	Activity after 120 hours of fermentation u/ml
DXXII	$65P_2/144P_5$	0	DXXIX	$65P_2/163P_9$	436
DXXIII	$65P_2/157P_8$	0	DXXXII	$65P_2/147P_9$	244
DXXIV	$65P_2/158P_6$	0	DXXXIII	$65P_2/162P_9$	307
DXXVII	$65P_2/148P_7$	0			

On the basis of a study of the genotype of recombinants obtained from diploid XXIX, SERMONTI has come to the conlusion that the two P genes (P_{1-8} and P_9) are in different chromosomes. The results presented above also allowed us to conclude that the mutations incapable of synthesizing penicillin are recessive in relation to the wild-type allele, i.e. to the capability of synthesizing the antibiotic.

Later on SERMONTI (1959) showed that not only negative, i.e. mutants with little activity are recessive in relation to the wild type, but also positive, i.e. highly active ones are recessive. He obtained a number of diploids among

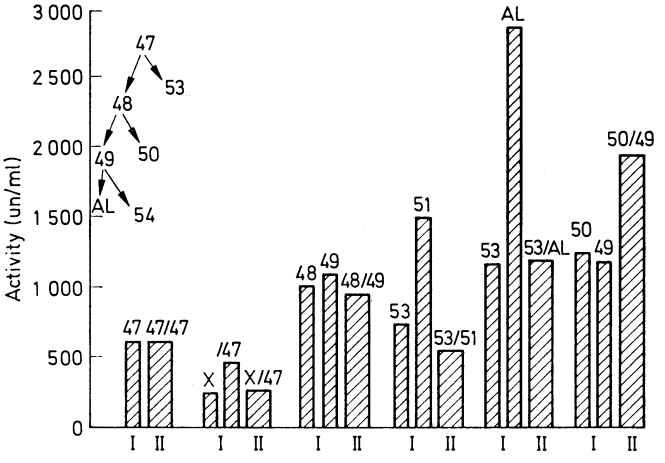


Fig. 11. Activity of penicillin formation of diploids (*II*) of *P. chrysogenum* and initial haploid strains (*I*). 47 = 47 1564 Wis; 53 = 53 844 Wis; 50 = 50 1247 Wis; 49 = 49 133 Wis; 51 = 51 20 Wis; AL = Ap 206

several strains of the Wisconsin series differing from each other in their activity. In all cases the diploid had the productivity of the less active parent (Fig. 11). The recessive character of positive mutations was also confirmed by the data of MCDONALD et al. (1963) who obtained diploids between a poorly active strain (derived from NRRL) and its highly active descendents D-734

Table 12. *Penicillin production by diploid strains of P. chrysogenum and their ancestors after 144 hours of fermentation* (MCDONALD et al., 1963)

Prototrophic ancestors		Auxotrophic parents		Diploids	
strain	activity u/ml	strain	activity u/ml	strain	activity u/ml
NRRL 1951	115	54	105	1	280
Wis 54-1255	3410	10	945		
NRRL 1951	115	54	105		
WD 734	3110	13	375	2	115
NRRL 1951	115	55	107		
WD 734	3110	51	3290	3	175

and Wis 54-1255 (Table 12). All three diploids had a productivity close to that of the original ancestor, independent of the activity of the auxotroph parents (diploids 2 and 3).

Aside from the great number of cases where the wild allele of penicillin production is dominant over the active mutant allele, there are some cases where heterozygote diploids are superior in their activity to the initial prototroph ancestors. For example, ELANDER (1966) obtained a diploid between two biochemical mutants of the highly active strain E-15, surpassing the

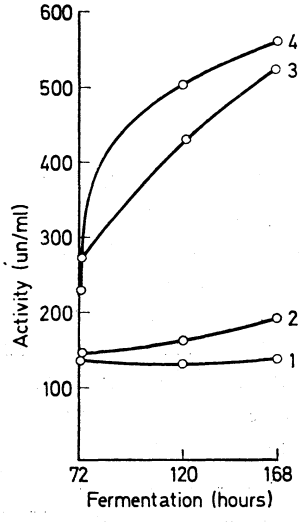
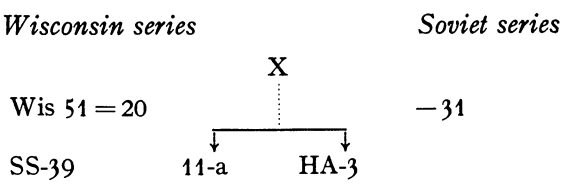


Fig. 12. Penicillin formation by *P. chrysogenum* E-15 strain (*1*), its two biochemical mutants (*2* and *3*) and the diploid obtained from them (*4*)

latter in penicillin production capacity (Fig. 12). SERMONTI also managed to obtain a diploid greatly exceeding both initial strains in a cross between strains Wis 50 and Wis 49 of the Wisconsin series. It is probable that, besides loci directly responsible for penicillin production, a number of other genes having no direct relation to antibiotic synthesis may govern the capacity for antibiotic production.

SERMONTI (1957) has made the assumption that the activity of diploids may be influenced by the genealogical relationships of their initial strains, i.e. more distant and consequently more heterozygotic strains may yield more active diploids than closely related strains. Studies of a large number of diploids between biochemical mutants of six *P. chrysogenum* strains of various genealogical relationships permitted KAMENEVA (1960, 1961) to reach the conclusion that the genealogical relationships of the initial strains did not influence the productivity of diploids. As participants in her crosses she used two strains obtained in our laboratory: "New Hybrid" and a strain obtained from it, as well as a descendant of the New-Hybrid strain, a strain of the Wisconsin series (Wis 51-20), and strain SS-39 of unknown origin (see diagram below).



The crosses were carried out in the following order:

1. H × HA-3,
2. HA-3 × 11 a,
3. HA-3 × Wis 51-20,
4. 11 a × Wis 51-20,
5. H × SS-39,
6. 11 a × SS-39.

The crosses thus differed from each other in genealogical divergence between initial strains. All the hybrid dioloids obtained were considerably superior to their parents (biochemical mutants) in penicillin production but did not exceed that of the prototroph ancestors (Table 13). As it evident from Table 13, the degree of genealogical divergence of the strain used to obtain diploids does not contribute to an increase in their activity. It is difficult to see any definite regularity in the inheritance of the capability to synthesize penicillin. In combinations where the initial prototroph strains did not greatly differ from each other in their activity, the diploids had an activity either lower than that of the least active ancestor (DIV, DVII, DXIII, DXIX), or intermediate (DVIII), or close to that of the more active ancestor (DV, DVI). In combinations where the prototroph ancestors greatly differed in their activity (3, 4), half the diploids (DIX, DXI) had an activity close to that of the more active ancestor, while the other half (DX, DXII) were close to that of the less active parent.

It is probable that the activity of diploids is influenced by the activity of their auxotroph parents. For example, in cross combination 4, the same mutant of strain Wis 51-20 (mutant No. 5), somewhat exceeding its parent in activity, was crossed with 3 mutants of strain 11 a, differing from each other in their activity. The most active diploid was obtained in the case where the most active mutant (No. 20) of strain 11 a was used (Table 13). Rather active biochemical mutants were also used in cross combination 3, where a diploid with an activity close to that of the more active parent was obtained.

The assumption that the activity of auxotroph parents may influence that of diploids obtained from them seems to be well founded, but in some cases no such regularity can be observed (see Table 12). The majority of biochemical mutants obtained from the active strains P have a lower penicillin production capacity. Such a phenomenon, as was shown by MACDONALD et al., is due to the pleiotropic effect of the biochemical deficiency mutation. Probably, this pleiotropic effect also occurs in cases where the mutation to auxotrophy is present in the heterozygote state, i.e. is carried by the diploid.

Table 13. *Penicillin production by strains of P. chrysogenum having different genetic relationships* (KAMENEVA, 1961)

Number of cross combination	Prototrophic ancestors		Auxotroph parents		Diploids		
	strain	activity u/ml	strain	activity u/ml	strain u/ml	activity	
						% in relation to the activity of the more active ancestor	
						auxotroph	prototroph
	HA-3	2382	534 2116	1035 371	DIV 1997	193	80
	H	2441	66 2035	1140 779	DV 2426	213	99
			66 48	1140 1156	DVI 2685	232	109
			66 2123	1140 371	DVII 1961	172	80
2	HA-2 11[a]	2382 2898	66 16	1140 655	DVIII 2538	222	87
6	HA-3 Wis 51-20	2382 1287	534 5	1035 1644	DIX 2030	123	85
4	11[a] Wis 51-20	2898 1287	16 5	655 1644	DX 1419	86	48
			20 5	1656 1644	DXI 2175	131	75
			113 5	967 1644	DXII 1196	72	41
6	SS-39 H	2609 2441	148 599	445 493	DXIII 1625	329	62
6	11[a] SS-39	2609 2441	16 70	655 789	DXIV 2010	254	69

Taking into consideration that positive mutations of the capacity for penicillin production, like negative ones, are recessive and that the pleiotropic effects of biochemical mutations express themselves in the heterozygote state too, it is predictable that it is hardly possible to obtain heterosis in penicillin production in diploids. However, in some cases, as, for example, in the case of the active diploids obtained by SERMONTI and ELANDER, heterosis is still possible due to concealment of "harmful" alleles.

Besides studying the capacity for penicillin production in diploids, SERMONTI also studied the activity of haploid recombinants, obtained as a result of mitotic segregation of prototroph diploid AL 206 pr Wis 50-1247, synthesizing 130 units of penicillin per ml. The parental strains of this diploid synthesized 3058 un/ml (AL 206 pr) and 1300 un/ml (Vis-50-1247). Of 50 auxtrophic haploid recombinants, 48 out of 49 requiring proline were intermediate in their penicillin production capacity between strain AL 206 pr and the diploid. A recombinant requiring nicotinamide did not differ in its activity from strain

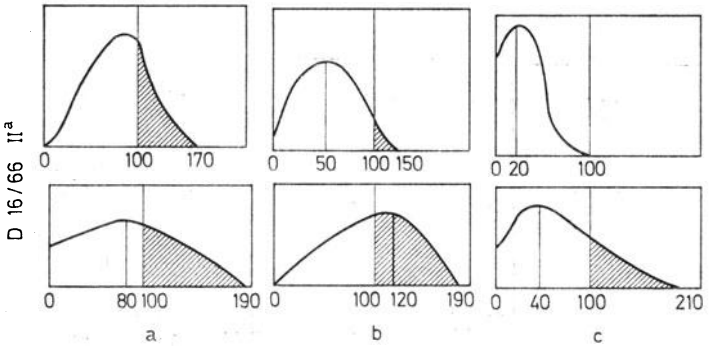


Fig. 13a—c. Variation in penicillin synthesis of haploid (11[a]) and diploid (D 16/66) strains *P. chrysogenum*. a Natural variability; b variability induced by 2000 erg/mm² UV; c) variability induced by 4000 erg/mm² UV

Wis-50-1247. Besides auxotroph recombinants, about 150 prototroph recombinants were isolated from the same plate culture of diploid conidia; these recombinants did not differ in their activity from strain AL 206. Most of them were haploids.

ELANDER (1966) has studied the capcity for penicillin synthesis in a number of recombinants selected from the previously mentioned diploids of strain E-15. The productivity of all recombinants was lower than that of E-15. Some of the diploid recombinants had an activity even lower than that of KAMENEVA's initial diploid. KAMENEVA (1960, 1961) studied the activity of 24 recombinants in different crosses (Table 13). She also was unable to find variants more active than their initial prototroph strains.

Diploid strains may also be selected by the use of mutagenic agents. Thus KAMENEVA (1961) studied variation in penicillin production in active strain 11a of *P. chrysogenum* and in the diploid obtained as a result of long-term selection and with the use of mutagenic agents. The diploid strains did not differ greatly from each other in regard to their penicillin production. The picture, however, changed greatly after their treatment with UV-light. As shown in Fig. 13, diploid cultures treated with mutagenic agents show a greater amplitude of variation in penicillin production than haploid ones.

Interesting results confirming the above conclusion were obtained by ELANDER (1966) in the selection of the most active diploid of strain E-15. After treating conidia of this diploid with a mutagenic agent, two variants

with increased activity, Nos. 2 and 3, were selected. One of these, No. 2, synthesized 25 % more penicillin than the diploid E-15 and 35 % more than its haploid ancestor E-15 (Fig. 14).

Attempts to use hybridization for the purpose of selection were made by FANTINI (1961), who obtained heterokaryons, diploids and recombinants in *E. mericellopsis salmosynnemata*, a microorganism producing the antibiotic synnematin. On the basis of the rather scanty data obtained by him one may

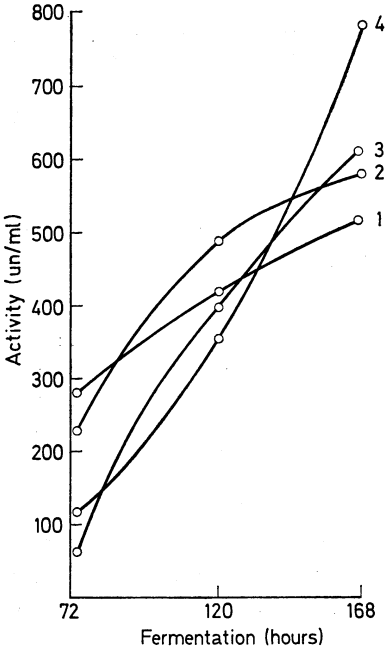


Fig. 14. Penicillin synthesis by *P. chrysogenum* E-15 strain (*1*), its diploid (*2*), and two variants (*3*, *4*) selected from the diploid

conclude that the regularities of the inheritance of the capacity for antibiotic production in *Emericellopsis* probably do not differ substantially from those observed in penicillin. The capacity for antibiotic production in diploids and somatic recombinants of *E. salmosynnemata* (FANTINI, 1961) was as follows:

Strain	Activity un/ml	Strain	Activity un/ml
Initial prototroph	625	Recombinant:	
Auxotroph parents	75	1.	0
Diploid 1	800	2.	30
Diploid 2	400	3.	400
		4.	400
		5.	0

More detailed results on the use of hybridization for purposes of selection were obtained in *Aspergillus sojae*, *A. niger*, *A. orysae* and *A. fonsecaens*, the fungi producing organic acids and enzymes (IKEDA, NAKAMURA, UCHIDA, ISHITANI, 1957). CIEGLER and ROPER (1957) obtained heterokaryons between active and inactive strains of *A. fonsecaens*, a producer of citric and glucoronic acids. Their activity was intermediate between the activities of the parental strains. Somewhat different results were obtained by IKEDA et al. (1957) (Table 14). On the basis of their results, the authors have come to the con-

Table 14. *Proteolytic activity of polyploid hybrids of A. oryzal* (IKEDA et al., 1959)

	Strain	Activity (colorimetric units) un/ml
Prototroph ancestor	51	25.3
Auxotroph parents	23...	22.7
	22...	33.1
Diploid	7...	45.7
	37...	33.0
	166...	45.1
Triploid	3...	25.1
Tetraploid	1...	50.9

clusion that high activity in citric acid synthesis in aspergilli is recessive to low activity. They explained the high activity of heterocaryons as being due to the fact that in the latter they had obtained a selective advantage for the nucleus of the highly active strain; this, in their opinion, appeared to be the cause of the high activity.

IKEDA et al. (1957) also studied the proteolytic activity of heterokaryons and diploids of *A. oryzae*. The heterokaryons obtained between two strains with low activity showed a certain degree of heterosis in their capacity to synthesize protease: during fermentation they began to produce the enzyme earlier than the parental strains and retained their activity for a longer time. Diploids, derived from a cross between poorly and highly active strains, exceeded in their activity both components of the cross. Some of the diploids obtained by IKEDA et al. also exceeded their auxotroph parents and prototroph ancestor in the activity of protease synthesis. Later on a number of triploid and tetraploid cultures were obtained from these diploids. Some tetraploids and diploids showed high activity in enzyme synthesis. Although they exceeded the activity of the poorly active parent, one of them reached the activity of the active parent (Table 14). From these poorly active diploids some recombinants varying greatly in their activity were obtained; some showed high activity, one of them synthesizing as much as 6000 mg of citric acid per ml. However, while studying the inheritance of proteolytic activity in diploids of another *Aspergillus* species, *A. sojae*, ODA and IGUCHI (1963)

obtained results differring from those described above. The heterokaryons and diploids obtained by them from biochemical mutants of two strains of *A. sojae*, KS and X816, with different activities (strain X816 was twice as active as strain KS) displayed activity of protease synthesis intermediate between the activities of their parents (Fig. 15). These heterokaryons and diploids were studied for their capability to synthesize another enzyme, amylase; here, too, the diploids showed an intermediate activity. Recombinants, obtained as a result of mitotic segregation of a diploid, were divided on the basis of protease activity into 3 groups: one did not differ in activity from the diploid, the two others did not differ from their two parents. Thus, the

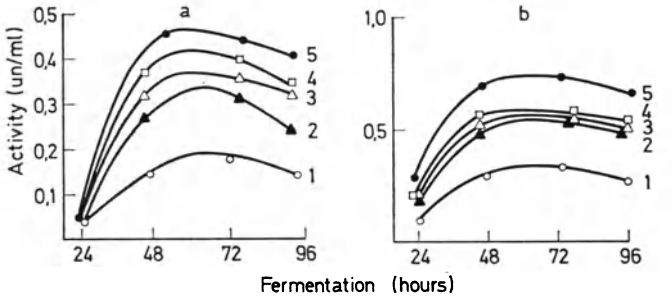


Fig. 15a and b. Synthesis of protease (a) and of amylase (b) by *A. sojae* strains K (*1*) and X816 (*5*), heterokaryons (*2*) and by two heterozygote diploids (*3*, *4*), derived from them

authors did not manage to obtain heterosis in enzyme production either in diploids or in recombinants of *A. sojae*.

Judging by the experimental data just presented, one might conclude that the pattern of inheritance of proteolytic activity *A. oryzae* and *A. sojae* is different, however, such a conclusion may be premature.

Paskova and Munk (1962) studied the synthesis of the enzyme glucooxydase in variants, isolated as a result of segregation of a heterokaryon, obtained between two strains of *A. niger*: R_{10} and N77. Judging by the data of these authors, heterosis occurs in regard to the capacity to synthesize glucooxydase and therefore heterokaryons may be useful in industrial fermentation.

The data presented here sugest that the pattern of inheritance of the capability to synthesize organic acids in *Aspergilli*, probably does not differ substantially from that observed in *Penicillia* with respect to penicillin synthesis. At the same time, however, the inheritance of high activity of enzyme synthesis probably follows some other pattern.

Finally, it is necessary to note that even if crosses of fungi seem at present to be of little use with respect to immediate practical results with hybrids, such experiments enrich our knowledge of the mechanism of biosynthesis of various metabolites and, what is still more important, contribute to our understanding of the nature of the genetic changes which take place in the process of selection.

ALIKHANIAN, MINDLIN, VLADIMIROV and BORISOVA (1957, 1959, 1960, 1961, 1962, 1966, 1967) as well as JARAI (1961) have reported on the hybridization of industrial strains of *Actinomycetes*. In contrast to *Penicillia* and *Aspergilli*, where one can obtain diploids and thereby possible heterosis, the main effects of hybridization among *Actinomycetes*, resemble those obtainable with eubacteria. Genetic recombination in *Actinomycetes* cannot be regarded as fully

Table 15. *Comparative antibiotic activity (in mg/ml) of initial strains, biochemical mutants and prototroph recombinants of Actinomyces rimosus* (ALIKHANIAN and MINDLIN, 1957)

Initial strain		Biochemical mutants		Prototroph recombinants		
				cross combination		
designation	activity	designation	activity	initial strains	biochemical mutants	activity[a]
Cross of active strains						
8229	1429	1361 thr	262			I 705
					310 × 870	II 1622
		311 ilv	97	8229 × 101 (2)		III 1555
101(a)	1234	870 his	28			I 663
						II 281
					1361 × 870	III 1269
Cross of highly active strains						
LS-T293	2650	1 ilv	7	LS-T293		I 2025
					1 × 49	II 2925
		49	115	X		III 1700
BS-21	1900	24 arg	0	BS-21	1 × 24	I 1500
						II 630
						III 1800

[a] Maximum of activities on two media.

equivalent to hybridization in higher organisms, whereas in fungi one can obtain diploid effects resembling those in higher organisms.

ALIKHANIAN and MINDLIN (1957) in their very first study on antibiotic activity of *Act. rimosus* recombinants, found that recombinants with different antibiotic activity occur in one and the same cross between biochemical mutants (Table 15). The authors crossed biochemical mutants of two *Act. rimosus* strains, differing insignificantly in their antibiotic activity. These were strains *Act. rimosus* 101 (2a) and 8229. Each cross yielded prototrophs of three types, characterized by different colonial features (the morphology and colour of colonies) and each morphological type and its own level of antibiotic activity. While all the prototrophs exceeded the biochemical mutants in their activity, only some of them reached the level of the initial strains, or exceeded

them in activity. (As to the total level of the activity, prototrophs obtained from two crosses combinations differed greatly from each other). A similar picture was observed in crosses of other strains of *Act. rimosus*, as well as in crosses of other *Actinomycetes* species, such as *Act. aureofaciens*, carried out by ALIKHANIAN and BORISOVA (1961) and BORISOVA (1962) (Fig. 16 and Table 16) and *Act. streptomycini* (Table 17), carried out by BORISOVA (1966).

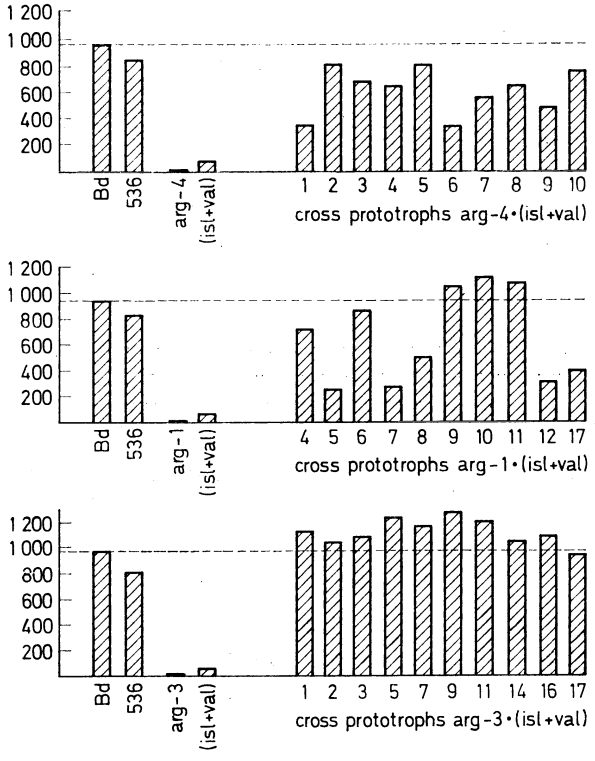


Fig. 16. Antibiotic production by strains of *Actinomyces aureofaciens*. The activity levels are given in absolute values. The maximal activity level is found with wild-type strains (ALIKHANIAN and BORISOVA, 1961)

On examining all these data, one cannot but notice the great variability and activity of the recombinants both when comparing recombinants from one and the same cross and the results of different crosses. A comparative analysis of antibiotic activity in genetic recombinations enables us to make some statements, applicable to different species of *Actinomycetes* (including *Act. antibioticus*, for which the available data are much more limited). These are:

1. Within identical crosses, prototroph recombinants vary greatly in their activity.

2. Considerable differences in the antibiotic activity level are also observed in different recombinants of one and the same cross.

Table 16. *Comparative antibiotic activity (mg/ml) of initial strains, biochemical mutants and prototroph recombinants of Act. aureofaciens* (BORISOVA et al., 1962)

Initial strain		Biochemical mutants		Prototroph recombinants		
designation	activity	designation	activity	cross combination		activity
				initial strains	biochemical mutants	
2201	3000	5, 8, 10, 13	50—70	2201 × 2201	1 × 13	600—3300
		26 arg			1 × 8	100—2700
			250—370		5 × 14	560—3500
		1 thr	600—1000		10 × 14	250—2500
		14 ala			26 × 14	400—2000
536	900	6 ilv	traces	2201 × 536	1 × 6	1000—2700
	900	4 his	traces	2201 ×	14 × 4	180— 880
					1 × 4	400—2500

Table 17. *Comparative antiobiotic activity (u/ml) of initial strains and biochemical mutants of Act. streptomycini* (BORISOVA, 1966)

Initial strain		Biochemical mutants	
designation	activity, in % of activity C-1	designation	activity, in % of activity C-1
3	10	1 try	0
		2 his	0
178	5	2—4 try	0
120	100	4 his	0
			20
		11 ilv-leu	42
78	100	3 B_6	0
32—13	150	1	0
		2 ilv-leu	34
		4 thr	36

3. All possible activities of initial strains and prototroph recombinants are observed in different crosses (as well as within one and the same cross). With respect to their activity, the prototroph recombinants may be: a) inferior to both initial strains; b) intermediate between the two strains; c) equal in their activity to the more active strain; d) superior to the more active strain.

However, the factors described do not answer the question: what determines the antibiotic activity level of recombinants in different crosses, and why do the recombinants with different genotypes from one and the same cross differ in their activity? In other words, what are the characteristics of

inheritance of the antibiotic production capacity of *Actinomycetes*? The overwhelming majority of facts accumulated by now points to the absence of any connection between the activity of biochemical mutations used for crossing, and the activity of prototroph recombinants.

An analysis of two different crosses (2201 × 6 ilv and 2201 × 4 his) of *Act. aureofaciens* with two combinations of biochemical mutants studied in each of them, yielded equal results: a higher level of activity is obtained when the less active of the two biochemical mutants of strain 2201 (1 thr) is used. The same mutant of the second strain 4 his (Table 16) was used in the two combinations of each cross. No connection between the two values can be traced in *Act. streptomycini* either, though the analysis in this case is difficult because of the absence of absolute data (Table 17).

In 1967 VLADIMIROV and MINDLIN carried out a special study of this problem using a large quantity of material. They studied 19 crosses between biochemical mutants of two *Act. rimosus* strains (LS-T293 and BS-21). Two groups of biochemical mutants of each strain differing sharply in their activity were used. Mutants with an activity of less than 1 % were placed in the group with low activity while a highly active group consisted of mutants with an activity ranging from 50 to 100% of that of the initial strain (the activity of one mutant was about 36% of that of the initial strain). That there was no connection between the level of activity of recombinants and the participants of the cross, i.e. biochemical mutants, was quite evident on the basis of an analysis of all possible variants of crosses (VLADIMIROV and MINDLIN, 1967) (see scheme p. 130). It is interesting that the most active recombinants are obtained in combinations derived from poorly active mutants of each strain, while the least active ones are obtained in crosses between poorly active mutants of the more active initial strain and highly active mutants of the other strain. Thus, one can say with certainty that it is not the activities of the markers (biochemical mutants) but some other factors, that are of decisive importance in the crossing of industrial strains.

Is there any relationship between the activity of initial strains and the activity of prototroph recombinants obtained as a result of crossing the initial strains?

There is no doubt that such a relationship must exist and it can be easily detected by comparing the activities of recombinants obtained in crosses between poorly active and highly active strains of the same species. Thus, a cross between the poorly active strains 101 (2a) and 8229 of *Act. rimosus* yields recombinants much less active than those obtained after crossing the highly active strains LS-T293 and BS-21 of the same *Actinomycete* (compare Table 15). Although the recombinants obtained in a cross between poorly active strains 536 and of *Act. aureofaciens* are somewhat more active than the initial strains, nevertheless activity as a whole remains at the same level. But at the same time the most active of the recombinants between biochemical mutants of the highly active strain 2201 are characterized by the activity of this highly-active strain (compare Table 16 and Fig. 16). It is more difficult

to predict the results of crosses between two strains having different activity levels. Since, as was shown above, a certain amplitude of recombinant variation is observed in each cross the question should probably be put as follows: is there any difference between the curves of variation in activity obtained in crosses of the same strain with others having different activity levels. There is almost no information on this problem. However, some reports, such as the one by BORISOVA (1966), present some data on the activity of more

Table 18. *Activity of Act. streptomycini prototroph recombinants in relation to the activity of the initial strain*[a] (BORISOVA, 1966)

Cross combinations designation of strains are given in large brackets)	Total number of prototrophs checked	Active prototrophs[b]					
		0		0 to 100		more than 100	
		number	%	number	%	number	%
1 [1005, 120] × [78, LS-1, 120]	83	5	6	78	94.0	—	—
2 [LS-1, 120, 1005] × [3]	78	20	25.6	56	71.9	2	2.5
3 32-13 × 32-13	317	2	0.6	306	96.6	9	2.8
4 32-13 × [1005, 120, 78]	364	11	3.0	352	96.7	1	0.3
5 32-13 × [3, 178]	285	181	63.5	100	35.0	4	1.4
Total number of prototrophs	1127	219	19.4	892	79.2	16	1.4

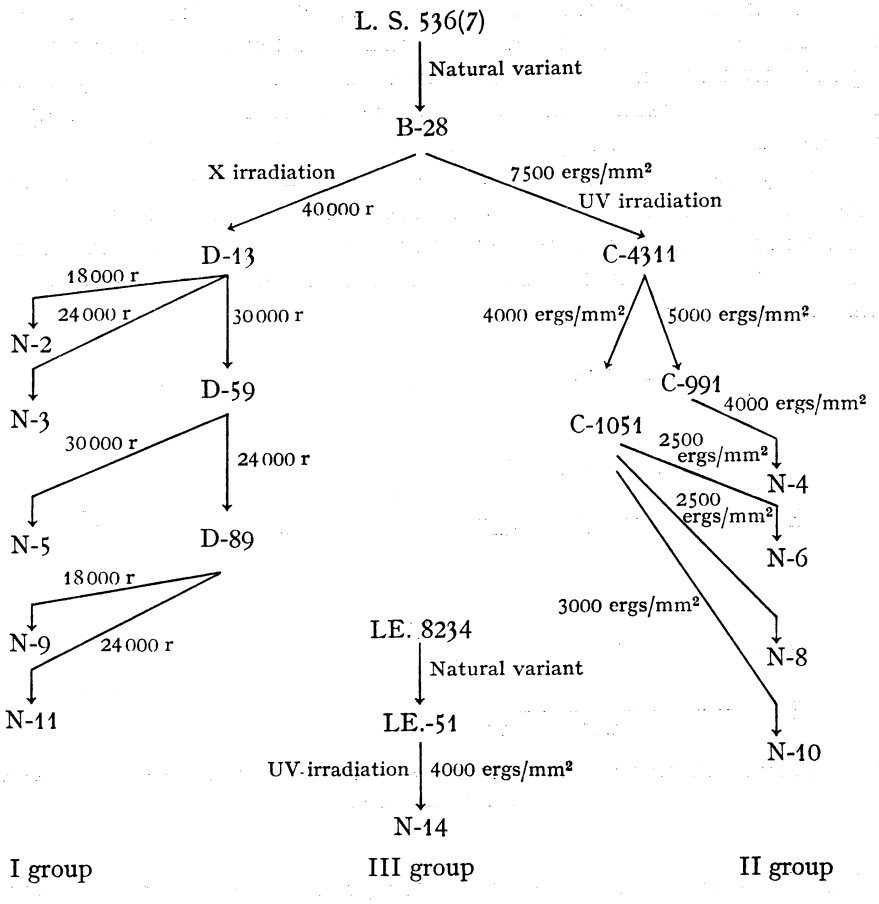
[a] The initial strains are divided into 3 groups (onn the basis of activity, calculated as percent of activity LS-1: 1. 3 and 178-5-100%; 2. LS-1, 120, 1005 and 78-100%; 3. 32-13-150%.

[b] In crosses 1 and 2 recombinant activity is calculated as percent of activity LS-1; in crosses 3—5 as percent of activity 32—13.

than 1100 recombinants from crosses of different strains of *Act. streptomycini*. The results are grouped in Table 18. Depending on the activity levels of the initial strains, these data show a tendency to a regular decrease in antibiotic activity of recombinants in crosses in which poorly-active strains take part.

Whatever the peculiarities of genetic recombination of microorganisms as compared to higher organisms, its function is the same in both cases and consists in obtaining organisms that combine the valuable features of their parent forms or have new and valuable properties, absent in their parents. On considering the crosses of industrial strains from that point of view, one

Table. 19. *Activity of prototroph recombinants and degree of relationship to initial strain of Act. aureofacient* (JARAI, 1961)



Related crosses: I×I and II×II
Non-related crosses: I×II, I×III and II×III

Cross	Total number of prototroph recombinants	Highly-active prototroph recombinants[a]	
		number	%
Non-related	132	26	19.7
Related	146	6	4.1
Total	278	32	11.5

[a] Increase of initial strain activity by 40—50%.

can formulate as one of the principal tasks the breeding of strains combining in their genotypes the capability of the two initial strains for antibiotic production.

The relationships between strains are probably of decisive importance. In cases of closely related parental strains, having consequently similar genotypes controlling antibiotic production, no significant effect of crossing can be expected. On the other hand, crossing of strains of different origin and selected independently from one another in different laboratories will greatly increase the chances of obtaining highly active recombinants. Unfortunately the experimental data on this question are scarce. The most interesting results, obtained by JARAI (1961) in *Act. aureofaciens* are presented in Table 19. Studies of 278 recombinants show a greater effectiveness of crossing non-related strains compared to related strains. According to the author's data, up to 20% of recombinants from non-related crosses exceed the activity of the initial strains 1.5 times.

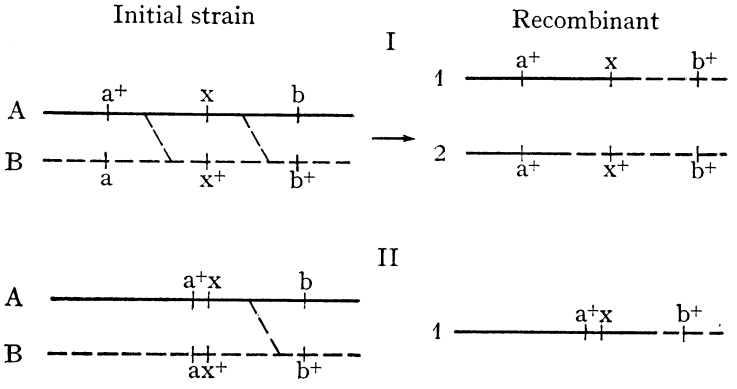


Fig. 17. Diagram illustrating the significance of the location of selected markers in industrial crosses

Some data on the effectiveness of using non-related strains for hybridization are also contained in reports concerning *Act. rimosus*. The maximum effect was obtained by MINDLIN et al. (1961) in crosses of strains LS-T293 (Soviet) and BS-21 (Hungarian). As a result of selection among a small number of genetic recombinants obtained in three combinations of crossing biochemical mutants of these strains, a strain, LS-T Hybrid, was obtained which not only exceeded in its activity the parent strain, LS-T118, but differed from it advantageously by causing less intense foaming of the fermentation medium. This property of LS-T Hybrid contributed to the use of concentrated media. The activity of strain LS-T Hybrid in such media reached 5000 mg/ml. VLADIMIROV and MINDLIN (1967) have shown that reverse crosses of LS-T Hybrid with one of the parent strains (BS-21) produce no effect. The data just presented are, in essence, the only information on the comparative effectiveness of hybridization depending on the degree of relationship between the parents. No data on the combination of other valuable properties (besides activity) with the help of hybridization of *Actinomycetes* are found in the literature.

As was repeatedly mentioned above, the choice of biochemical mutants of the participant strains is of extreme importance for hybridization. Since biochemical deficiency markers act as antiselective markers in the course of selection of prototroph recombinants (their normal alleles being selected), the question arises whether the localization of these markers on the chromosome of the initial strains is of importance. It is important to ask this question because the localization of selected markers determines the frequency of transmission of nonselected ones, including factors controlling antibiotic production. It is evident that in the case of a tight linkage of the latter with biochemical deficiency markers the probability of their transmission to the recombinants will be insignificant, since the recombinants will inherit the wild-type allele of the marker from the other parent. On the other hand, where there is a weak linkage of selected markers with the factors of antibiotic production, the frequency of their transmission to the recombinants will be high. Both possibilities are shown in Fig. 17. In case I, where the selected and non-selected markers are not linked, recombinants of two types carrying the non-selected factors of both parents (x and x^+) appear in the posterity. In case II, where the selected and non-selected markers are tightly linked, the majority of recombinants will inherit the non-selected marker (x) from only one part (A). The consequences of such a situation are very important when strains differing greatly in their activity participate in the cross, and it is the factors of antibiotic production which are the tightly linked non-selected markers.

The propositions just presented were assumed as a basis for the analysis of the inheritance of the capacity for antibiotic production in *A. rimosus* carried out by MINDLIN et al. (1962). The initial strains, participating in the cross, namely LS-T Hybrid and BS-21, differed not only in their activity (the activity of BS-21 was about 50% that of LS-T Hybrid) but also in their sensitivity to streptomycin. Strain BS-21 was streptomycin-resistant (str), while

Table 20. *Frequency of streptomycin-resistant and streptomycin-sensitive prototrophs in different crosses of Act. rimosus* (VLADIMIROV and MINDLIN, 1967)

Cross combination LS-T Hybrid × BS-21	All	str^+		str^-	
		number	%	number	%
F-36 × BS-231	300	230	76.7 ± 24	70	23.3 ± 24
F-91 × BS-231	565	560	99.2 ± 0.3	5	0.8 ± 0.3
F-43 × BS-231	168	160	95.3 ± 1.7	8	4.7 ± 1.7
F-36 × BS-240	651	607	93.3 ± 0.9	44	6.7 ± 0.9

strain LS-T Hybrid was streptomycin-sensitive (str^+). Hence recombinants of both types appeared in the posterity. However, the ratio between str and str^+ recombinants in different combinations of biochemical mutants of the two strains varied greatly (Table 20) and there was a definite connection

Scheme

Cross type	Recombinant activities (% of activity of LS-T Hybrid)
Active LS-T Hybrid × active BS-21	72.9—85.4
Active LS-T Hybrid × inactive BS-21	77.9—88.9
Inactive LS-T Hybrid × active BS-21	41.0—81.2
Inactive LS-T Hybrid × inactive BS-21	64.7—96.6

between the number of str$^+$ recombinants and the average activity of recombinants from the cross. The more str$^+$ recombinants appeared in a cross the more frequent were highly active recombinants in this cross, and *vice versa*. In this respect combination F-36 × BS-21 was the most effective of the four studied.

A study of the activity of a great number of str$^+$ and str recombinants of four cross combinations (Table 21) confirmed that both features are linked: str$^+$ recombinants are more active than str recombinants, in correspondence with the combination of these features of their parents (LS-T Hybrid — highly active, sensitive to streptomycin; BS-21 less active, resistant to streptomycin). A similar finding was made in the genetic analysis of inactive mutants. To explain the difference in the correlation of str and str$^+$ recombinants in different cross combinations, it was assumed that there is a different linkage of biochemical deficiency factors with the factor of streptomycin-sensitivity in the highly active LS-T strain.

Table 21. *Average antibiotic activity of streptomycin-resistant and streptomycin-sensitive recombinants in different crosses of Act. rimosus* (VLADIMIROV and MINDLIN, 1967)

Cross combination Hybrid × BS-21	Number of recombinants studied		Average antibiotic activity, as % of activity of C-T Hybrid × BS-21		
	str$^+$	str	initial strain BS-21	recombinants	
				str$^+$	str
F-36 × BS-231	47	48	23.7	86.8	53.1
F-91 × BS-231	5	43	52.7	91.0	51.3
F-43 × BS-231	8	10	25.5	90.8	72.5
F-30 × BS-240	50	50	45.3	81.4	68.1

In analogy with the cases illustrated in Fig. 17, it was confirmed that the biochemical deficiency factor of mutant F-36, being unselected, is linked slightly with the factor of streptomycin sensitivity (case I), i.e. white prototrophs sensitive to streptomycin appear alongside prototroph recombinants resistant to streptomycine. Contrary to that, the linkage of other mutants

(F-91, F-43, F-36) is rather high (case II) and recombinants in most cases inherit the factor of streptomycin resistance and also the antibiotic-producing factor of the less active strain BS-21 (Table 20). The different localization of auxotroph factors of mutants F-36 and F-91 was confirmed by special tests. Thus it is evident that nowadays the planned selection of parent forms for crosses is not possible without a genetic linkage map of industrial producers.

Many questions concerning industrial hybridization, both specific ones and general ones, are not cleared up as yet. One particular question concerns the reasons for the considerable variety of recombinants in closely related inter-strain crosses. One would think that on crossing two biochemical mutants of the same strain, the recombinant genotype, of haploid origin should not differ greatly from the genotype of the initial strain. Nevertheless, a wide range of differences in antibiotic activity is found among recombinant individuals. A more general question concerning the prospects for hybridization of actinomycetes and fungi can be formulated in the following way: what opportunities for hybridization does the characteristic offer in the way of production of new forms possessing valuable features and suitable for practical use? In view of lack of published articles, the answer can be only preliminary. Probably, even nowadays, hybridization can be considered as one of the methods of selecting for antibiotic production, giving favorable effects with a large variety of producer strain.

Another aspect of the application of hybridization to the selection of microorganisms concerns the use of recombinant forms with characteristics of the initial material for the selection of induced mutants.

Practical Use of Auxotrophic Mutants

The use of mutations to auxotrophy for the isolation of highly productive microorganisms is the newest technique of microbial selection. In spite of the novelty of this method great success has been achieved in this way, outstripping the success of many years of selection of antibiotic producing microorganisms by the use of mutagenic factors. The most effective results have been obtained in the selection of strains producing amino acids and nucleotides.

The molecules of secondary metabolites are more complex than those of primary metabolites. The mechanisms of biosynthesis of many of them have been studied only in general, and at present there are very few data about enzyme systems controlling individual biochemical reactions on the pathway of synthesis of these substances. On the contrary, the main pathways of biosynthesis of such primary metabolites as amino acids, nucleotides and to some extent vitamins have been studied in microorganisms rather thoroughly. It is known that the concentration of primary metabolites in the cell is under the strict control of the well-studied mechanisms of repression and feed-back inhibition, which under normal conditions prevent the primary metabolites from accumulating in the cell in large quantities. Secondary metabolites, on the

other hand, are often formed in large amounts; the investigation of the mechanism which regulates their biosynthesis is only in the very early stages.

The vast amount of information on the pathways of biosynthesis of amino acids and nucleotides and on the mechanisms controlling their rate of synthesis, as well as on the application of genetic methods for blocking different links in this process, confirms the specificity of the methods for selection of microorganisms producing corresponding primary metabolites. These methods are based on the use of auxotroph mutants and mutants with an impaired system of regulation.

We have already indicated that a characteristic peculiarity of auxotroph mutants is their capability to accumulate intermediate products of biosynthesis preceding a particular genetically blocked reaction, the intermediate products of biosynthesis being able to undergo further biochemical transformations. On the other hand, the biosynthetic pathways of different metabolites are closely connected with each other, a fact which means that the blocking of one chemical reaction very often leads to a change in the speed of other reactions that are connected with the first one. Thus, one biochemical mutation may cause many different changes in the process of metabolite biosynthesis. Nevertheless the use of auxotroph mutants of microorganisms has proved to be an extremely valuable method in the selection of microorganisms producing certain metabolites. As mentioned already, two main mechanisms regulating the biosynthesis of primary metabolites are known: repression and feed-back inhibition. In the case of repression, the accumulation of the final product of biosynthesis in an excessive quantity stops the synthesis of enzymes participating in the corresponding biosynthetic pathway. In the case of feed-back inhibition, the accumulating final biosynthetic product inhibits the activity of an enzyme acting at the beginning of the biosynthetic pathway; as a result of this the subsequent biochemical transformations are also halted. The presence of the two regulatory mechanisms provides the possibility of fast and effective adaptation of microorganisms to changing environmental conditions. The presence of the final product in the medium immediately stops its further synthesis by the organism in question as a result of the action of feed-back inhibition. Subsequently the mechanism of repression comes into effect and arrests the synthesis of new molecules of enzyme proteins. Conversely, in conditions of a deficiency of the given metabolite, both mechanisms stop acting, and derepression takes place, leading to intense synthesis of the deficient product. However, one can obtain mutations which disturb these normal regulation mechanisms. Mutants, characterized by hereditary disturbances of the regulatory mechanisms differ from their parent strains by being able to synthesize excessive amounts of a given final product of biosynthesis. Such derepressed mutants can be used in the selection of microorganisms producing primary metabolites. The specific methods for obtaining them are based on the use of antimetabolites, i.e. substances similar in their chemical structure to the natural metabolites but incapable of substituting for them as components

of macromolecules. The antimetabolites, like natural metabolites, produce the effects of repression or feed-back inhibition and these form the basis of their bacteriostatic (or bactericidal) action. In the presence of an antimetabolite (an analogue of the normal biosynthetic product) in the medium the growth of the initial microbial culture is inhibited and only colonies of mutants resistant to the antimetabolite grow up. Some of these synthesize the final product (natural metabolite) in excess because of derepression (or lack of feed-back inhibition) of the corresponding enzyme system. Thus, ADELBERG (1958) has shown that, as a result of selection by the use of a medium containing 5-methyltryptophan, one can obtain mutants synthesizing and excreting a large amount of tryptophan in the presence of ethionine.

The use of such specific methods in selecting microorganisms producing primary metabolites considerably reduces the duration of the selection process and makes it much less laborious. In practice, a selection process is in some cases reduced to obtaining highly productive single or double biochemical mutants, while in the selection of microorganisms producing secondary metabolites (e.g. antibiotics) the selectionist has to carry out a many-stage procedure using mutagenic factors. This does not mean that the stage-by-stage selection of induced mutations, as well as other methods of selection, such as the isolation of microbial strains from natural sources, cannot be used in the selection of microorganisms producing primary metabolites. They are indeed used but less frequently than specific methods of selection.

In 1956, KINOSHITA isolated a soil microorganism, named *Micrococcus glutamicus*. This microorganism was capable of synthesizing large amounts of glutamic acid. In the course of isolation of this microorganism, Petri dishes with colonies of soil microorganisms were irradiated with bactericidal doses of UV and then covered by an agar layer containing a test microbe requiring glutamic acid (UDAKA, 1960). After incubation, a zone of growth of the test microbe developed around the colonies that produced glutamic acid. Of 500 bacterial strains tested in such a way, about a dozen synthesized glutamic acid, including *M. glutamicus*.

Later on bacteria capable of synthesizing considerable amounts of glutamic acid were found by MIYAI et al. (1962) and SHIIO et al. (1963) among microorganism related to *M. glutamicus* and belonging to the genera *Brevibacterium*, *Mycobacterium* and *Corynebacterium*. All of them possessed some common properties, and in particular all of them were auxotrophic to biotin, and under favorable fermentation conditions produced about 25 to 30 mg of L-glutamic acid per ml. Another common feature of these microorganisms was their ability to give high yields of glutamic acid only under very specific fermentation conditions. Two factors were of special importance: the content of biotin in the medium, and the rate of aeration during fermentation. KINOSHITA (1961) and ASSAI (1963) have shown that an excess of biotin and a diminished aeration rate reduce the yield of glutamic acid several times, whereas other products of metabolism (α-ketoglutaric, lactic and succinic acids) accumulate in the fermentation medium.

In connection with the necessity for strict regulation of the process of glutamic acid biosynthesis in industrial processes, we set out to improve some properties of the producing microorganism isolated from nature. In addition to increasing total productivity, we attemped to select strains with a reduced demand for aeration and reduced sensitivity to biotin. We (ZHDANOVA, BALITSKAYA and ALIKHANIAN, 1967) used the method of stage-by-stage selection of variants induced by UV and by diethylsulfate. The high level of

Table 22. *Glutamic acid biosynthesis by Micrococcus glutamicus 13032 and its mutants. Comparative productivity on a medium with 25% of molasses* (ZHDANOVA, BALITSKAYA and ALIKHANIAN, 1967)

Strain	Productivity fluctuation mg/ml	Average output mg/ml	Activity %
13032 (initial)	25.0—44.5	32.7	100
1544	44.6—52.6	50.6	153.0
1439	27.0—51.0	40.8	125.0
1378	38.2—45.3	42.6	130.0
1423	30.0—33.2	31.8	96.5

Table 23. *Influence of aeration on biosynthesis of Micrococcus glutamicus* (ZHDANOVA, BALITSKAYA and ALIKHANIAN, 1967)

Strain	Glutamic acid output, volume of medium (ml)			
	10	20	30	50
13032 (initial)	33.0	33.0	11.7	4
1544	40.3	34.6	16.5	5
1439	28.5	32.9	38.7	27.8
1378	33.9	41.8	30.7	—
1423	36.4	43.0	28.8	—

variation induced by diethylsulfate allowed the selection, from *M. glutamicus* 13032, of mutants with improved properties. The productivity of one mutant reached 50 mg/ml, that is 1.5 times the productivity of the initial strain. Two other mutants also had a higher productivity (Table 22).

Some interesting peculiarities of these mutants were revealed in the course of studying their physiological properties. Among the mutants there were some that were either more or less sensitive to aeration as compared with the initial strain (Tables 22 and 23). The most productive mutant, 1544, produced the most of glutamic acid under conditions of most intense aeration (the volume of the medium in the flasks being 10 ml). All other mutants differed from the initial strain, 13032, by their lesser sensitivity to aeration. This feature

was especially pronounced in mutant 1439, which was characterized by high and stable yields of glutamic acid under conditions of both intense and moderate aeration (with 20 or 50 ml of medium in the flasks).

A great shift also took place in the mutants with respect to their sensitivity to biotin. While the optimal concentration of biotin in the medium was 2.5 mg per litre, the maximal yield of glutamic acid was observed in the mutants with a biotin content of 5—7 mg per litre (Fig. 18). Since biotin plays a primary role in the regulation of glutamic acid synthesis, the possibility of obtaining mutants with changed sensitivity to biotin is of prime significance for selecting strains for industrial production of this amino acid.

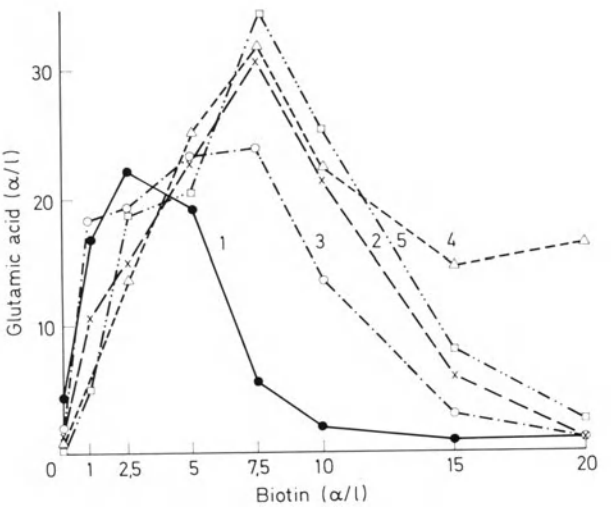


Fig. 18. Influence of different concentrations of biotin on glutamic acid synthesis of *M. glutamicus* culture 13032 and its mutants. 1-strain 13032; 2-strain 1378; 3-strain 1423; 4-strain 1439; 5-strain 1544 (ZHDANOVA, BALITSKAYA and ALIKHANIAN, 1967)

The discovery in nature of highly productive strains synthesizing glutamic acid is one of the cases where economically valuable forms of microorganism have been obtained as a result of natural selection. At the same time we cannot overlook the fact that all microorganisms producing glutamic acid are biotin-deficient auxotrophs. In this case natural conditions have selected types analogous to those that the selectionist sometimes obtains (e.g. in selection of microorganisms producing other amino acids). That is why the task of the selectionist who works with glutamic acid producers consists in further improvement of natural forms. As shown above, this task is being solved successfully by means of induced mutagenesis.

The ability to excrete free amino acids into the medium is widespread among representatives of various groups of microorganisms such as bacteria, actinomycetes and fungi. Microorganisms producing alanine and glutamic acid are frequently found whereas microorganisms producing other amino acids are less numerous. The discovery of "ready" natural glutamic acid producers with

a very high productivity motivated investigators to search for microbial strains producing large amounts of lysine, but nothing resembling the glutamic acid producers was found. KINOSHITA (1959) and NAKAYAMA et al. (1961) obtained auxotroph mutants of a number of bacterial species, including *E. coli* and *M. glutamicus* and observed that, as expected, some of these auxotroph mutants acquired a capability to release considerable amounts of free lysine into the medium; the best producers were mutants with a hereditary impairment in the pathway of aspartic acid transformation. As indicated in the scheme of amino acids of the aspartic acid group (Fig. 19), aspartic acid is the initial product for the biosynthesis of many amino acids, such as methionine, threonine, isoleucine, as well as lysine, the chain of biosynthesis being

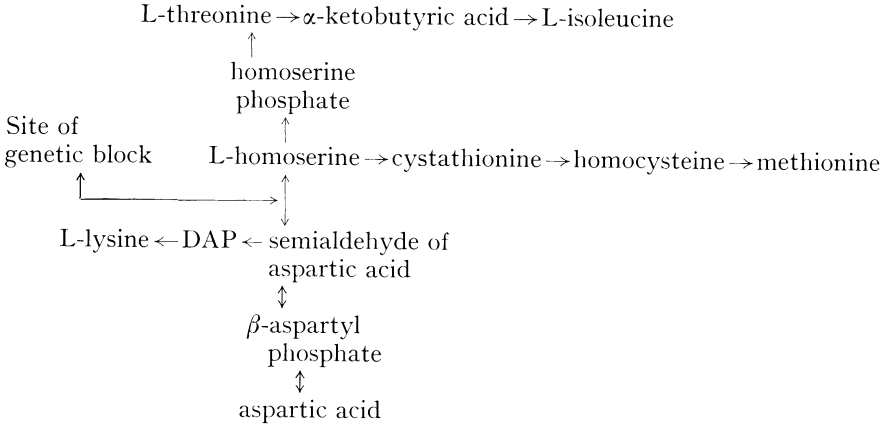


Fig. 19. Biosynthesis of amino acids of the aspartic acid family

branched. The brach ending with lysine formation deviates first, the two other branches lead respectively to the formation of methionine and threonine, as well as isoleucine. Consequently the accumulation of excessive amounts of lysine may take place in all cases where the genetic block involves any or all of the stages of methionine, threonine and isoleucine biosynthesis (Table 24). It is also evident that the maximal effect may be produced by a genetic block in the biosynthetic pathway at a stage preceding the formation of homoserine, the key amino acid for the synthesis of both methionine and threonine. And indeed, homoserine-deficient auxotroph mutants were characterized by having the highest productivity with respect to lysine.

Strain *M. glutamicus* 13032, which was the initial material for the isolation of homoserine-deficient mutants, was capable of accumulating glutamic acid in large amounts. With the help of various mutagenic agents (UV-light, ^{60}Co, fast neutrons, sarcolysine), some biochemical mutants were obtained from that strain and, from these, homoserine-deficient mutants were selected by the use of the penicillin method (KINOSHITA, 1961; LEGCHILINA, SHISHKINA, 1965). All these mutants synthesized about 20 mg/ml of L-lysine under favorable fermentation conditions. A simple calculation shows that in comparison with

the initial strain of *M. glutamicus*, which synthesizes about 0.1 mg/ml of L-lysine, the homoserine-deficient auxotrophs show a 200 times higher productivity. Such a steep increase in productivity as a result of only one mutation is one of the best achievements of microbial selection.

In the course of further selection work with the homoserine-deficient auxotroph *M. glutamicus*, both specific and non-specific selection methods were used. Such work was necessary because the homoserine-deficient mutants had some shortcomings, producing, in addition to lysine, some other amino acids

Table 24. *Lysine production by different auxotrophs of M. glutamicus* (NAKAYAMA et al., 1961)

Strain	Necessary growth factor	L-lysine (lysine-HCL) mg/ml
901	homoserine (methionine and threonine)	12.9[a]
702	homoserine (methionine and threonine)	11.4[a]
602	methionine	1.6[a]
703	methionine	1.6[a]
707	leucine and isoleucine	2.3[a]
543-13	threonine	1.8[b]
534-62	threonine	2.6[b]
615-313	isoleucine	3.2[b]
615-396	isoleucine	2.3[b]

[a] Medium with 10% glucose.
[b] Medium with 7.5% glucose.

(mainly alanine and valine). This was especially manifest under poor aeration conditions, when the amount of lysine produced fell practically by half with a simultaneous increase in alanine and valine content. Another disadvantage of homoserine-deficient mutants is their sensitivity to the accumulation of the final biosynthetic products, i.e. threonine and isoleucine, in the medium. If the latter accumulates in excess, the synthesis of lysine is sharply impeded because of the action of feed-back inhibition on the first enzyme in the chain of aspartic acid transformation. Thus it was necessary to select a strain: a) producing lower quantities of additional amino acids; b) less sensitive to the concentration of threonine and isoleucine in the medium; and c) more productive than the homoserine-deficient mutant. An attempt to use induced mutations for this purpose brought very poor returns. After four stages of selection, strain 95 was selected from 2350 variants induced by fast neutrons and UV. This strain was only 5 to 10% more productive than the initial homoserine-deficient mutant (LEGCHILINA and SHISHKINA, 1965). The cause of this poor effect of selection following exposure to mutagenic agents was probably an insignificant increase in the extent of natural variation after irradiation. In an attempt to utilize an alternative way of selection, the possibility of inducing further changes in metabolism by means of additional biochemical mutations

Table 25. *Amino acid synthesis by single and double biochemical mutants of M. glutamicus (volume of medium = 25 ml)* (MINDLIN, 1966)

Strain	Biochemical properties	Lysine		Alanine		Valine	
		mg/ml	%	mg/ml	%	mg/ml	%
N 95	homoserine unnecessary	20.6	72.9	1.7	0.9	1.8	7.1
T-3	homoserine, isoleucine and valine necessary	22.1	86.2	1.4	5.6	traces	—

Table 26. *Lysine biosynthesis, at different aeration levels, by homoserineless strains of M. glutamicus and by their dwarf mutants* (MINDLIN, 1966)

Strain	Lysine (mg/ml) in the presence of different volumes of medium per flasks (ml)		
	10	20	30
N 95	26.2	25.0	15.8
UV-14	22.8	22.0	18.4
T-3	25.4	23.8	16.6
DES-56	21.2	25.8	21.2

was explored. Among others, an attempt was made to obtain auxotroph mutants carrying, in addition to a homoserine deficiency, some other mutation involving the synthesis of another amino acid of the aspartic acid group, such as methionine, threonine or isoleucine. The level of lysine production in these double mutants obtained in our laboratory was virtually unchanged as compared with the single mutant (LEGCHILINA and SHISHKINA, 1965). But at the same time the double mutants with an additional valine or isoleucine deficiency differed advantageously from the single mutant in having a reduced synthesis of additional amino acids. Moreover it was shown later that the double mutant T-3 produced almost no valine which had been one of the main impurities (Table 25).

The inclusion of a third mutation into the genome of a double mutant was explored in an effort to reduce the producers sensitivity to aeration. Accordingly, mutations leading to a respiratory deficiency were induced in mutant T-3 with the help of diethylsulfate vapor. These mutations, similar to aerobic mutants of yeast, were characterized by reduced rates of growth and formation of dwarf colonies. The great extent of variation in lysine production which characterized the dwarf colonies suggested that, apart from lysine production, selection among them should also take into consideration the sensitivity of the dwarf colonies to aeration and the content of amino acids in the fermentation medium. In line with this consideration, MINDLIN

(1966) selected mutant DES-56, which is as active as strain T-3 but less sensitive to aeration and accumulates almost no alanine in the culture broth (Fig. 20 and Table 26). Similar properties were shown by dwarf mutant UV-14, induced in strain 95 by UV light.

The third way of selecting lysine-producing microorganisms was to select mutants resistant to threonine analogues. As already mentioned above, the synthesis of lysine is sharply inhibited by an increase of threonine concentrations in the medium. To obtain mutants with reduced sensitivity to threo-

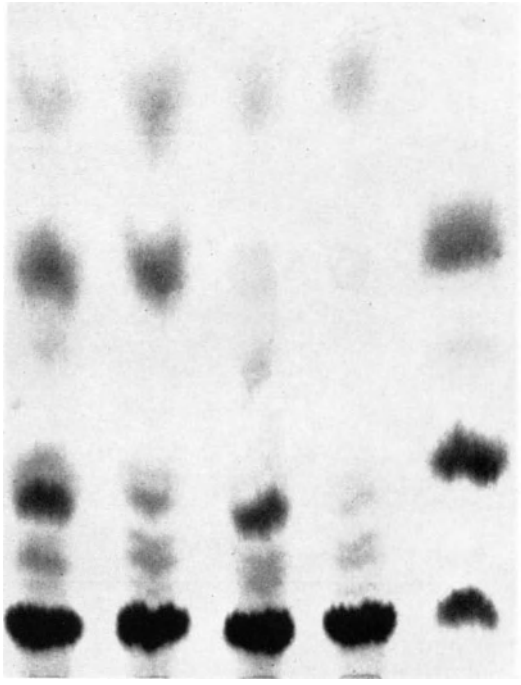


Fig. 20. The amino acid content of culture liquid after growth of the parental strain of *M. glutamicus*, and after growth of its air-mutants. Chromotographic analysis of the amino acid content of the culture liquid of different strains. The upper spot is valine. The spot in the middle is alanine. The bottom spot is lysine

nine, MINDLIN and ZAITSEVA (1966) treated the cells of homoserine-deficient strain 95 with UV and then transferred them to media containing the threonine analogues allothreonine, and serine. Thus the growth of unchanged cells was inhibited and only allothreonine or serine-resistant mutant forms could grow. In contrast to strain 95, the mutants resistant to threonine analogues were characterized by an increased resistance to threonine itself (Fig. 21). Maximal accumulation of lysine by these mutants was observed with higher threonine concentrations as compared with the initial strain, while the maximal level of lysine production did not change. Consequently the selection of mutations interfering with the normal regulation of lysine synthesis has failed in

this case to increase the yield of the final product. The failure probably can be explained by the fact that threonine resistance developed as a result of a derepression of enzymes involved in isoleucine biosynthesis, but not of enzymes specific for lysine biosynthesis.

Summing up the results of selection work on lysine production and the comparative efficiency of different methods of selection, we must say that

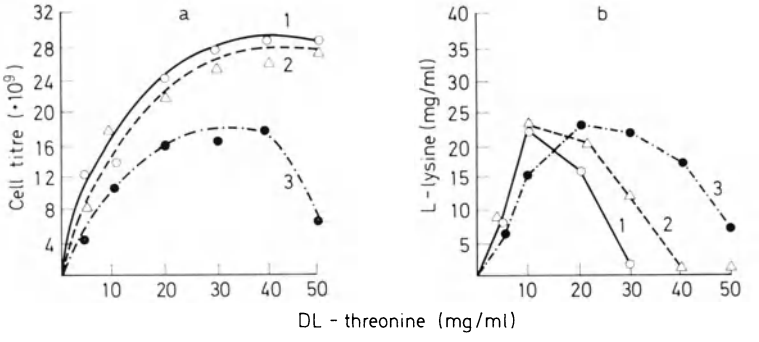


Fig. 21a and b. Dependence of growth and L-lysine biosynthesis on threonine concentration in the case of *M. glutamicus* strain 95, (*1*) its allothreonine-resistant mutant AT-102 (*2*) and the serine-resistant mutant C-112 (*3*). a Growth; b L-lysine biosynthesis

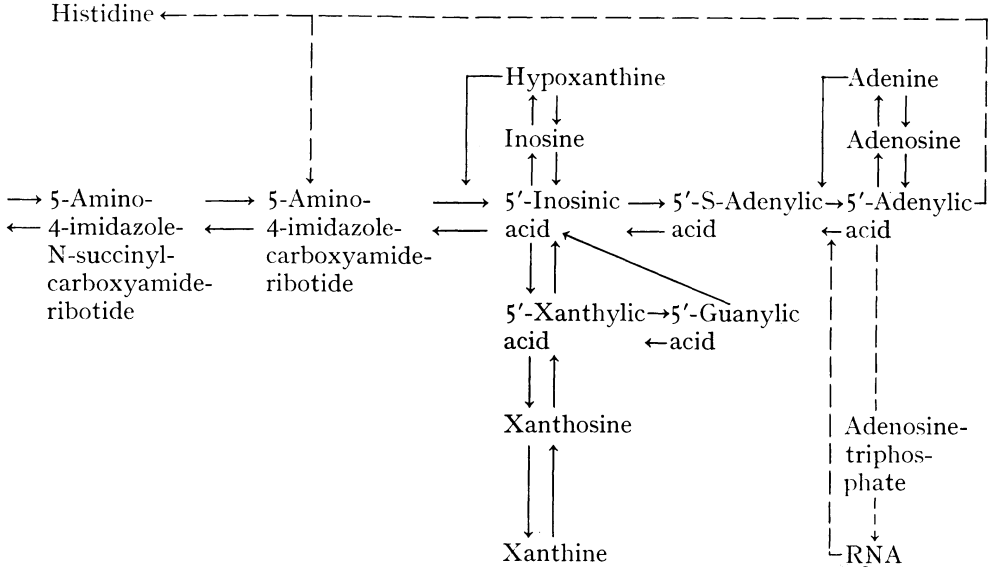
Table 27. *Amino acid production by auxtrophic strains of microorganisms* (ASSAI, 1963; DULANEY, 1963)

Microorganism	Required growth factor	Amino acid synthesized	Productivity mg/ml
M. glutamicus	threonine	homoserine and lysine	13—15
M. glutamicus	arginine	ornithine	26
M. glutamicus	isoleucine or leucine	valine	8
E. coli		threonine	3.7
E. coli	lysine		23.6
E. coli	tyrosine	phenylalanine	2
E. coli		tryptophan	traces
E. coli	phenylalanine	tyrosine	traces
Ustilago maydis	leucine	methionine	6

the main jump in productivity was obtained as the result of a single mutation — a mutation of homoserine deficiency. A second and third biochemical mutation did not greatly change the productivity of homoserine strains but led to the formation of new strains with other valuable properties.

Attempts to obtain strains with depressed lysine synthesis were unsuccessful, similar to the lack of success in step-wise selection following exposure to mutagenic agents. Three amino acids — glutamic acid, methionine and lysine have a leading place in our industry. Only two of them, glutamic acid and lysine, can be obtained by microbial methods (methionine is produced with

the help of chemical synthesis), nevertheless the geneticists and selectionists of microorganisms have now in their arsenal highly productive mutants, offering an opportunity of organizing large-scale production of other amino acids as soon as it becomes necessary. Some of them are listed in Table 27. Auxotroph mutants of various microorganisms and *Ustilago maydis* are among them. It is interesting to note that the leucine auxotroph of this fungus possesses high methionine productivity. So there is, in principle, an opportunity for methionine production bn a microbial method. The auxotroph



Scheme

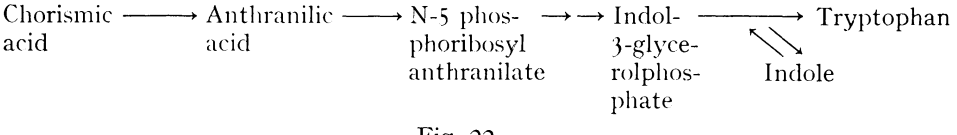


Fig. 22

mutants given in Table 27 can be divided into groups depending on the localization of the genetic block in the chain of biosynthetic processes. In some mutants, a genetic block of biochemical reactions leads to the accumulation of an intermediate biosynthetic product, namely the substance prior to the impaired reaction; such are the mutants synthesizing ornithine and diaminopimelic acid. The first of these, requiring arginine, accumulates its precursor, ornithine; the second, due to impairment of the last stage of lysine synthesis, accumulates its precursor, diaminopimelic acid. Some other mutants are characterized by a genetic block in the synthesis of one final product in the branched biosynthesis chain and that is why they accumulate another final product of this chain. Tyrosine and phenylalanine mutants are of this type,

the first accumulating tyrosine and the second phenylalanine, as both these amino acids have a common precursor. A mutant which is a double auxotroph for diaminopimelinic acid and methionine also belongs to this group. In this case the sequential occurrence of two auxotroph mutations, described by HUANG (1961), blocking the lysine and methionine branches of one biosynthetic pathway, cause an excessive accumulation of the third branch of this pathway, threonine (Fig. 19). Homoserine mutants, accumulating lysine, also belong to this group of mutants.

Finally, an auxotroph for leucine, accumulating methionine, and an auxotroph for isoleucine, accumulating valine, represent the type of mutant whose genetic blocking of the final product of one biosynthetic pathway leads to the accumulation of the final product of another, related biosynthetic pathway.

The *E. coli* mutant accumulating tryptophan in excess stands separately. As seen from Fig. 22, tryptophan is the final product of a non-branched biosynthetic chain and that is why there are great difficulties in obtaining it by the usual method with the use of auxotrophs. But as DULANEY (1963) showed, a large amount of tryptophan or its precursors, indole and serine, can be synthesized by a mutant whose initial stages in tryptophane biosynthesis are genetically blocked, so that it needs anthranilic acid or indole. In connection with the damage of a specific system of regulation such a mutant synthesizes more tryptophan than the initial prototroph strain (see Fig. 22).

Mutant strains of *Hansenula* yeast carry out the synthesis of tryptophan from its precursor, anthranilic acid, very efficiently. A selection of prototroph and induced auxotroph mutants was used in the isolation of these strains. TERUI and ENATSU (1962) first obtained, with the help of UV, a diploid prototroph strain of HANSENULA, synthesizing about 1 mg/ml of tryptophan. Subsequently, haploid strains isolated from the diploid one were irradiated with UV, and after that auxotrophs requiring amino acids, purines and vitamins were selected. Some of them (three requiring histidine and one methionine) synthesized about 6 mg/ml of tryptophan on a medium deficient in the necessary growth factor.

It was mentioned several times that the synthesis of amino acids by auxotrophic organisms depends very much on the content of the necessary growth factor in the culture medium. An extremely high concentration of growth factor inhibits the synthesis of the required amino acid and moves the biosynthetic reactions into another direction. Such a picture can be found in the case of glutamic acid biosynthesis, where an excess of succinic biotin moves the process into the direction of lactic acid accumulation.

Similarly, as shown above, the process of lysine biosynthesis in *M. glutamicus* homoserine mutants was greatly inhibited by an excess of threonine or homoserine. Comparable phenomena are characteristic for other auxotrophic mutants of microorganisms and apart from allowing a phenotypic control of biosynthetic processes, permit the isolation of mutants capable of derepressing the synthesis of necessary amino acids.

LIM and MATELES (1964) described an interesting case of isolation of a prototroph strain of *E. coli* capable of accumulating large amounts of indole and tryptophan. The isolation of such a strain was made possible by isolating mutants with derepressed tryptophan synthesis, i.e. mutants with an impaired regulation of the final biosynthetic product. The actual selection of the mutants was based on their resistance to the tryptophan analogue, α-5 methyltryptophan, and also on their ability to release tryptophan into the medium. Strain E-271 was selected as a result of a four-stage selection process, yielding cells that produced about 350 mg of indole and 50 mg of tryptophane per litre of medium during a period of nine hours. The mutant's ability to form large amounts of indole and tryptophan was associated with a derepression of the synthesis of the final enzymes on the pathway of tryptophan biosynthesis. In comparison with the initial *E. coli* strain K-12, the activity of these enzymes of the mutant increased 13 and 15 times respectively. Thus, although in this case no strain fit for practical use was obtained, the possibility of great increase of output of the final product in prototroph organisms with the help of derepressed (constitutive) mutations was demonstrated in principle.

In 1959/1960 a new branch of microbial industry began to develop in Japan: the production of certain purine compounds such as 5-inosinic acid (5-inosine monophosphate) and 5 xanthic acid (5-xanthosine monophosphate), used as additives in the food industry.

The history of the isolation of strains producing purine compounds repeats exactly the history of isolation of strains used for amino acid production. Some microorganisms accumulate about 0.3—0.5 mg/ml of these compounds, in the culture medium, but neither inosinic nor xanthic acids are found among them (ARIMA et al., 1963; IKEDA, 1967). Consequently microbial genetists used the method that had proved to be effective in the selection of amino acid producers, the method of obtaining auxotroph mutants having a genetic block in the biosynthesis of purine and its analogues. The results again confirmed the important role of auxotroph mutants in the selection of producers of primary metabolites. The best results in the selection of purine producing compounds were achieved with auxotroph mutants of *B. subtilis strain K* (IAM1145) which played the same role in the production of nucleotides as *M. glutamicus* in amino acid production. The initial prototroph strain of *B. subtilis* did not utilize analogues of xanthine and hypoxanthine to any significant extent. But according to the results of UCHIDA et al. (1961), the adenine mutant of strain 2 was able to accumulate inosine, 5-inosinic acid and hypoxanthine, the latter in considerable amounts. As the productivity of the adenine mutant was insufficient for its utilitation in industry, AOKI et al. (1963) did further selection work, obtaining additional auxotroph mutations which were induced by X-rays and were selected by the penicillin method. The results are given briefly in Table 28. An increase in inosine productivity affecting amino acid synthesis was shown by many mutations. As the initial stages in amino acid and purine biosynthesis are common, it is quite natural that the impairement of biosynthesis should move the metabolism in the

Table 28. *Formation of inosine and hypoxanthine (in mg/ml) by different auxotroph mutants of Bacillus subtilis K.* (AOKI et al., 1963)

Strain	Necessary growth factor	Inosine	Hypo-xanthine
2	Adenine	0.21	1.03
B-4	Adenine, histidine	4.46	0.1
S-26821	Adenine histidine	0.70	0.2
B-1	Adenine, histidine, aspartic acid	2.45	0.1
C-30 (obtained from B-4)	Adenine, histidine, tyrosine	6.30	0.1
A-43	Adenine, methionine	2.10	0.9
S-3291	Adenine, amino acids	1.56	0.1
A-28	Adenine, amino acids	1.11	0.26
A-9	Adenine, X[a]	1.59	0.51

[a] Unknown requirement.

Table 29. *The formation of xanthosine by auxotrophs of B. subtilis K.* (FUJIMOTO et al., 1966)

Strain	Required growth factor	Accumulation mg/ml
Gu-3	guanine	4.04
Gu-Ad-3-11	guanine, adenine	8.06
Gu-Ad-3-35	guanine, adenine	8.80
Gu-10	guanine	4.41
Gu-Ad 10-13	guanine, adenine	7.82
Gu-Ad-10-37	guanine, adenine	7.92

direction of excessive accumulation of purines. The extremely high production of inosine was examined in double mutant B-4, needing histidine as well as adenine and having some common precursors with purine. The increase in the output of hypoxanthine analogues in the double mutant B-4 was followed by a change in favour of inosine. This phenomenon played a great role in the industrial application of the double mutant, because inosine can be easily transformed into 5-inosinic acid by phosphorylation (see Fig. 22). The triple mutant C-30, carrying an additional genetic block in tyrosine synthesis, had an even higher productivity than the double mutant. In order to obtain xanthosine analogues, another line was selected, starting with the *B. subtilis* wild strain. FUJIMOTO et al. (1966) using UV, obtained guanine-deficient auxotroph mutants synthesizing about 3—5 mg/ml of xanthosine. An additional genetic block in alanine doubled mutant productivity (Table 29).

Some investigators have tried to increase the output of 5-nucleotides with the help of derepressed mutations. But according to IKEDA (1967), this type of selection for production of purine compounds was not very successful.

Future prospects for the use of purine-deficient mutants of microorganisms are based on the fact that purine analogues affect precursors of riboflavin.

Thus a genetic block in purine biosynthesis, affecting early stages, can be used to obtain mutants producing riboflavin in excess. A mutant of *Candida guillermondii* Y-4 requiring purines, gives about 39 mg/ml of riboflavin in a medium with low iron content. SHAVLOVSKI et al. (1966) stated that the purine biosynthesis of this mutant is impaired at a stage preceding the formation of 5-amino-4 imidazole carboxamide ribotide.

Summing up the results obtained in the selection of strains with high production of purine and its analogues, a field that constitutes the youngest branch of microbial selection, we must stress the following two circumstances: 1. the main successes in selecting these producers were achieved with the aid of auxotroph mutants of microorganisms; 2. unlike many amino acid producers, differing by one mutational step from wild strains, the producers of purine compounds are double and triple auxotroph mutants, obtained gradually from wild type protrotroph strains.

The various examples of the isolation of super-active strains producing amino acids or purines confirms the great significance of data on the genetic apparatus, and its manipulation, for the isolation of mutants with desired characteristics. The activity of the amino acid and purine producers is nowadays in the neighborhood of 30 mg/ml (lysine) and 50 mg/ml (glutamic acid), and thus is far superior to that of numerous strains producing antibiotics, whose best performance after many years of selection is in the range of about 5—15 mg/ml (penicillin, streptomycin, terramycin) and many of which now produce only about 1.5—2.0 mg/ml (oleandomycin, erythromycin).

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New Ideas and Data on Competence and DNA Entry in Transformation of Bacillus subtilis

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With 3 Figures

Contents

I. Introduction

The steps involved in bacterial transformation can be divided into two main categories, those leading to the juxtapositioning (synapsis) of the donor and recipient genes and those involved in the subsequent recombinational processes. The elucidation of the mechanism by which the latter step is accomplished is of general importance to the field of genetics since similar mechanisms could quite possibly be responsible for genetic exchange reactions in all living systems. On the other hand, one might consider studies concerned with the juxaposition reactions of a more limited value in view of the uniqueness of bacterial transformation as regards the ability of purified DNA to transfer information and thus to induce hertiable genetic alterations. But, to the contrary, the understanding of the basic mechanisms involved in the process of DNA transport in bacterial transformation might be of great importance since it may provide insights into the manner in which the surfaces of more

complex cells can interact with and respond to informational macromolecules in the environment.

Most of the original investigations into the nature of the phenomenon of transformation have been done with two organisms, *Diplococcus pneumoniae* and *Hemophilus influenzae*. From these studies a rather generalized view of competence [previously defined as the ability of a cell to irreversibly bind highly polymerized DNA (LERMAN and TOLMACH, 1957)] and the total transformation process has been presented as a representation of bacterial transformation (for example see HAYES, 1964). More recently a very efficient and integrated model of some of the early stages in transformation has emerged from the study of pneumococcal system. This interpretation is exciting not only because it explains many facets of the system so well and argues for a significant role for genetic transformation in the evolution of transformable bacteria, but it also is a demonstration that, under the proper conditions, the population of bacteria in a liquid culture can act as a biological unit (TOMASZ, 1965a).

Similar mechanisms are now being suggested for other transformable bacterial species. One of the aims of this paper will be to evaluate the applicability of the pneumococcal juxtaposition reactions to the *Bacillus subtilis* transformation system and, in a sense, this article may serve as the devil's advocate to those reports that would extend the pneumococcal results to all transformation systems. In addition, an entirely different view of competence in the *B. subtilis* system will be presented which suggests that, in this organism at least, transformation is a relatively inefficient, nonspecific mechanism of genetic exchange, the evolutionary role of which is questionable. This interpretation implies that although the end result is the same in all systems, the means may be quite diverse and bacterial transformation may be an example of convergent evolution.

Since this article will be concerned primarily with the juxtaposition reactions and the nature of the competent state, the topic will be approached from a perspective quite different than that of previous reviews, the present review placing less emphasis on the transforming DNA molecule and stressing the relationship between the structural organization of the recipient cell and the process of transformation. For this reason many aspects of the study of bacterial transformation will not be reviewed (e.g., historical development, relation between structure and function of the transforming DNA molecule, the mechanism of recombination) and the interested reader should consult the previous reviews for a more basic detailed analysis of the total process of transformation (RAVIN, 1961; SCHAEFFER, 1964; HAYES, 1964; BRAUN, 1965; SPIZIZEN et al., 1966).

II. A Definition of Transformation

Bacterial transformation is a process of intercellular transfer of information in which an extracellular DNA molecule, produced by natural means (secretion or cell lysis) or by chemical extraction, can bind to competent

recipient cells, penetrate the surface layers of that cell and, providing a significant degree of homology exists, physically replace the homologous region of the recipient genome (modified from BRAUN, 1965).

This definition is purely descriptive and presents the numerous problems with which this paper will be concerned, namely the nature of the competent state and the competent recipient cells, the factors responsible for the competent cell's ability to interact with the transforming DNA molecule and the manner through which the adsorbed DNA molecule can actually penetrate the surface layers of the recipient cell.

III. The Transforming DNA Molecule

Bacterial transformation differs from all other known sexual mechanisms by the fact that purified DNA can act as the vector in information transfer. The extracellular DNA may result from artificial means (chemical extraction and purification) or from natural causes [cell lysis (OTTOLENGHI and HOTCHKISS, 1962; TAKAHASHI, 1962) or release of DNA from actively growing cells (EPHRATI-ELIZUR, 1968)]. The extracellular DNA, no matter by what route it gains entry into the exocellular environment, is subjected to degrading forces. Naturally occurring nucleases can attack the DNA as it is released from the cell, while it is in the suspending medium or during purification procedures. The transforming DNA is also degraded in the course of purification and manipulation by hydrodynamic shearing forces generated during homogenization and pipetting (LEVINTHAL and DAVISON, 1961). This degradation redistributes the genome into a large number of smaller DNA fragments. If these ruptures in the double helix occur at random along the bacterial genome then the number of possible distinct DNA molecules in a DNA preparation can be extremely large and recombinational data obtained using transformation as the analytical system should be relatively accurate. On the other hand, if preferential points of breakage do exist then mapping data based on transformation should be distorted and inconclusive. Thus, the term linkage, when applied to bacterial transformation, is best defined as the probability that two distinct genetic loci will be situated on the same DNA fragment after release or purification (KENT and HOTCHKISS, 1964) and linkage maps of the entire bacterial genome obtained using transformation results may not be true representations of the sequence of cistrons in the physical structure.

The dimensions of the transforming DNA molecule and the total *B. subtilis* genome can be used to calculate some interesting data in this regard. The size of the genome can be assessed either by direct physical measurement or by calculations based upon the mass of a single, non-replicating genome; however, both methods have produced conflicting results. Physical measurements of well-spread autoradiographs have given evidence for structures varying in length from 700 μ (GANESAN, 1967) to 1300 μ (DENNIS and WAKE, 1968), while direct chemical measurements have ranged from a molecular weight value of 1.2×10^9 daltons (corresponding to an approximate

length of 700 μ) (GANESAN, 1967) to 3.9×10^9 daltons (corresponding to an approximate length of 2000 μ) (EBERLE and LARK, 1967). DENNIS and WAKE (1968) who assumed that a *B. subtilis* spore contains a single genome[1], have determined the DNA content of spores of several strains of *B. subtilis* and have concluded that the molecular weight of an entire genome is 3.3×10^9 daltons or about 1700 μ in length, a value that will be used in the following discussion.

BODMER (1966) has subjected a preparation of transforming DNA [extracted by the method of MARMUR (1961)] to zonal centrifugation and has determined that the weight average molecular weight of the double-stranded DNA molecules was 2.1×10^7 daltons. This means that the *B. subtilis* genome is broken into about 160 fragments with an average length of 10 μ. The competent *B. subtilis* is smaller than an average vegetative cell, being about 1.4 μ in length (JAVOR and TOMASZ, 1968) and, thus, the initial reaction involves the recipient cell and a linear molecule approximately 7 times its length. If the DNA extraction procedure is relatively gentle (i.e., a minimum of disruptive steps) the relative size difference can increase greatly and it has been suggested that two competent recipient cells may compete for the same transforming molecule and cause a further destruction of linkage (KELLY, 1967).

The fact that there may be 160 DNA fragments/genome will put a limit on the number of transformants for a specific marker if the number of penetration sites on each competent cell is significantly less than 160. For example, if we assume that once the donor molecule has penetrated the surface layers of the recipient cell the probability of integration for each marker is 1.0 and that there are 32 active sites per competent cell [an estimate of FOX and HOTCHKISS (1957) for pneumococcal transformation], then only 20% of the competent cells will be transformed, on the average, for this specific marker.

The transforming DNA molecule must also posses certain biophysical and biochemical attributes in order to participate in the transformation reaction. Most important among these are: 1. a molecular weight greater than a minimal magnitude [estimates range from 3.0×10^5 daltons (CATO and GUILD, 1968) to 10×10^6 daltons (SZYBALSKI and OPARA-KUBINSKA, 1965) and the nature of the size-dependent reaction is not agreed upon (see CATO and GUILD, 1968)]; 2. the native double-stranded configuration; and 3. origin from a related genus or species. These aspects of the transformation process have been reviewed recently (SCHAEFFER, 1964).

IV. Competence

The penetration process described in the definition not only places restrictions upon the DNA molecules that can participate in the transformation reaction but also requires the recipient cell to possess certain physiological and structural characteristics that are made manifest by the uptake and

[1] YOSHIKAWA (1968) has presented evidence that each *B. subtilis* spore contains 2 complete genomes, each with a molecular weight of 2.7×10^9 daltons.

expression of the exogenous DNA. This peculiar state of the recipient cell has been designated "competence". The competent state remains ill-defined and the best all-inclusive statement concerning the nature of competence may still be that "it is not unreasonable to suppose that ... reversible alterations at specific sites on the surface of ... cells can result from enzymatic action, and that these alterations make possible the adsorption or penetration of the specific desoxyribonucleic acid" (McCarty et al., 1946).

A. Attainment of the Competent State

The significance of the required physiological state of the recipient cells was hard to demonstrate in the early investigations on pneumococcal transformation because of the elaborate requirements (e.g., agglutinating antibodies, serum albumin and pyrophosphate) (see Ravin, 1961, for review). It soon became apparent, however, that the recipient population had to undergo a period of growth and multiplication before a rather short period of susceptibility to transformation was attained (McCarty et al., 1946). Subsequent work demonstrated quite clearly the transitory nature of competence and it was calculated that the length of time that an individual pneumococcal cell remained competent was approximately 15 minutes (Thomas, 1955).

Spizizen (1958) was the first to describe a procedure that permitted the transformation of germinating spores of *B. subtilis* 168, a UV-induced mutant isolated by Burkholder and Giles (1947). Another mutant of common origin, strain W23, has been found to be non-transformable by conventional transformation procedures (Sueoka and Yoshikawa, 1963). Following the initial demonstration of the transformability of *B. subtilis*, Anagnostopoulos and Spizizen (1961) defined conditions for the transformation of vegetative cells. They observed that when grown in a semi-defined minimal medium competence began to appear in the culture during the transition from logarithmic growth to the stationary phase of the growth cycle. Unlike the pneumococcal system where the state of competence is limited to only a few minutes, the duration of the competent state, in most cases, was observed to be from 3 to 4 hours, and although the appearance of competence is somewhat asynchronous [i.e., different cells become competent at various times during the competence of the culture (Jensen and Haas, 1963b)], it appears as though an individual cell may remain transformable for approximately the same interval (Nester, 1964; Kammen et al., 1966b).

Since *B. subtilis* can be grown and transformed in a defined, simple medium the nutritional factors affecting the development of the competent state have been studied quite profitably. Yeast extract was shown to contain inhibitory factors for the transformation of vegetative cells and could be replaced by low concentrations of acid-hydrolyzed casein (Anagnostopoulos and Spizizen, 1961). In addition to supplying the auxotrophic requirements, the casein hydrolysate contained L-histidine, an amino acid that proved to be necessary to chelate the inhibitory Cu^{++} ions present in the medium. Other chelating compounds (e.g., EDTA) were able to replace the histidine. The reported

stimulation of transformation by polyphosphate (KOHIYAMA and SAITO, 1960) may also be due to its chelating capacity (RAVIN, 1961).

Recently it has been demonstrated that the competence of the culture varies with the composition of the commercial casine hydrolysate (WILSON and BOTT, 1968). Certain amino acids were shown to be stimulatory in the transformation process (arginine, histidine, threonine, glycine, valine, aspartic acid, lysine, and methionine) and others inhibitory (alanine, glutamic acid, leucine, isoleucine, and proline) and the relative concentrations of these were shown to vary considerably in 4 separate batches of casein hydrolysate. The inhibitory amino acids were shown not simply to interfere with the interaction of recipient cell and DNA molecule but actually to impede the development of the competent state. Substitution of a mixture of the stimulatory amino acids for the casein hydrolysate was shown to increase the level of competence of the recipient population.

YOUNG and SPIZIZEN (1961, 1963b) have studied the physiological requirements of transformation using both frequency of transformation and DNA incorporation as measurable parameters. The inhibition of transformation noted in rich media and in the presence of Cu^{++} was shown to be due to a decreased uptake of ^{32}P-labeled transforming DNA per cell. The optimal temperature range was between 34° C and 37° C and the pH optimum was found to be between 6.9 and 7.4. Agitation of O_2 or air increased DNA uptake and transformation relative to an atmosphere of N_2 or to results in non-agitated recipient cultures. The nature of the carbon source (e.g., glucose, pyruvate or lactate) had no influence on either parameter. Neither transformation or uptake occurred in tris buffer containing glucose and the presence of Ba^{++}, Ca^{++}, Sr^{++}, or Mg^{++} was required. The optimal concentration for the cation was 0.005 M. Other cations, monovalent of divalent, did not support DNA uptake (YOUNG and SPIZIZEN, 1963b) and the presence of Fe^{++} and Mn^{++} (two cations associated with sporulation) destroyed competence (BOTT and WILSON, 1968).

The original growth medium described by SPIZIZEN (1958) contains 0.14 M potassium phosphate and it has been reported that the phosphate anion might act as a competetive inhibitor of transformation (STEWART, 1968). It was found that the omission of the potassium phosphate from the minimal medium did not alter the pH and that in the linear response range of DNA concentration the frequency of transformation was 5 times greater in the phosphate-free medium.

There are probably as many methods of obtaining a competent culture of *B. subtilis* as there are laboratories engaged in studying the phenomenon. Since, as we shall discuss shortly, the ability to become competent is genetically determined, the obvious first step in initiating a study utilizing bacterial transformation is to select a highly transformable strain. YOUNG and SPIZIZEN (1963) have found that the probability of successfully isolating such strain is enhanced by picking colonies that have been allowed to age on enriched agar plates for 14 days or more and testing a number of isolates for frequency

of transformation under defined conditions. Once a good strain has been found it is best to prepare a large stock spore suspension to ensure genetic stability as well as to reduce the work necessary to maintain a large bacterial collection (EPHRATI-ELIZUR, 1965).

In order to begin an experiment it is best to begin to grow the recipient culture the night before the transformation experiment since it has been found that optimal results are obtained with primary cultures that have been in a log phase of growth for many generations. This is usually accomplished by either streaking the recipient strain on an enriched agar plate for overnight growth before transfer of a small inoculum to the primary growth medium (YOUNG and SPIZIZEN, 1963 b) or by inoculating the primary growth medium with so few cells that the culture is still in the log phase of growth after overnight incubation (J. C. COPELAND, personal communication).

Since it was originally demonstrated that high concentrations of amino acids were inhibitory to the development of competence (ANAGNOSTOPOULOS and SPIZIZEN, 1961), many investigators have employed the "step-down" procedure which involved dilution of the primary culture into a nutritionally poorer medium (but having a higher Mg^{++} concentration) a short time before the addition of transforming DNA. It now seems apparent, however, that the decreased frequencies of transformation were due to presence of inhibitory amino acids in the casein hydrolysate (WILSON and BOTT, 1968) and the semi-starvation conditions are, in fact, not required for competence development. Other methods have proved equally efficient and it makes little difference whether the cells are diluted into a step-down medium or an enriched medium (ERICKSON, unpublished observations) or whether they remain in the primary growth medium (BOTT and WILSON, 1967). In fact, the method outlined by BOTT and WILSON, which involves the addition of transforming DNA 3 hours after the cessation of logarithmic growth, may well be the method that induces the highest percentage of cells in the total population to become competent (WILSON and BOTT, 1968).

The time at which the cells are transferred to the final growth medium (or exposed to transforming DNA) is very important. Many studies described in the literature have utilized cells from a primary culture of a definite age in time (e.g., 4 to 5 hours growth at 34° C) and, since the growth rates of the various mutant strains used as recipients undoubtedly varies significantly, the results have been that the age of culture (i.e., age with respect to where it may be in the growth cycle of the culture) has not been constant. For this reason it is best to use routinely cultures at a specific point in the growth cycle of the recipient culture.

The time of addition of transforming DNA varies from procedure to procedure and is best determined experimentally by either observing when the highest percentage of cells becomes transformed. The duration of exposure is dictated by experimental design. When short periods of exposure are required (i.e., less than 10 minutes) the reaction is usually terminated by the addition of DNAase or by simple washing, the goals of the experiment again

dictating the proper procedure. For normal genetic analyses a 30 minute exposure is most often used and DNAase addition may be omitted since the initial binding reaction is completed by this time. It should be noted, however, that if the period of incubation is extended much beyond 30 to 45 minutes the frequency of transformation drops, the decrease being due to a decrease in the total number of transformed cells and not to an increase in the total population. It is not clear whether this is due to a release of previously bound transforming DNA that is rendered genetically inactive (KAMMEN et al., 1966) or to actual destruction of "potential" transformants (JENSEN and HAAS, 1962).

The relative concentration of transforming DNA used is again dependent on experimental design. Although it may vary according to the method of competence development, the response curve of the number of transformants as a function of DNA concentration is linear to a concentration of about 0.1 μg/2$\times 10^8$ recipient cells/ml (the frequency of transformation being approximately 0.008) (ANAGNOSTOPOULOS and SPIZIZEN, 1961). For most experiments a saturating or just saturating concentration of from 1 to 2 μg DNA/ml is adequate, especially in competitive inhibition studies (SCHAEFFER, 1964). On the other hand, concentrations well within the linear response range are required for many studies (e.g., genetic linkage studies or experiments in which a quantitative estimate of transforming DNA is desired).

The methods used in dilution and plating of the recipient cells after exposure to DNA has also been found to affect the frequency of transformation significantly. The dilution medium should be ionically favorable to the survival of the potential transformants (especially with respect to divalent cation concentration) and perhaps should include certain amino acids (KAMEN et al., 1966a). In addition, it has been reported that expression of the transformed character (or alternatively, survival of the potential transformant) is enhanced if the recipient population is first allowed to be in contact with an enriched suspending medium before being plated on the appropriate minimal medium (JENSEN and HAAS, 1962).

It is obvious from this discussion that there exists wide lattitude in the choice of methodology for transformation of *B. subtilis*. It appears to this author, however, that the method described by BOTT and WILSON (BOTT and WILSON, 1967; WILSON and BOTT, 1968) should be considered as the system of choice for future investigations, especially by those investigators interested in elucidating the basic mechanisms involved in bacterial transformation. The outstanding features of this method are its basic simplicity and its obvious relationship to the growth cycle of *B. subtilis* grown under a defined laboratory environment.

B. The Nature of the Competent State

In the past the competent state was exclusively associated with genetic transformation and, for this reason, competence has been simply defined as a cell's capacity to bind DNA irreversibly (LERMAN and TOLMACH, 1957).

Irreversible binding, in this context, means that the transforming DNA is bound to the cell surface in such a way that treatment of the DNA-cell complex with DNAase does not reverse the genetic event (Fox and Hotchkiss, 1960). The dissociation of incorporation and integration, and the justification for describing competence only in terms of the former, is based upon two observations. First, uptake of non-homologous DNA occurs in both pneumococcus (Lerman and Tolmach, 1957) and *B. subtilis* (Bodmer and Ganesan, 1964) without phenotypic expression and without any demonstrable recombination (Lacks et al., 1967; Lorkiewicz et al., 1961). Second, cells that are not competent in transformation tests are still capable of undergoing genetic recombination with genetic markers introduced by transduction (Takahashi, 1961). Quite recently the capacity to bind transforming DNA irreversibly was shown to be a characteristic of only cells exhibiting properties associated with the competent state. Based upon the observation that competent *B. subtilis* cells are less dense than normal vegetative cells and could be separated by zonal centrifugation (Singh and Pitale, 1967), Singh and Pitale (1968) and Cahn and Fox (1968) have shown that only cells banding at a position expected for the less dense competent cells were able to bind isotopically labeled DNA.

Fox and Hotchkiss (1957) found that competent pneumococcal cells could be frozen, stored for prolonged periods before thawing, and could regain the competent state under certain growth conditions. These conditions included the presence of serum albumin, Ca^{++}, amino acids and glucose. These requirements suggested to the investigators that the attainment of the competent state required protein synthesis, a hypothesis strengthened by the observed inhibitory activity of chloramphenicol on competence development. The results of Kammen et al. (1966b) show a direct dependence of expression of competence on protein synthesis in the *B. subtilis* system. The addition of actinomycin D or puromycin to recipient *B. subtilis* cultures not only inhibited the development of competence but also destroyed this physiological state after it was achieved. When puromycin was added simultaneously with the transforming DNA there was no decrease in the uptake of isotopically labeled DNA but as the interval between the addition of antibiotic and DNA was increased, there was a drastic decrease in amount of label bound by the recipient cells. These results suggested that both the development and maintenance of the competent state were dependent upon messenger-RNA directed protein synthesis and that the protein product had a very short half-life.

The nature of the protein required for attainment of the competent state in the pneumococcal system has been studied by Tomasz and associates. This series of investigations has shown that a macromolecular cell product, presumably protein in nature (Tomasz and Mosser, 1966), can be extracted from competent cells and will induce competence in non-competent cultures during any stage of growth (Tomasz, 1965a) but not in cells having recently lost the property of competence (Tomasz, 1966). Antiserum produced against competent cells (but not antiserum against non-competent cells) can, when

added to a pre-competent culture, inhibit the appearance of the wave of competence or, if added to a competent culture, inhibit the initial binding of DNA to recipient cell (TOMASZ and BEISER, 1965). Antiserum to competent pneumococci does not interfere with transformation of *B. subtilis* or *H. influenzae* but does inhibit transformation of a related streptococcal strain, a result indicating that if competence is attained by asimilar mechanism in all transformable bacteria, then the activator is species-specific.

TOMASZ (1966) has proposed that this activator substance is first produced by, or first reacts with, a small number of cells and that these cells are induced to form additional activator which then induces more cells to become competent. This autocatalytic spread of activator would induce a synchronous-like appearance of competence in the recipient culture. He has also suggested that after the majority of cells in the population have reacted with the activator, an inhibitor may appear in the culture capable of combining with and inactivating the activator. This event may deplete the pool of endogenous activator and inhibit the continued expression of competence. The inhibitor has been assumed to have no effect on cells already competent (TOMASZ and HOTCHKISS, 1964). Thus, it appears that a few exceptional cells can be first induced to form activator, that this protein can then induce other cells to elaborate the new surface constituent and that this activator substance may itself be part of the surface site involved in the binding of DNA to recipient cell.

A soluble factor has also been isolated from competent cultures of *B. subtilis* that can induce the appearance of competence in non-competent cultures. This factor is present in the supernatant, in phosphate-extracted acetone powders (CHARPAK and DEDONDER, 1965) and in aqueous extracts of competent cultures (AKRIGG et al., 1967). AKRIGG and associates have chromatographed concentrated aqueous extracts of competent and non-competent cells and have demonstrated characteristic UV-absorbing peaks unique to the competent cell extracts. The active material is heat-sensitive and inactivated on exposure to trypsin or chymotrypsin but not after exposure to RNAase. No inhibitor of this substance has been reported or postulated but the fact that a competent *B. subtilis* cell appears to retain its capacity to incorporate transforming DNA for 3 to 4 hours (NESTER, 1964; KAMMEN et al., 1966b) may indicate the absence of inhibitor and the natural decay of the activator. Although these results are compatible with the results of the pneumococcal system many more data must be analyzed before a definite conclusion can be drawn regarding the importance of identity of this factor.

Although protein and RNA synthesis appear to be requirements for the development of competence, DNA synthesis does not appear to be necessary and, as will be emphasized in a later discussion, the absence or decreased rates of DNA synthesis may be characteristic of cells destined to become competent. BODMER (1965) found that during the period of competence development in the secondary growth medium the total recipient culture synthesized negligible quantities of DNA. More recently ARCHER and LANDMAN (1969) have shown

that thymine-starved cells, in which there was no net DNA synthesis, could develop competence after quite lengthy periods of incubation. It should be mentioned that the original demonstration of transformation in *B. subtilis* utilized germinating spores (SPIZIZEN, 1958), another demonstration that DNA synthesis is not critical to competence development.

The capacity to enter the competent state appears to be under genetic control. In pneumococcus transformation, the classic studies of AVERY et al. (1944) demonstrated the variants isolated from a transformable rough (R) strain were unable to be transformed and TOMASZ (1965b) has identified some heritably incompetent pneumococci as cells having lost the ability to synthesize the activator substance. YOUNG and SPIZIZEN (1961) isolated a non-competent mutant from a highly transformable strain of *B. subtilis* 168 which was characterized by a decreased capacity to bind transforming DNA and a transformation frequency of about 4×10^{-7}. If one of the non-competent cells that had been transformed was cloned and transformed for a different marker the frequency of transformation remained at a low level (REILLY, 1965).

As regards cell structure, it appears as though the cell wall is required for some step in the juxtaposition reactions. It was originally found that lysozyme treatment of the recipient cell-DNA complex greatly reduced the frequency of transformation (MILLER and LANDMAN, 1963), that this reduction was due to a decreased ability of the cells to bind the transforming DNA and that lysozyme-induced protoplasts were non-transformable (MILLER and LANDMAN, 1966). Experiments by others have contradicted these data and demonstrated that lysozyme-treated cells could retain cell-associated DNA (YOUNG, 1967; BODMER and LAIRD, 1968; ERICKSON et al., submitted for publication), that lysozyme treatment enhanced transformability (PROZOROV, 1965) and that protoplasts were transformable (HIROKAWA and IKEDA, 1966; TICHY and KOHOUTOVA, 1968). TICHY and LANDMAN (1969) have suggested a reason for these apparent contradictions. They have found that a lysozyme-treated population is heterogeneous and contains lysozyme-resistant bacilli, osmotically sensitive rods, "quasi-spheroplasts" and protoplasts. Their data show that all osmotically sensitive structures can bind the DNA and that lysozyme treatment does enhance the binding reaction but that the transport of the molecule across the membrane requires the presence of cell wall. Thus, "quasi-protoplasts" exposed to transforming DNA and allowed to re-synthesize cell wall demonstrate the highest degree of transformability. In addition, the authors suggest that DNA bound to the protoplast membrane is completely sensitive to DNAase degradation[2].

One may cautiously conclude that protoplasts can bind transforming DNA but cannot transport the macromolecule into the cell interior and one might

[2] Work of LANDMAN and KNOTT (1968) has demonstrated that DNA exposed protoplasts can be adsorbed to cellulose anion exchange columns and that the binding is sensitive to DNAase. After DNAase treatment, however, the protoplasts again bind to the column suggesting that DNA has remained associated with the protoplast after DNAase treatment.

postulate that the binding properties of DNA to the membrane may change with time.

In view of the preceding discussion a number of attributes of the competent *B. subtilis* cell can be deduced (e.g., a competent cell should synthesize protein and messenger RNA but little or no DNA, be deficient in cell wall synthesis and be slow or non-growing). The study of the physiological basis for competence in the *B. subtilis* transformation system, however, has been hampered because competence has not been treated as a stage in the growth cycle of the laboratory grown culture and because the percentage of competent cells in a typically grown recipient culture is quite small.

The demonstration of the heterogeniety of a competent population can be approached by two different methods, one statistical and the other involving autoradiography. In using the statistical method one assumes that each of the 160 or so fragments of the genome produced during isolation of the transforming DNA has an equal probability of interacting with a competent recipient cell and that all subsequent events are independent (i.e., the transformation of one non-linked marker is in no way related to the transformation of a second non-linked marker). Thus, if we are dealing with two genetic markers, A and B, then the frequency with which A transformants will occur is (a) and the frequency with which B transformants will occur is (b). Since we are assuming that the events occur independently then the probability of events A and B occurring will be (a) (b). Now this relationship will only be valid if all members of the population participate in the transforming event and, if this is true, then the experimental values (a) (b) and (ab) should be equal [i.e., (a) (b)/(ab)=1]. This relationship appears to be valid for the pneumococcal transformation system (FOX and HOTCHKISS, 1957); however in the *B. subtilis* system one often observes that (a) (b)$<$(ab) (ANAGNOSTOPOULOS and CRAWFORD, 1961; NESTER et al., 1963) and the most plausible explanation is that only a fraction of the total recipient population is participating in the transformation reaction (i.e., only a minority of the cells are competent) (NESTER and STOCKER, 1963). NESTER and STOCKER further point out that the fraction (a) (b)/(ab) should be a good approximation of the fraction of the recipient population that is competent. In most studies this value for the *B. subtilis* system is less than 10%.

There is an obvious pitfall to this method (NESTER and STOCKER, 1963). The total population that we are dealing with does not correspond to the number of colony forming units or even the number of individual bacteria in the culture but to the number of genomes actively involved in the transformation reaction. Since we are measuring the number of colony forming units, this value must be directly related to the number of transformable nuclei. If each colony forming unit corresponds to two transformable genomes (one bacterium with two nuclear bodies) then our estimate would be twice the actual percentage of competent cells. If, on the other hand, only one of the two genomes participated, then the estimate would be valid with respect to competent cells. It must be emphasized that this method is only a simple

approximation and double transformants involving two unlinked genetic markers may be dependent on other undefined variables (see page 183).

Autoradiography appears to be a much more reliable method. JAVOR and TOMASZ (1968) have added ^{3}H-labeled transforming DNA to competent populations of both pneumococcus and *B. subtilis*, washed and treated the cells with DNAase, and then processed the cells for autoradiography. As predicted from the statistical method, 100% of the pneumococcal cells appeared competent while only 15% of the *B. subtilis* population bound the ^{3}H-DNA. Surprisingly, comparison of the autoradiographic and statistical data for the *B. subtilis* system showed that the statistical method produced a lower estimate of competence than the percentage of cells actually associated with activated silver grains. Thus, one may infer that either an undefined factor decreases the frequency of (ab) relative to (a) (b) or that some non-transformable bacteria can bind DNA.

It cannot be concluded that only a small fraction of the *B. subtilis* cells in a culture have the capacity to become competent. The possibility exists that the precise conditions required for maximal expression of competence have not been established. WILSON and BOTT (1968), employing the previously described conditions for attaining competent populations, have used transfection with ϕ29 bacteriophage DNA in single burst experiments and, by analyzing the data with the Poisson distribution function, estimated that 50 to 80% of the cells in the stationary culture can interact with the phage DNA.

Nonetheless, using the accepted transformation procedures one is evidently dealing with a heterogeneous population of cells in a competent culture of *B. subtilis* and, for this reason, experiments concerned with elucidating the nature of the competent *B. subtilis* cell have approached the problem in three general ways: 1. comparing the properties of competent and non-competent cultures and deducing some of the characteristics of the competent cell; 2. studying the properties of transformed cells and the factors affecting their survival; and 3. attempting to isolate a pure population of competent cells and studying their properties directly. The first method has the disadvantage that such extrapolation must be done with caution and interpretation of such experimental data is subject to bias by the procedure employed to induce competence. In using the second method one assumes that the physiological state of all classes of transformants is identical and that the genetic marker selected does not prejudice the results. In addition, this method involves only those cells that have survived to form a colony on the agar medium and completely neglects the "potential transformants" that did not survive. The last method is theoretically most attractive but it must be recognized that manipulation during isolation may alter the cells in numerous ways.

The first property of competent cultures (besides their transformability) described in the literature was their capacity to bind exocellular highly polymerized DNA (LERMAN and TOLMACH, 1957; YOUNG and SPIZIZEN, 1961). Both the rapid initial binding of transforming DNA and the subsequent

DNAase-resistant state demonstrated concurrent changes with the appearance and loss of transformability. Recently it has been shown that competent cultures bind other macromolecules besides DNA (ERICKSON et al., submitted for publication). When *B. subtilis* cultures were grown according to the step-down procedure of competence development, binding of DNA and transformability reached a maximum at about 60 minutes after transfer of early stationary cells to the secondary growth medium. When identical cultures approaching maximal competence were exposed to ^{131}I-labeled rabbit gamma-globulin or DNAase I, maximal binding of the proteins was observed about 15 to 20 minutes before the peak of transformability. The binding of DNA that had been heated to temperatures above T_m and rapidly chilled showed similar behavior while the binding of rabbit serum albumin was observed to decrease during the time span studied. Thus, the concept that a competent *B. subtilis* cell has elaborated a specific protein involved solely in the binding and uptake of DNA may be questioned.

The initial interaction between the recipient cell and the DNA molecule must be ionic in nature and must therefore be dependent upon the interrelationship between the surface charges of the two reacting bodies and the balancing ionic charges of the medium. The charge density at the cell surface, as measured by electrophoretic mobility, has been shown to vary with the physiology of the bacterial culture (PLUMMER and JAMES, 1961) and such alterations of surface charges have been implicated in conjugation in *E. coli* (TURRI and MACCACARO, 1960). JENSEN and HAAS (1963b) have studied the surface charge of *B. subtilis* in relation to the appearance of competence. The ability to penetrate cellulose acetate filter discs of varying pore size was used as a measure of the electrokinetic potential at the cell surface since this method proved to fractionate particles as a function of charge rather than size (JENSEN and HAAS, 1963a). By analyzing two sets of data, one obtained from cultures of varying competence and the other obtained from a single culture approaching the time of maximal competence, the following conclusions were reached: 1. competence is directly related to the surface charge of the recipient cell; 2. competence is characterized by an abrupt increase in electronegativity; 3. the appearance of competence, under the defined conditions, is asynchronous; and 4. the negative charge is slowly discharged. Hence, transformation is associated with recipient cells possessing a definite range of surface charge values and previously mentioned binding characteristics may be a reflection of the changing electronegativity of the cell surface.

The physiology of the recipient culture has been studied by BOTT and WILSON (1968). As the culture nears the stationary phase of growth there is a marked increase in respiratory activity and this increase in O_2 utilization is related to a shift from the rapid degradation of glucose via the Embden-Myerhof glycolytic pathway to the tricarboxylic acid cycle. Thus, just prior to the onset of competence the pH of the medium is relatively low because of the accumulation of acid and during the rise of competence the increase in CO_2 produced via the TCA cycle causes an increase in pH. BOTT has sug-

gested that this increase in respiratory activity is related to the preparation of the culture to commence sporulation.

An inter-relationship between sporulation and competence was first suggested by SPIZIZEN (SPIZIZEN, 1958, 1961). This hypothesis was based upon the observation that many mutations altering the capacity to undergo sporulation also altered the transformability of the strain. Some of the genetic loci concerned with competence and spore formation have been shown to be linked to the cistrons concerned with protease and antibiotic formation (SPIZIZEN et al., 1966), the antibiotic and the enzyme being cell products that are released from the cells during early stationary growth (COLEMAN, 1967) and are perhaps related to the sporulation process. In addition, BOTT and WILSON (1968) have observed that if the ions required for spore formation (i.e., Fe^{++}, Mn^{++}, in addition to Mg^{++}) are added to the transformation medium, competence is immediately abolished and spores are efficiently formed. Since these ions are normally omitted from the transformation medium it appears as if competence may be correlated with an abortive attempt of some cells to sporulate.

SCHAEFFER (1964) has pointed out that many transformable species are non-sporulating and that this correlation cannot be of general significance. In *B. subtilis*, however, competence always appears associated with either the lag or the early stationary phase (EPHRATI-ELIZUR, 1968), phases in which the generation times of the bacterial population are changing and the processes of sporulation or germination may be involved (HORVATH, 1968).

The physiology of the recipient culture during the "step-down" procedure of competence development has not been critically examined. BODMER (1965), as mentioned previously, has shown that DNA synthesis in the recipient culture is taking place at a very low level (if all cells were synthesizing DNA during the time span studied only about 3% of the genome is synthesized in 30 minutes). MCCARTHY and NESTER (1967), on the other hand, have presented data that suggest that a significant amount of DNA synthesis is occurring in the total population. They added ^{3}H-thymidine to competent cultures, froze the cells and then observed the suicide rate of the frozen cultures. During the time interval studied (90—120 minutes after transfer to the secondary growth medium) they found the death rate to be exponential until 0.05% of the cells survived, a result suggesting that the population was heterogeneous and some cells were not synthesizing DNA. More recent work of BODMER (BODMER and LAIRD, 1968; LAIRD et al., 1968) also suggests that the recipient culture is synthesizing appreciable amounts of DNA. These tests employed density transfer experiments (see discussion on page 180) and showed that a round of replication was completed in about 60 minutes at 30° C, indicating a relatively high rate of growth. Their data also suggests heterogeneity with respect to DNA synthesis in that DNA associated with donor marker activity was replicating at a much lower rate than the bulk of the recipient DNA. The discrepancy in the above results could be due to the lack of constancy in the transformation procedures. MCCARTHY and NESTER followed growth

of the primary culture turbidimetrically and transferred the culture at the end of the log growth while BODMER transferred his primary culture after a defined period of incubation. Using the latter procedure one may be transferring cells well into the stationary phase of growth and the lag period in the secondary growth medium may greatly influence the experimental results.

A correlation has also been suggested between structural changes in the cell surface and competence. The cell wall of *B. subtilis* is a very complex structure consisting of a mucopeptide (composed of N-acetyl glucosamine, N-acetyl muramic acid, alanine, glutamic acid and diaminopimelic acid), teichoic acid (a polyglycerol phosphate with D-alanine or D-glucose on glycerol carbons 1 or 2 and a poly-N-acyl hexosamine on the terminal phosphate), insoluble protein and a few minor heteropolymers (see ROGERS, 1963; YOUNG, 1966). The mucopeptide is a polymer of repeating units of N-acetyl muramic acid and N-acetyl glucosamine with the three amino acids attached as a tripeptide to the muramic acid. The muramic acid and glucosamine are linked by (1—6) and (1—4) glycosidic bonds, the latter being susceptible to cleavage by lysozyme. Cross-linking is thought to occur by peptide bond formation between the terminal amino acids of adjacent polymers linking two muramic residues together. On the cell surface the microfibrils of mucopeptide might be arranged in parallel with the cross-linked peptide chains giving the entire structure a net-like appearance. The teichoic acid and other cell wall constituents are associated with this basic structure in an unknown manner. ROGERS (1963) has suggested that there may be several layers of this type with peptide bonds between the layers resulting in a very strong three-dimensional structure.

It is obvious that this rigid cross-linked surface will present an obstacle to the transforming DNA molecule and that the cell wall must be altered before transformation can proceed. This problem has been investigated in the *B. subtilis* system by YOUNG and associates. A morphological and biochemical study showed no gross structural differences between genetically competent (C^+) and non-competent (C^-) cells, but there proved to be significant biochemical differences (YOUNG et al., 1963). The C^+ strains contained more alanine and galactosamine and less diaminopimelic acid and glucosamine. The amount of labile alanine (i.e., alanine released by partial hydrolysis) was relatively high in the C^+ strain and decreased with growth. The galactosamine content of the C^+ strain increased with growth and was highest during the period of maximal competence (YOUNG, 1965). These studies also revealed that if cell wall preparations were suspended in phosphate buffer instead of H_2O and placed at 35° C, the C^+ wall preparation lysed 3 to 4 times faster than a similar C^- preparation (YOUNG and SPIZIZEN, 1963a). The enzyme responsible for this autolysis was identified as an N-acylmuramyl-L-alanine amidase (YOUNG et al., 1964) and rapidly solubilized the cell wall by hydrolysis of the amide bond between L-alanine and muramic acid (YOUNG, 1966). Maximum activity of the amidase was observed during early logarithmic growth (YOUNG and SPIZIZEN, 1963a). A biosynthetic role for such an enzyme

is not easy to visualize. Undoubtedly addition to the highly cross-linked wall structure would first require slight disruption of its three-dimensional structure before insertion of a new unit could take place through the action of a transpeptidase (ROGERS, 1963; YOUNG, 1966) but hydrolysis of the bond between the peptide and the muramic acid residue would not appear to be involved in such cell wall expansion. However a breakdown in the harmonious interactions of some enzymes involved in cell wall synthesis could result, depending on physiological conditions, in either partial hydrolysis or complete autolysis (YOUNG, 1966) and such a partial degradation of wall structure could produce discontinuities in the surface area and these "gaps" might allow the negatively charged DNA molecule to by-pass the negatively charged cell wall (YOUNG et al., 1964). This interpretation of competence is strengthened by some of the data discussed previously (see page 159), most noticeably the increase in transformation frequency with mild lysozyme treatment (PROZOROV, 1965; TICHY and LANDMAN, 1969).

If such partial degradation of cell wall has occurred one might expect the competent cells to be osmotically fragile relative to the total population. It has in fact been found that when competence appears during the early log phase of growth of a *B. subtilis* culture, that transformed cells are more prone to lyse than the total population. If, on the other hand, competence is allowed to develop in the late stationary phase, this proneness to lyse is not observed (EPHRATI-ELIZUR, 1968). This observation is of interest since it suggests that competence in the early log phase and competence in the early stationary phase may differ in some important aspects.

Osmotic fragility has also been described in the pneumococcal system (THOMAS, 1957) and EPHRUSSI-TAYLOR and FREED (1964), upon observing disturbances in cell wall synthesis, have also suggested that a temporary breakdown in wall structure may be intimately connected with the ability to bind transforming DNA. Recently TOMASZ (1968) has demonstrated that the replacement of cell wall choline by the structural analogue ethanolamine rendered the pneumococcal cells completely resistant to autolysis. These resistant cells could not be transformed and could neither produce nor be induced by the activator substance. TOMASZ concluded that the substrate for the activator resided in the cell surface.

STOCKER (1963), using micromanipulative techniques, observed that *B. subtilis* cells transformed with respect to motility or tryptophan independence did not express these properties for about 3 hours after the addition of transforming DNA. Also, if transformed cells were plated on solid media and the kinetics of increase of both the total number of viable cells and transformants was followed for 5 hours, the total number of cells was found to increase 16-fold while the number of transformants remained constant (NESTER and STOCKER, 1963). Recombinational events were observed to occur as early as 30 minutes after DNA exposure (as measured by establishment of linkage between a donor marker and recipient marker), a result that apparently ruled out both segregation lag and delayed recombination as the causes of the lag

in transformant expression. It was also observed that newly transformed cells were both resistant to the action of penicillin and devoid of tryptophan synthetase activity (in cells transformed to tryptophan independence) for approximately 3 hours after DNA addition (NESTER and STOCKER, 1963). All of these results indicated that the newly transformed cells were latent in certain biosynthetic functions. It was subsequently shown that this latency was independent of DNA contact and was a property of competent cells (NESTER, 1964).

MCCARTHY and NESTER (1967) studied the extent of synthesis of DNA, stable RNA, unstable RNA and protein in transformed cells by following the kinetics of suicide after exposure of competent populations of *B. subtilis* to tritiated precursors of each of these macromolecules. Using ^{3}H-thymidine they observed a biphasic survival curve which suggested that 50% of the transformants were synthesizing DNA at a rate equal to about one-half that observed for the total population and the remaining 50% were not synthesizing DNA at an appreciable rate. They also observed that DNA addition was not responsible for the cessation or decreased rate of DNA synthesis in the transformant population. Studies utilizing ^{3}H-uracil suggested that net RNA synthesis was negligible in the transformants relative to the non-transformed cells. In studies with tritiated amino acids (histidine, tryptophan and leucine) it was found that transformed cells synthesized extensive amounts of protein and, from inferences from the action of actinomycin D on the rate of survival of transformants exposed to the antibiotic at different times, they suggested that the protein synthesis was mediated by unstable messenger RNA.

Data presented by BODMER (1965) indicated that transformed cells are resistant to thymineless death and, therefore, are presumably not synthesizing DNA. GANESAN (1967) has shown that this resistance is only conferred to the competent cells in the presence of the transforming DNA. Since, as pointed out above, DNA addition is not responsible for the reduced rate of DNA synthesis, it may be argued that the adsorbed DNA can be hydrolyzed and subsequently utilized for DNA synthesis. YOUNG (1967a) has shown that shortly after the addition of transforming DNA to recipient cells about 25% of the DNA is degraded into fragments not precipitable by acid. Since a competent *B. subtilis* cell may bind more than one cell equivalent of DNA (BODMER and GANESAN, 1965) there would be enough DNA precursors localized in the competent cell's immediate environment to synthesize substantial amounts of the genome. For these reasons, experiments measuring the extent of DNA synthesis in competent thymine-requiring mutants of *B. subtilis* in the presence of transforming DNA are open to criticism.

Biosynthetic latency has been implicated as the cause of nonphotoreactivability of competent *B. subtilis* cells. It is known that both *D. pneumoniae* (REBEYROTTE and LATARJET, 1960) and *H. influenzae* (GOODGAL et al., 1957) lack the enzyme involved in photoreactivation. KELNER (1964) has shown that although vegetative cells of *B. subtilis* are photorestorable (although not

by the usual mechanism KELNER, 1965), cells that have been transformed lack this mechanism. This transient loss of photoreactivability was suggested to be due to either the general biosynthetic latency described by NESTER and associates or to the specific absence of the repair enzyme. Another possibility, namely that temporarily defective cell wall leads to lethal UV-induced membrane damage while nuclear damage remained photorestorable, was rejected by KELNER on the grounds that transformed cells were not more UV-irradiation-sensitive than the total population (KELNER, 1964; MAHLER, 1965). There may be a great difference between a competent cell and a cell that has expressed its transformed character, however, and to base generalizations of the state of competence upon a cell that has survived to the transformant stage may be deceiving. Nonetheless, the general correlation between nonphotoreactivability and transformability is noteworthy.

Since competent cells were known to bind DNA and it was assumed that the remainder of the population did not interact with the macromolecule, some investigators have labeled the competent cells with tritiated transforming DNA and examined thin-section autoradiographs of cells that have interacted with the labeled DNA by electron microscopy. YOUNG (1967a) observed that in 88% of those cells that bound the tritium label (about 20% of the total population) the disintegration tracts were associated with the cell wall-plasma membrane complex and that these cells were frequently in the process of sporulation [stage III as described by RYTER (1965)].

WOLSTENHOLME et al. (1966), in a similar study, did not observe the relation to the sporulation process but did confirm the site of binding as being in the region of the wall-membrane complex. The tritium disintegration tracts, in this study, were found to be associated with the mesosomes and statistical treatment of the data suggested that the adsorbed DNA molecule was near or adjacent to these membranous structures and not located directly inside the well-packed lumen. It was also noted that during the time of maximal competence the number of mesosomes increased about twice that seen in the log phase of growth and connections between the plasma membrane and nuclear region increased 30 times that observed in the log phase. Moreover, if transforming DNA was added to the competent recipient culture before processing, the previously observed increases were only one-half as great as in the absence of transforming DNA. These observations are very interesting and suggest that the transforming DNA molecule may induce biosynthetic or structural changes in competent cells of *B. subtilis*, a hypothesis advanced early in the study of transformation (KOHIYAMA and SAITO, 1960) and never seriously considered. SCHAEFFER (1964) has pointed out that since DNA is released into the medium during the development of competence (OTTOLENGHI and HOTCHKISS, 1962; EPHRATI-ELIZUR, 1968) the effect of exocellular DNA on competent cells should be re-examined.

The suggestion that the mesosome is involved in DNA uptake has been made (MILLER and LANDMAN, 1966; BODMER, 1966; TICHY and LANDMAN, 1969) and is based more upon inference than concrete experimental data.

The temptation to implicate the mesosome in the transformation process is related to its well substantiated structural relationship to the bacterial nucleoid (JACOB et al., 1963; RYTER, 1968) and its less definitive role in the uptake of macromolecules (FITZ-JAMES, 1960; TOMASZ et al., 1964; BLADEN et al., 1964). The mesosome has also been shown to be the site of the respiratory enzymes (VAN ITERSON, 1965) and related to transverse septum formation (ELLAR et al., 1967). These structures may be multifunctional and the existence of specialized mesosomes is quite probable; however, since binding occurs in the early stationary phase when respiratory activity is increasing (BOTT and WILSON, 1968) and since the site of binding is in the area of cell division [i.e., the middle and tips of cells (JAVOR and TOMASZ, 1968)], the relation between the site of binding and the mesosome is not surprising and its importance to the transformation process may only be fortuitious or secondary.

SINGH and PITALE (1967) reported a significant breakthrough in the study of the competent cell in observing that transformed cells could be separated from the bulk of the population by zonal centrifugation on linear gradients of sucrose. The frequency of transformation was increased 4—8-fold in relation to the original cultures. It was subsequently observed that the fractionation on sucrose was due to differences in the densities of normal vetatative cells (density between 1.127 and 1.136 g/ml) and the transformed cells (density between 1.105 and 1.115 g/ml) (HADDEN and NESTER, 1968). This allowed the isopycnic separation of competent *B. subtilis* cultures on preformed gradients of Renografin-76 (HADDEN and NESTER, 1968; CAHN and FOX, 1968), a radiopaque substance that had been previously employed to separate spores from vegatative cells of *B. megaterium* with minimal loss in viability (TAMIR and GILVARG, 1966). By determining a density of Renografin that would allow the separation of the lighter competent cells from the heavier non-competent cells, a step-gradient could be prepared and an enriched population of competent cells or transformed cells could be obtained rather easily.

These investigations showed that about 95 % of all competent cells appeared in the light fraction indicating that the density distributions of competent and non-competent cells did not overlap. Calculations estimating the percentage of competent cells in the enriched population showed in one study that 35 to 50% of the cells were competent (CAHN and FOX, 1968) and in the other study 145 % of the lighter cells were competent[3] (HADDEN and NESTER, 1968). This separation was only successful at the time of the maximal competence; fractionation of cells at an earlier stage in the development of competence produced two populations of cells of equal transformability upon further incubation (CAHN and FOX, 1968).

This method of separation allows the direct investigation of properties of competent recipient cells. Initial investigations have suggested that the

[3] Percentages greater than 100% have been reported to be normal for cultures exhibiting high degrees of competence (WILSON and BOTT, 1968) and have been discussed on page 160.

majority of competent cells appear to be uninucleate[4] (SINGH and PITALE, 1968) and are the only cells capable of binding transforming DNA (SINGH and PITALE, 1968; CAHN and FOX, 1968). Work in progress in this laboratory (ERICKSON and COPELAND, in preparation) has confirmed previous postulates concerning the nature of competency and has also produced some interesting new data. These experiments involve the separation of maximally competent cultures on Renografin, washing the cells in a medium containing relatively high amounts of Mg^{++} (to counteract the EDTA in commercial preparations of Renografin) and subsequent growth in an enriched medium. Direct chemical measurements have shown that DNA and RNA increase at a much slower rate in these cells compared to the total mass of the culture. Attempts to estimate at what point in the growth of the culture the reduction in rate of DNA synthesis takes place in the competent cells has been difficult since, as mentioned previously, the cells cannot be separated efficiently until maximal competence has been achieved. We have tried to circumvent this difficulty by adding ^{3}H-thymidine to a series of thymine-requiring cultures at different times during the development of competence, allowing all the cultures to reach maximal competence and then separating the cells on Renografin. The distribution of label in the light competent cells and heavier non-competent cells should be a rough estimate of the relative amount of DNA synthesis occurring in the two fractions at the various times studied. The results suggested that about 45 to 50 minutes prior to maximal competence, DNA synthesis in the cells destined to become competent is decreasing significantly. These results are in agreement with those of LAIRD et al. (1968) which showed that in cells that have integrated transforming DNA the extent of synthesis is retarded and that this reduced rate commences about one generation before DNA addition (BODMER and LAIRD, 1968).

The stage of replication of the genome has also been investigated (ERICKSON and COPELAND, in preparation). These studies were based upon the work of YOSHIKAWA and SUEOKA (1963b) which will be discussed in a later section (see page 180). In essence, the work of YOSHIKAWA and SUEOKA assumed that if replication of the bacterial genome was sequential, semi-conservative and always beginning at a specific site on the linear (or circular) structure, then transfer of a synchronized culture to a growth medium containing density isotopes, subsequent isopycnic separation of DNA preparations and analysis of the fractions by transformation would enable the investigator to demonstrate synchrony in DNA synthesis and establish a replication map based upon the temporal sequence in which the genetic markers are transferred to the hybrid density region of the CsCl gradient.

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[4] Investigations in our laboratory (ERICKSON and COPELAND, in preparation) do not support this result. We have separated cells on RENOGRAFIN, fixed the cells with osmium tetroxide, hydrolized and stained with thyionin. In our preparations the cells appear smaller than normal vegatative cells, have fewer than 4 nuclear regions, but few uninucleate cells are observed.

In the work using cells separated on Renografin, the cells were collected, washed, and then allowed to grow in an enriched medium containing D_2O and deuterated algal extract and algal hydrolysate. Aliquots of the culture were removed at various times, lysed and fractionated on a CsCl gradient. As in the experiments of YOSHIKAWA and SUEOKA, the order of replication of various genetic markers can then be assessed and the existence of synchrony and the approximate point of re-initiation of replication could be determined. The results demonstrated that at the time of separation (maximal competence) the genome of competent cells appears to be partially replicated and upon subsequent incubation DNA replication is quasi-synchronous, replication commencing at about the mid-point of the replication map (see Fig. 1). These results are significant in the light of recent results of ERICKSON and BRAUN (1968) which suggest that DNA synthesis in transformed cells is synchronous and that the efficiency of transformation is, in some way, dependent on genome replication (see page 179). In addition, the density transfer experiments showed that only 50% of the genomes in the competent cell population were being replicated, a result similar to those reported by MCCARTHY and NESTER (1967).

V. The Interaction between Transforming DNA and the Competent Cell

A. Binding of DNA by Competent Cells

As mentioned previously, the addition of transforming DNA to a competent recipient culture results in the rapid binding of the DNA to the cells (FOX and HOTCHKISS, 1957; GREEN, 1964; LEVINE and STRAUSS, 1965). The extent of this initial binding is proportional to the competence of the culture (LERMAN and TOLMACH, 1957). This rapidly bound DNA forms a reversible complex with the competent cell, reversible in the sense that it can be removed by simple washing, environmental manipulation of DNAase treatment (FOX and HOTCHKISS, 1957; LERMAN and TOLMACH, 1957; LEVINE and STRAUSS, 1965). Washing and enzyme treatment have been shown to act on the DNA-cell complex at different stages of the transformation process. LERMAN and TOLMACH (1957) studied transiently bound DNA in the pneumococcal system and found that after washing by centrifugation the remaining ^{32}P-labeled DNA was released into the medium at a rate proportional to the temperature. This rate of release was greatly increased by the addition of DNAase. The DNA released by warming the culture remained acid-precipitable in contract to the acid-soluble material released after DNAase treatment. In the *B. subtilis* transformation system a washing procedure allowed a linear rate in the increase of transformed cells beginning at the time of DNA addition while after DNAase treatment a lag of 1.0 to 1.5 minutes was observed and the rate of appearance of transformants was non-linear (LEVINE and STRAUSS, 1965). The length of this lag period was shown to be independent of the marker studied, the concentration of donor DNA and the presence

of genetically unmarked competitive DNA, but dependent on the temperature, a decrease in the temperature increasing the lag period.

If the transformation reaction is treated as a simple enzyme-substrate reaction it can be calculated that the rate constant for the dissociation of the competent cell-DNA complex is 30 times greater than the rate constant for irreversible incorporation (Fox and Hotchkiss, 1957). The temperature dependent rate at which the transforming DNA attains a DNAase-insensitive state is indicative of the involvment of an enzyme reaction or series of reactions. Calculations based on an Arrhenius plot of the relationship between length of lag and temperature in the *B. subtilis* system permitted an estimation of the activation energy required for this reaction (i.e., reversible binding DNAase insensitive binding), the calculated energy of activation being 13.9 kcal (Levine and Strauss, 1965).

The configuration that the DNA molecule assumes during the process of attainment of the DNAase-insensitive state has been investigated in both the *B. subtilis* and the pneumococcal transformation systems. Strauss (1965) has pointed out that the lag period observed in *B. subtilis* transformation may represent the time required for a DNA fragment to assume a structure whereby it can be taken up *en masse* or it may represent the time required for a sufficient length of DNA molecule to gain the DNAase-insensitive state, the latter explanation implying a lengthwise attainment of such a state (Strauss, 1965). The latter event, believed to reflect the mode of entry, was suggested by the observation that after exposure of the DNA-bacteria complex to DNAase at various times after the addition of transforming DNA, the rate of appearance of transformants was non-linear.

Proof for a linear entry of the DNA molecule into the competent cell was obtained by two simple, but elegant, techniques. As stated previously, the duration of the lag period characterizing the appearance of various transformant classes after DNAase treatment was not a function of the marker studied but was a constant and at 25° C was equal to 2.5 minutes (Strauss, 1965). If similar studies were carried out with linked genetic markers, it was observed that although there was a 2.5 minute lag for the appearance of the individual single transformants, the lag period required for the joint entry of the two linked markers varied directly with the map distance between them. In other words, the farther apart the individual loci were on the DNA molecule, the more time required for both markers to reach the DNAase-insensitive state. Since the individual transformant classes demonstrated equal lag periods, it appearred as though the DNA molecule attained the DNAase-insensitive state in a lengthwise manner and the direction of entry was randomly determined (Strauss, 1965).

Gabor and Hotchkiss (1969) used a slightly different technique in demonstrating a linear entry of DNA into competent pneumococci. The transformation reaction was terminated by two procedures, DNAase treatment and swamping the recipient culture with genetically unmarked DNA. These procedures differentiated between markers having entered the cell and those either

unattached or attached but not DNAase-insensitive. The results obtained for a number of linked markers showed that the rates at which single markers escaped the action of DNAase varied and that the order of this escape was related to their linkage relationship. Thus, in pneumococcus, not only do the molecules enter in a lengthwise manner, but the direction of entry may be predetermined.

The pneumococcal system also differs from the *B. subtilis* system in the fact that, although there is a similar lag in appearance of transformants using DNAase inactivation, the rate of single marker transformations is linear (KENT and HOTCHKISS, 1964). These kinetics have been used in the argument for a single DNA fragment causing a transforming event. STRAUSS (1965), on the other hand, has presented three other factors that could explain the non-linear kinetics observed in *B. subtilis* transformation. First, as incubation time increases the probability that a fragment of DNA carrying the genetic marker has entered the DNAase insensitive state increases. Second, the larger the DNA fragment the greater the efficiency of the recombinational event. And third, the rate of incorporation may vary from cell to cell.

The nature of the DNAase-insensitive state has not been thoroughly investigated. It apparently has been assumed that it involves entry into the interior of the cell, implying a lengthwise penetration of the cell wall and plasma membrane. Thus, accepting this interpretation, "uptake" or irreversible binding are synonymous with entry into the cytoplasm. As previously described, autoradiographs of tritium-labeled transforming DNA irreversible bound to competent cells show activated grains in the region of the plasma membrane (WOLSTENHOLME et al., 1966; YOUNG, 1967a) and it has been demonstrated in these studies that the majority of the disintegration tracts remain at membrane sites for a considerable length of time after DNA addition. If one assumes that these autoradiographs represent potential transformants, then it appears that in the *B. subtilis* transformation system the DNAase-insensitive location is on, or within, the membrane structure and not within the cytoplasm or nucleoid body.

Such results are difficult to explain in light of the work of other investigators. BODMER and GANESAN (1964) exposed competent recipient cells (labeled with ^{32}P, ^{2}H and ^{14}N) to transforming DNA (labeled with ^{31}P, ^{2}H, ^{3}H, and ^{15}N) for 30 minutes, subjected the donor recipient complex to pycnographic fractionation on CsCl and assayed the resulting fractions on a recipient strain that allowed them to differentiate donor and recipient genetic activity. Their results showed physical association of the donor and recipient genetic material after this brief incubation time and the genetic studies utilizing DNA re-extracted from the recipient cells at various intervals after DNA exposure suggested that maximum linkage was extablished after only 10 minutes incubation and that donor material not integrated after 30 to 60 minutes was rapidly destroyed. In addition, similar genetic studies by NESTER and STOCKER (1963) demonstrated that linkage between donor and recipient DNA was constant by 30 minutes. The latter authors point out, however, that

these interpretations are based on the assumption that the DNA extraction procedure does not discriminate between the exogenotic and endogenotic markers and that both DNA specimens in the re-extracted preparation are equally efficient in the ensuing transformation reaction. The validity of this assumption has never been critically examined and has been questioned by EPHRUSSI-TAYLOR (1960) in the pneumococcal system. She has suggested that the donor DNA might form a strong complex with some cellular constituent which would decrease its extractability or transformability. These alternatives are especially pertinent to *B. subtilis* transformation in view of the association of DNA and membrane in autoradiographs and the lack of unequivocal evidence for single-stranded DNA after exposure of transforming DNA to competent recipient cells.

If the interpretation that linkage is established rapidly is correct, then it implies that the autoradiographic studies may have been of cells destined to result in abortive transformation or that the activated grains resulted from non-transformable DNA fragments. Recent work in a number of laboratories, however, strongly suggests that the actual integration of transforming DNA does not occur immediately in *B. subtilis*. If genetically inert DNA is added to the recipient cell-DNA complex up to 4 hours after the initial time of donor DNA exposure, a very significant reduction in the frequency of transformation is observed (REILLY, 1965; KAMMEN et al., 1966). KAMMEN and associates labeled this phenomenon "displacement" and noted that while denatured DNA had little effect, *E. coli* DNA was a better competitor for the cell binding sites than genetically unmarked *B. subtilis* DNA. These experiments are strong support for the hypothesis that the majority of DNAase-insensitive transforming DNA molecules remain at membrane sites for a considerable length of time before they actually enter the interior of the recipient cell. If this is not the case, then non-homologous DNA is capable of replacing integrated regions of the genome with great efficiency, a very unlikely alternative.

There has been some disagreement regarding the nature of the initial association between the transforming DNA and the recipient cell. YOUNG (1967a) has exposed competent cells to ^{3}H-labeled DNA, washed the cells thoroughly and then removed the cell wall with lysozyme in the presence of stabilizing concentrations of sucrose. He then has pelleted the protoplasts and has determined the distribution of label between the sedimented protoplasts and the solubilized cell wall material. The data showed that the removal of the cell wall does not significantly reduce the amount of adsorbed DNA on the protoplasts and YOUNG concluded that DNAase resistant DNA is bound firmly to the membrane. These results have been confirmed in similar experiments by others (BODMER and LAIRD, 1968; ERICKSON et al., submitted for publication). In another related experiment, however, MILLER and LANDMAN (1966) reported that most of the labeled transforming DNA was released upon protoplasting. The major differences in these studies were that in the latter experiments the transforming DNA was labeled with ^{32}P [and therefore may

have been contaminated with other cell products (YOUNG and JACKSON, 1966)] and the recipient cells were allowed to incubate for 40 minutes after DNAase treatment. If such an experimental variable is responsible for the conflicting experimental results, the experiments may indicate that the binding properties of the transforming DNA-recipient cell complex may change with time and what has previously been thought of as "irreversible binding" may actually become reversible after an undefined period of incubation (e.g., after at least 40 minutes).

Such a hypothesis is compatible with the explanation of the displacement of DNAase-resistant DNA by genetically inert DNA proposed by KAMMEN et al. (1966b). They suggest that cell-associated DNA exists in two states, firmly bound to membrane sites and compartmentalized between cell wall and plasma membrane. Transgression of the membrane may be the rate-limiting step in the transformation reaction and the number of sites on the membrane at which penetration may occur may be rather small. This means that at any time after DNA addition the majority of the DNA fragments are not firmly bound to the membrane but are compartmentalized in such a way as to render them inaccessible to the DNAase molecules. This association is not irreversible and the adsorbed DNA molecules might compete with the subsequently added DNA for the limited number of membrane sites. It is of interest to note that the phenomenon of displacement does not appear to exist in the pneumococcal transformation system. In the studies of GABOR and HOTCHKISS (1966) on the linear entry of the DNA molecule into competent pneumococci, the reaction was terminated by either the addition of DNAase or an excess of genetically unmarked DNA to the recipient culture. The two methods differentially reduced the further production of transformants shortly after DNA addition, but after a short incubation period (only about 10 minutes) both methods produced identical results. Thus, in this system, DNAase-resistant binding also signifies refractivity of the bound DNA to competition for binding sites by exocellular DNA.

The structure of the membrane-DNA complex has been investigated by a number of methods. VENEMA et al. (1965a) have reported that shortly after the adsorption of DNA to the recipient cells they were unable to recover donor DNA transforming activity. Upon further incubation (approximately 20 minutes) the donor marker recovered its transforming activity and, at this time, it also demonstrated linkage to the recipient cell's markers. Before the donor marker regained its genetic activity it was shown to demonstrate altered heat resistance and renaturation abilities relative to the recipient markers (VENEMA et al., 1965b). It was suggested that this could be due to the denaturation of the transforming DNA upon irreversible binding. ERICKSON et al. (1968) have presented additional data that suggests that cell-associated DNA exhibits single-stranded characteristics. They employed antibodies specific for single-stranded DNA [the specificity appears to reside in the base moieties of the polynucleotides (PLESCIA et al., 1964)] in studies on the inhibition of transformation in *B. subtilis*. As expected, the antibodies did not react with

native transforming DNA and maximal inhibition required a pre-exposure of the recipient cells to the antibody. These results suggested that the inhibition reaction occurred within the cell boundaries and that at some step in the transformation reaction the DNA exhibited denatured characteristics (i.e., the bases were exposed) (BRAUN et al., 1965; ERICKSON et al., 1968). Subsequent work has shown that if the recipient cell-DNA complex is first exposed to DNAase, reincubated and then exposed to antibody at the optimal time of binding of the protein, up to 90% of the expected transforming events could be reversed (ERICKSON et al., submitted for publication). This result implies that the structure of the cell-DNA complex is such that the phosphodiester bonds of the polynucleotide chain are protected from enzymatic hydrolysis while the purine and pyrimidine bases are exposed and capable of interaction with antibody. Such a configuration might result from ionic bonds between the phosphate groups on the DNA backbone and charged proteins on the membrane, perhaps mediated by Mg^{++} ions. This type of binding would weaken the α-helical DNA structure since the hydrophobic bases would be repelled by the hydrophilic outer membrane layer, and the resulting configuration might expose the bases to the antibody molecule.

This interpretation does not require that the entire DNA molecule unwinds or becomes single-stranded, but rather that short segments lose their rigid α-helical configuration and become partially denatured. This consideration is important since pyconographic separation of differentially labeled donor and recipient DNA has shown no significant material corresponding to the density expected of denatured donor DNA (PENE and ROMIG, 1964; BODMER and GANESAN, 1964). This might indicate that the re-isolation and deproteinization procedures allow the adsorbed DNA molecules to regain their original configuration. It is also possible that the deproteinization procedures could have removed all membrane-associated transforming DNA (including single-stranded DNA) and only a portion of the total adsorbed DNA is being analyzed in the reported data.

In contrast to these results, data accumulated on the pneumococcal system again suggest a more efficient genetic exchange mechanism. A definite "eclipse" period (i.e., the inability to recover donor DNA transforming activity shortly after DNA addition) has been shown to exist and has been characterized quite well. In this system the recovery of donor transforming activity has a half-time of about 3 minutes (FOX, 1960). This eclipse period has been explained by LACKS and associates as being due to the immediate degradation of one strand of the double helix and the conservation and subsequent utilization of the remaining strand. With the aid of chromatographic separation (LACKS, 1962) or by the use of alkaline CsCl gradients (LACKS et al., 1967), it was shown that pneumococcal and *E. coli* DNA are rapidly degraded into *equal* amounts of dialyzable fragments and single-stranded DNA. The donor pneumococcal DNA isolated during the eclipse period could be renatured to single-stranded "helping DNA" (i.e., DNA not carrying the specific marker being studied) and regain its activity. This renaturability decreased rapidly

with time, indicating completion of the integration process (GHEI and LACKS, 1967) if one assumes single-stranded integration (for justification of this assumption see BODMER and LAIRD, 1968).

Integration in the pneumococcal system has been demonstrated to occur within 10 minutes after the initial binding reaction (FOX, 1960) and the transformed region appears to become functional at this time (LACKS and HOTCHKISS, 1960). In addition, the newly transformed DNA segment replicates at the same rate as the total DNA of the recipient population [using increase in transforming activity of donor and recipient markers as a measure of DNA synthesis (FOX and HOTCHKISS, 1960)]. There have been numerous reports in the literature, however, suggesting that the transformed pneumococci demonstrate a lag in division and that some transforming events take place a number of generations after the binding of transforming DNA (HOTCHKISS, 1953, 1957; EPHRUSSI-TAYLOR, 1959).

The mechanism by which the transforming DNA becomes bound in a DNAase-insensitive manner is not known but the results presented for the pneumococcal system are highly suggestive of the involvement of a nuclease. The demonstration of the immediate conversion of native donor DNA to single-stranded DNA and dialyzable fragments [identified first as oligonucleotides (LACKS, 1962) and more recently as 5′-deoxynucleotides (LACKS et al., 1967)] strongly suggests that a cell-associated nuclease hydrolyzes one strand of the donor material while transferring the sister strand to the DNAase-resistant location. An enzyme with this specificity has been characterized in *E. coli* (RICHARDSON et al., 1964) and has been isolated from transformable pneumococci (LACKS and GREENBERG, 1967). KOHOUTOVA (1966) has also presented evidence which suggests a relationship between DNAase activity and penetration in pneumococcal transformation.

The case for a similar mechanism in *B. subtilis* rests on much less experimental data. The linear entry and required energy for DNAase-resistant binding appear somewhat analogous, as does the equivocal demonstration of an eclipse period; however, the contrasting results in efforts to find single-stranded material renders any attempt at complete correlation questionable. There is evidence for degradation of DNA by competent cells (BODMER and GANESAN, 1964; KAMMEN et al., 1966; YOUNG, 1967a) and a membrane-associated DNAase that preferentially degrades single-stranded DNA has been partially characterized (BIRNBOIM, 1966), but the quantitative results obtained by LACKS have not been reported with *B. subtilis* transformation.

B. Retention of Bound DNA by Competent Cells

If penetration of the plasma membrane and DNAase-resistant binding are synonymous or occur almost simultaneously, then conditions that stabilize the intermediate ionic complex are of secondary importance. On the other hand, this paper has emphasized the importance of the membrane-DNA complex in the *B. subtilis* transformation system and many workers have stressed the significant relation between the frequency of transformation

and environmental factors after the initial binding of DNA to the recipient cells has taken place (JENSEN and HAAS, 1962; KAMMEN et al., 1966). It is a common observation that if the DNA-exposed cells (potential transformants) are allowed to incubate in the transformation medium for extended periods there is a significant loss in the number of transformants with time. A similar reduction in the frequency of transformation is observed if the cells are subjected to increased ionic conditions at times well removed from the initial binding event (ERICKSON, unpublished results). If the potential transformants are diluted into fresh media (or, perhaps, placed on the appropriate agar medium for transformation assay), this loss of transformants can be reduced and after 3 to 4 hours incubation the cells can begin to divide (NESTER and STOCKER, 1864; KAMMEN et al., 1966a).

The observed loss of transformants could be due to two independent factors, release of DNA or death of potential transformants. KAMMEN and associates (1966a) have demonstrated that much of this reduction can be attributed to viable transformants that release bound DNA back into the medium. They exposed the recipient cells to ^{32}P-labeled DNA and observed a correlation between DNA release and transformant survival. In exploring the conditions that enhanced the cells ability to retain the bound transforming DNA they found that addition of a mixture of amino acids (containing both inhibitory and stimulatory amino acids as defined by WILSON and BOTT (1968) for the attainment of the competent state) appeared to stabilize the potential transformant-DNA complex for extended periods. In addition, high concentrations of puromycin were found to be bacteriostatic to the total culture yet stabilized the potential transformants in a manner similar to amino acid supplementation. Addition of the amino acid mixture to cultures exposed to lower, bacteriocidal concentrations of the drug protected only the transformant portion of the population from the lethal activity. These results suggested to the authors that polypeptide synthesis is required for the retention of cell-associated DNA and that the synthesis of this molecule, unlike the protein required for reaching the competent state, was not mediated by messenger RNA. The role of this polypeptide remains unknown but the authors hypothesized that it might be related to the polypeptide described by LARK (1966) for the conversion of a proreplicator to a replicator, conceivably involving membrane attachment site alterations.

McCARTHY and NESTER (1969) have recently demonstrated that a step involved with either the initial binding of DNA to the cell or the retention of the DNA at the surface site is temperature-dependent. The heating of competent cells at 50° C for 5 minutes reduced transformability by 90% and this loss was not due to cell death. In cultures that were exposed to the transforming DNA before heating the loss in transformants was much less, a result indicating that some degree of heat protection is conferred upon the donor molecule after binding. The authors suggested that a heat-sensitive factor is destroyed at 50° C and cannot be re-synthesized for about 3 hours. The recipient cultures had been diluted after heating, however, and it is difficult

to distinguish between re-synthesis of a "factor" and re-attainment of the proper physiological and environmental conditions for competence (i.e., early stationary phase of growth).

C. Penetration of the Plasma Membrane and Synapsis

Two very important juxaposition reactions remain to be discussed, the penetration of the donor molecule through the plasma membrane and the subsequent synapsis of the donor and recipient material. These problems are experimentally difficult to approach since, at present, they cannot be studied directly. In the past the terms binding and uptake were used interchangeably and though treatments that inhibited the binding of the DNA to the recipient cells were easily classified, those that allowed binding but not penetration and those that inhibited synapsis were indistinguishable. Thus, such treatments as subjecting the transforming DNA to subcritical heat inactivation (LACKS and HOTCHKISS, 1960; ROGERS and HOTCHKISS, 1961), UV-irradiation (SZYBALSKI and OPARA-KUBINSKA, 1965) and exposure to mild nitrous acid treatment (LITMAN, 1961) all block or inhibit post-binding events. Investigations of this nature could be of value in studying the penetration process if such treatments could be shown to differentially inactivate various markers on the transforming DNA molecules. For example, if a number of treatments could be shown unequivocably to inactivate genetic markers on the basis of their relative A-T content, then those treatments that continually inactivate all linked markers equally would be disrupting the penetration process (i.e., the DNA fragment as a whole cannot enter the recipient cell) while those treatments that differentiate between linked markers would be interfering with synapsis. From some of the data cited above, it appears as though UV-irradiation and subcritical heat inactivation might allow penetration and interfere with synapsis while treatments such as nitrous acid treatment or exposure of the recipient cell-transforming DNA complex to antibodies specific for single-stranded DNA (ERICKSON et al., 1968) may not allow the DNA molecule to transverse the membrane. These observations are of interest since they suggest that structural alterations that interfere with either the denaturability of DNA or its single-stranded structure inhibit the penetration of the DNA molecule through the membrane.

Recent work has also demonstrated that cell structure can be altered in such a way as to allow binding of the transforming DNA to recipient cells but not penetration. RICHARDSON and LEACH (1969) observed that the addition of 0.05 % phenethyl alcohol to competent cultures did not affect viability or growth but inhibited transformation from 50 to 70 %. This treatment was shown not to inhibit the initial binding reaction but the subsequent steps to DNAase-resistant binding (DNAase treatment removed 95 % of the bound DNA). The presence of the phenethyl alcohol reduced the transport of many small molecules (e.g., serine, uracil and thymidine) but after washing, the transport of these molecules returned to normal while DNA penetration remained inhibited. This report indicates that the binding and penetration

events are dissociable and further experiments into the nature of the membrane alterations induced by phenethyl alcohol would be of considerable importance in defining the steps involved in DNA entry.

In the previously discussed work of TICHY and LANDMAN (1969) it was reported that protoplasts can bind transforming DNA but that no transformants are found in such tests. From this data they have suggested that removal of the cell wall might disorganize the membrane structure (specifically the mesosomes) and the cell wall is required for maintaining the proper membrane configuration. Many interpretations of data pertaining to DNA entry assume that the competent cells elaborate a specific mechanism solely for the binding and uptake of highly polymerized exocellular DNA and that the respective treatments that inhibit transformation (e.g., heating, antibiotic treatment, enzyme exposure, etc.) destroy or impair this specialized structure. Although such models have undeniable relevance to the penetration process, it should be emphasized that normal membrane structure is sensitive to such treatment (CRONAN, 1968) and that the stability and permeability of such "untreated" cell surfaces may be responsible for DNA uptake.

Whatever the actual uptake mechanism may be, it is apparent that reorganization of the plasma membrane is a required step for DNA entry. It is known that membrane alterations may greatly influence the reaction of a cell to its environment (HENNEMAN and UMBREIT, 1964). The biochemistry of membranes, especially bacterial membranes, is poorly understood at present and is undoubtedly of profound complexity (WEISS, 1963), a fact which may account for the avoidance of any serious consideration of membrane transitions in DNA binding and penetration. A new concept of membrane structure is replacing the older unit membrane theory, a view which no longer assigns definite sites for the protein and lipid components, but proposes that the structure is composed of lipoprotein subunits, the composition of which may vary from cell to cell and from location to location within an individual cell (KORN, 1968). Some studies have been reported in which the composition of the *Bacillus* membrane has been investigated and it is of interest to note that membrane composition may vary with the growth conditions (OP DEN KAMP et al., 1965) and that membrane stability is dependent upon the presence of Mg^{++} (BROWN, 1965). It would be of great value to the further understanding of bacterial transformation if a detailed study was initiated on the relation between membrane structure and DNA binding.

VI. The Relation between Efficiency of Transformation and the Replication Map of *Bacillus subtilis*

Results discussed up to this point have suggested that in the *B. subtilis* transformation system the transforming DNA molecule remains at the membrane binding sites for prolonged periods. Since a membrane-associated DNAase has been characterized in *B. subtilis* (BIRNBOIM, 1965), it appears feasible that DNA degradation may well account for the previously described

reduction in transformants and DNA release during extended periods of incubation after DNA addition (KAMMEN et al., 1966a). If this is true, and if we assume that each DNA fragment has an equal probability of transversing the membrane once bound, then the relative efficiency of transformation for individual unlinked genetic markers should be constant and independent of the age of the recipient culture. This has not been found to be the case (ERICKSON and BRAUN, 1966, 1968; COOPER and EVANS, 1968). These temporal changes in the efficiency of transformation were shown to be related to the order in which the markers are replicated on the *B. subtilis* chromosome. Since the following discussion regarding penetration and synapsis is based on the replication of the *B. subtilis* genome, it will be efficacious to first digress and briefly describe the mechanism of DNA replication in bacteria.

A. DNA Replication in *Bacillus subtilis*

The classical experiments of MESELSON and STAHL (1958) demonstrated that DNA replication in *E. coli* takes place in a semi-conservative manner and the labeling pattern produced implied an orderly unidirectional process. A replicating, complete *E. coli* genome isolated by CAIRNS (1963) showed that the structure was circular, was replicated from one growing point and that the process appearred to be sequential.

These observations suggested to YOSHIKAWA and SUEOKA (1963a, b) that the *B. subtilis* genetic system could be used to construct a replication map of the bacterial chromosome by two independent means. The first method, called marker frequency analysis, is based upon the prediction that if the genome does replicate from a fixed origin toward a single terminus, then in an exponentially growing culture the numbers of copies of markers situated close to the origin should be twice the number of copies of markers located close to the terminus. An approximation of the number of copies of each marker could be obtained by extracting DNA from an exponential culture and using the DNA to transform various auxotrophs. The complicating variable of integration efficiency could be removed by using DNA prepared from stationary cultures in which the majority of genomes (about 80%) are complete and not replicating, and using this preparation to normalize all transformation frequencies. These normalized values for the frequency distribution of various genetic markers could then be used to construct a map of the *B. subtilis* genome using the following mapping function: $g(X)=2^{1-x}$ (SUEOKA and YOSHIKAWA, 1965). If there exists only one replication point per genome and if the rate of synthesis per round of replication is constant, then the values for $g(X)$ can vary from 2 (markers near the origin of replication) to 1 (markers near the terminus of the replication map) and the position of the markers on the replication map [i.e., the value X which will vary from 0 (the origin) to 1.0 (the terminus)] will be an accurate representation of the linkage map.

The second and more direct method is based on the assumption that if genomes of stationary cells are in the completed form, then dilution into

fresh medium should imitiate synchronous replication of DNA molecule from the origin. Accordingly, YOSHIKAWA and SUEOKA (1963b) grew cells in an isotopically heavy medium (containing ^{15}N and D_2O) to the stationary phase of growth and then transferred the cells to light growth medium (containing ^{14}N and H_2O). Both heavy and light media contained ^{32}P-labeled phosphate of equal specific activity. Samples were removed at various times during the second growth period, lysates prepared and centrifuged in a CsCl solution. Transforming activity of the radioactive gradient fractions could be determined and the rate of transfer of various genetic markers from heavy, parental molecules to intermediate, hybrid molecules could be related to the rate of transfer of the total ^{32}P-labeled material to material of hybrid density, the data producing a map representing the sequence in which the genetic markers were replicated. Results of the marker frequency analysis and the isotopic

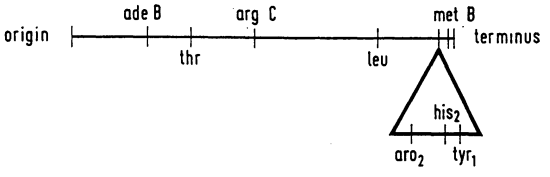

Fig. 1. Replication map of the *Bacillus subtilis* genome. This linear map represents the sequence in which the various genetic markers are replicated and does not imply that the *B. subtilis* genome is comprised of a single linkage group. The direction of replication is from origin to terminus. (See DUBNAU et al., 1967, for details and an expanded map)

transfer experiments were in complete agreement and, with some modification, the results seem to correspond to the *B. subtilis* linkage map (DUBNAU et al., 1967) (see Fig. 1).

Investigations into the nature of the replication point have yielded a number of interesting results. GOLDSTEIN and BROWN (1961) found that the major portion of DNA synthesis in *E. coli* occurred in a "particulate fraction", an observation suggesting disorganization of the α-helix in the replicating region. Subsequently it was shown that the DNA polymerase was bound quite firmly to the DNA in both *E. coli* (KADOYA et al., 1964) and *B. subtilis* (GANESAN and LEDERBERG, 1965). GANESAN and LEDERBERG grew thymine-required *B. subtilis* in ^{14}C-thymidine for a number of generations and then exposed the cells to a very short pulse of ^{3}H-thymidine before stopping further synthesis with azide. Fractionation of cell lystaes on sucrose gradients showed that the newly synthesized DNA was associated primarily with the particulate, rapidly sedimenting fraction. These investigators also showed that if the ^{3}H-thymidine pulse was subsequently chased by further growth in a medium containing ^{14}C-thymidine, the tritium label was displaced from the rapidly sedimenting fraction and appeared in the supernatant. In addition, treatment of the particulate fraction with a proteolytic enzyme altered the nascent DNA material to a more slowly sedimenting complex (GANESAN, 1967).

Electronmicrographs of lysed protoplasts of *B. subtilis* show the bacterial chromosome to be firmly bound to the plasma membrane (GANESAN, 1967). In some cases in which membranous material is not evident, flagella are found in the area of the DNA masses. GANESAN has suggested that this is an indication of the presence of small fragments of cell membrane and concludes that the membrane-DNA complex may be the site of DNA synthesis. His observation coincides with the previously mentioned association of nucleoid body with the mesosome (JACOB et al., 1963; RYTER, 1968) and also may explain how the large polymerase molecule can function in the compact nuclear region (MAALØE and KJELDGAARD, 1966). The machinery for replication of the genome appears to remain fixed to specific membrane-associated sites and the DNA duplex appears to pass through this enzyme complex during replication, as first suggested by JACOB.

One of the remaining problems in bacterial DNA replication is related to the biosynthesis of the anti-complementary strand of the Watson-Crick α-helix. If the replication of the genome takes place at one site, from origin to terminus, then the chains must be extended in both directions (i.e., 3′ to 5′ and 5′ to 3′) simultaneously; however, there is no evidence for synthesis taking place in the 3′ to 5′ direction. Two diverse theories have been presented to explain the dilemma. One proposes that the polymerase molecule is a dimer (or tetramer) and the functional groups are oriented in such a way that synthesis can take place in both directions simultaneously (CAVALIERI and CARROLL, 1968). The other model suggests that although DNA synthesis is sequential, it may be discontinuous (OKAZAKI et al., 1968). In other words, unwinding of the α-helix takes place initially, followed by 5′ to 3′ synthesis toward the replication fork on one strand and 5′ to 3′ synthesis away from the fork on the complementary strand. Evidence for this model is found in the observation that if one isolates the growing point of the genome, the nascent DNA is found in short unconnected chains. This material also appears to be single-stranded, implying that either the newly formed units are single-stranded or are abnormally unstable. These shorter segments may then be united to form a continuous structure by a ligase type enzyme (GELLERT, 1967).

B. Studies on the Temporal Changes in Efficiency of Transformation

ERICKSON and BRAUN (ERICKSON, 1968; ERICKSON and BRAUN, 1968) studied the dependence of transformation on stages in DNA replication of the recipient cells. Their studies were conducted in the following manner: cells were made competent by transfer of a stationary phase, spore-derived culture to fresh enriched medium, aliquots of the culture were then removed at various times after transfer, transformed by exposure to DNA, for a variety of non-linked markers (e.g., *purB, thr, argC, leu, metB*), and the relative efficiency of transformation was calculated for each marker at each time interval as a function of the total number of transformants for the interval. The data showed that, during the time period tested, markers located near

the origin of replication (*purB*, *thr*, and *argC*) were all increasing in relative efficiency of transformation while the terminal marker (*metB*) was decreasing in efficiency. Since the temporal pattern of transformation efficiencies corresponded to the replication order, the authors hypothesized that some early event in the transformation reaction was dependent on DNA synthesis and that genome replication was synchronous in the competent cells.

Some predictions could be based on such an interpretation of the data. For example, linked markers should demonstrate "in-phase" fluctuations in efficiency of transformation when compared to a non-linked reference marker. Also, one should observe a definite pattern of congression [i.e., the co-transformation of two physically non-linked markers (NESTER et al., 1963)]. This latter prediction was first advanced by BODMER (1966) who reasoned that if some step in the process of transformation was dependent on the stage of replication of the recipient genome, then one would anticipate that a co-transfer of two non-linked markers would be less likely to occur when one of the marker regions had passed the replication point in contrast to the situation in which both the marker regions are approaching the replication point. Both of these predictions have been shown to be correct (ERICKSON and BRAUN, 1968).

The one discrepancy that was observed in these tests was that the relationship between congression and the estimated stage of DNA replication was not always as unambiguous as that initially presented (ERICKSON and BRAUN, 1968). This problem could be overcome by studying the pattern of congression during an extended interval and could be due to either the replication point being at an intermediate position [i.e., just passing the region in question (ERICKSON, 1968)] or to the dependence of the efficiency of congression on the map distance between the markers being investigated. This latter possibility would suggest that a linkage map (or replication map) could be constructed from transformation data in a more classical manner by simply studying the frequency of single and double transformant classes for a variety of non-linked markers. Preliminary results (ERICKSON and COPELAND, unpublished observations) indicate that this is the case and fairly accurate representation of the linkage map is derived from such data. In addition, the more recent results explain why our original observations were not affected by this factor since the three markers studied in the earlier investigators, *thr*, *leu*, and *metB*, are almost equi-distant on a circular replication map.

The relation between the changes in efficiency of transformation and the replication order of the genome has suggested two important properties of competent *B. subtilis* cells, namely an occurrence of marker decay at membrane binding sites and synchrony of DNA synthesis in competent recipient cells. Evidence for the first assumption is not concrete and is based on the observation that the co-transfer index (NESTER and LEDERBERG, 1961) for two closely linked markers is not a constant but varies with the time of DNA addition. Thus, if one can calculate the expected position of the replication point by unlinked marker analysis, then one should observe that when the

region of the linked markers appears to be transforming most efficiently, the co-transfer index should be at a maximal value and then drop abruptly. This appears to be the case for the markers *his2* and *tyr1* (ERICKSON, 1968). Evidence for the synchrony with respect to DNA synthesis has been presented previously (page 169) and, additionally, GANESAN and BUCKMAN (1969) have recently reported that transformants divide synchronously for at least two generations.

The data on the replication of the bacterial genome suggest two attractive interpretations concerning the relation between DNA synthesis and transformation. First, it is possible that the membrane-bound transforming DNA is pulled into the recipient cell during replication of the recipient genome by regions of homology between donor and recipient molecules. Second, integration may take place during replication. Both hypotheses are theoretically attractive but there is some evidence that neither is correct. In the following section the cases for and against these theories will be presented.

Whether penetration or integration is the DNA synthesis-dependent step, both explanations imply that synapsis only occurs at the membrane site during some stage in the replication process. This implication is interesting for a number of reasons. Synapsis may only be able to take place with structures exhibiting single-stranded characteristics. The evidence for the single-stranded nature of the donor molecules has been presented and evidence for partially denatured regions of DNA *in vivo* has been described (ROSENBERG and CAVALIERI, 1964; OKAZAKI et al., 1968). Denaturation of the recipient DNA may only occur during transcription and/or replication and, perhaps, these processes may only take place on DNA regions that are in contact with the cytoplasm (i.e., regions at the surface of the nucleoid body). This consideration is based on calculations of MAALØE and KJELDGAARD (1966) on the dimensions within the bacterial nuclear region. They have reasoned that since the nuclear region appears to be a highly ordered structure (FUHS, 1965), one could construct a model of nuclear organization and then calculate certain nuclear dimensions. Their data suggest that within the nuclear body the distance between the axes of adjacent DNA strands is roughly to Å, or only 30 Å of free space. These dimensions create a number of organizational problems including the possibility that the polymerase molecules might be too large to function within the nucleus, a consideration that led MAALØE and KJELDGAARD to propose that these enzymes may only be active at the external boundaries of the nuclear region. In view of the fact that a DNA molecule has a diameter of about 25 Å and a length many times the size of the nuclear body, it is difficult to envision the donor molecule "searching out" its homologous segment on the recipient genome by simple and random movement. A mechanism whereby the donor molecule waits for the homologous region at the membrane binding sites and, when both molecules exhibit denatured characteristics, synapsis occurs sequentially with replication, is very plausible.

Such an interpretation is analogous to a proposed mechanism of DNA transfer in the *E. coli* conjugation system. PITTARD and WALKER (1967)

observed that markers near the origin of the donor DNA are integrated with a much higher efficiency than markers distant from the origin and they suggested that this fact may reflect an obligatory interaction between the leading edge of the donor DNA and recipient DNA. CURTISS (1966) has found that the rate of DNA transfer during conjugation is independent of the physiology of the donor cell and dependent upon the physiology of the recipient. He suggests that the donor material must first interact with the homologous DNA segment of the recipient and is then pulled into the recipient cell. This "winding in" of the donor material requires energy but no DNA, RNA or protein synthesis. The independence of transfer and DNA synthesis is difficult to reconcile with the transformation model discussed above unless one assumes that even in the absence of DNA synthesis the genome is constantly in motion at a membrane site. In contrast to these results, BONHOEFFER (1966), using temperature-sensitive mutants controlling DNA synthesis, has found that DNA synthesis in the recipient is essential for transfer.

A similar mechanism of DNA entry could be operative in bacterial transformation. Binding of the transforming DNA could be localized near the region of DNA synthesis and the interaction between donor and recipient material may induce membrane transition that would allow the donor strand to penetrate the membrane subunits. If homology is lacking, the donor DNA would be bound by the recipient cell but would not penetrate the membrane. It is known that competent *B. subtilis* cells do bind non-homologous DNA but do not integrate significant amounts of the exocellular material (LORKIEWICZ et al., 1961; BODMER and GANESAN, 1964). In addition, the exposure of competent *B. subtilis* cells to homologous transforming DNA greatly increases the rate of mutation while non-homologous DNA has no such effect (YOSHIKAWA, 1966). Since this increased mutability is most likely due to intracellular events, it may be that non-homologous DNA does not enter the cell.

The most convincing data indicative of an involvement of DNA synthesis in the penetration process is related to the studies on the inhibition of transformation by nucleic acid-specific antibodies (BRAUN et al., 1965; ERICKSON et al., 1968). In these studies it was observed that after addition of antiserum at different times in relation to the addition of transforming DNA, certain markers always "escaped" the action of the inhibitory antibodies at a faster rate than other genetic markers. As previously discussed, pre-exposure of the recipient cells to antibody was required for maximal inhibition of transformation and as the interval between antibody addition and DNA addition became less, the percentage inhibition decreased (i.e., the specific genetic marker being assayed escaped the action of the antibodies). It was noted that the rate of decrease was greatest for *leu* marker (relative to *ade*, *thr*, and *metB*) and it was initially proposed that different DNA fragments entered the cell at different rates (ERICKSON et al., 1968). It soon became apparent, however, that the age of the recipient culture determined which marker demonstrated the greatest rate of escape from inhibition. If DNA and antibody addition

were delayed it was observed that the *metB* marker escaped the action of the antibody at the greatest rate (ERICKSON, 1968). It can be seen from Fig. 1 that the replication of this marker occurs after the *leu* marker and supports the model. Extension of this study to different time periods proved impossible since inhibition of transformation by antibody only occurs during the early stages of competence.

Subsequent work utilizing a more reproducible test system has supported these results. The work of REILLY (1965) and KAMMEN et al. (1966b), suggested that the displacement reaction should produce results similar to the antibody results since the non-homologous DNA appears to compete for entry with the transforming DNA (or at least for membrane penetration sites). The results of such displacement experiments on recipient cultures of varying age have shown that if a genetic marker is being transformed most efficiently it is less likely to be inhibited by subsequently added *E. coli* DNA than those markers being transformed less efficiently (ERICKSON and BRAUN, in preparation). The results were compatible to a model that assumes that as a recipient marker passes a membrane-associated transforming DNA fragment, the latter is pulled into the cell.

An alternative interpretation of the changing efficiencies of transformation is that integration takes place during replication and, regardless of the mechanism of penetration, the most efficient time for the transformation of a given marker is the time its homologous segment on the recipient genome is being replicated. This relationship was first suggested by BODMER (1965) when he observed, in a series of physical and genetic studies using differentially labeled donor and recipient DNA, that donor material first became associated with recipient DNA exhibiting an anomalous density. He initially postulated that this material might have represented density transition points implying that integration occurred preferentially at pre-existing replication points creating recombinant molecules that were partially hybrid. More recent experiments did not confirm his original hypothesis and BODMER now considers the intermediate density material to represent either replication points that have been blocked at the point of integration or replication points initiated at the site of integration (BODMER and LAIRD, 1968; LAIRD et al., 1968).

A possible relationship between replication and integration allows the construction of a number of possible mechanisms of integration. One model is based on the recent discovery that newly synthesized DNA may be thermally unstable and fragmented (OKAZAKI et al., 1968). If both the transforming DNA and the recipient DNA are partially denatured and in small fragments, then it is plausible that the homologous single-strands (donor and recipient) may compete for pairing with the complementary strand before the ligase enzyme covalently links the strands together.

A related model is based on the demonstration that about 5% of the recipient DNA in competent *B. subtilis* cells is single-stranded (HARRIS and BARR, 1969). Since DNA synthesis in competent cells is retarded or stopped (see page 163), this denaturation may be related to the DNA degradation

observed when DNA synthesis is halted in *B. subtilis* by exposure of the cells to high concentrations of actinomycin D (FARMER, 1968). FARMER suggested that this degradation occurs at the replication point and destroys one of the daughter strands. Hence, one might postulate that the transforming DNA fragment replaces the degraded section on the recipient chromosome by some type of repair process (HARRIS and BARR, 1969). On the other hand, integration may take place during replication since the transforming DNA segment represents a preformed completed piece of suitable DNA.

Implicit in these interpretations is the prediction that the integrated donor material should be found in regions of the recipient genome that have been replicated after the addition of transforming DNA. GANESAN (1967) has presented evidence that this may not be the case. He added 5-bromodeoxyuridine to a (*thy*$^-$, *trp*$^-$, *phe*$^-$) auxotroph during competence in the presence of low concentrations of tritium labeled (*thy*$^-$, *trp*, *phe*$^-$) donor DNA and the absence of the required amino acid. These conditions would limit the use of donor material as DNA precursors, halt DNA replication in all cells after completion of one round of genome replication and also label all newly synthesized DNA with the base analogue. DNAase was added after 10 minutes, the cultures were washed and reincubated in the presence of the same concentration of 5-BdU. After 300 minutes incubation it was reported that the cells had finished one cycle of DNA replication and had not started another. Pycnographic separation of cell lysates showed both tritium activity and donor (*trp*) genetic activity equally distributed between light and hybrid density bands. Since the density distribution of genetically recombinant molecules was similar to the donor marker activity, the investigator concluded that integration occurred randomly with respect to regions of DNA synthesis. This intepretation assumes that competent cells have insignificant thymidine pools and that amino acid deprivation in *B. subtilis* does not allow re-initiation of DNA synthesis (see ANRAKU and LANDMAN, 1968 and COPELAND, 1969).

GANESAN and BUCKMAN (1969) studied the effect of 5-fluorodeoxyuridine on transformation and noted that inhibition of DNA synthesis had little effect on transformation or DNA uptake, results indicating the independence of these reactions from DNA synthesis. On the other hand, STEWART (1968) has reported that phleomycin [a selective inhibitor of DNA synthesis (TANAKA et al., 1963)] reduced the frequency of transformation 10-fold. These discrepancies may be due to the length of time DNA synthesis was retarded since, as the displacement phenomenon suggests, the integration of much of the adsorbed DNA does not take place for 2 to 3 hours after DNA addition and it is possible that differences in experimental proceudre could greatly influence the results.

ARCHER and LANDMAN (1969a, b) have presented data that they claim "clearly rule out the hypothesis that DNA enters only by a process involving homology at the replication fork". They starved a recipient population for a required amino acid for many hours and found that such a population could become competent (ARCHER and LANDMAN, 1969a). After demonstrat-

ing that the recipient population of genomes was aligned with respect to the origin of replication, they then exposed the cells to transforming DNA and compared the results of such synchronized cells with non-synchronized systems. By testing transformation for three non-linked markers they reasoned that if DNA synthesis was involved in the entry process they would observe differences in the relative frequencies of transformation of the various classes of transformants. In a more critical test, they protoplasted DNA-exposed recipient populations, treated with DNAase and again assayed the various classes of transformants. In both cases they observed no differences between the synchronized and non-synchronized populations and concluded that homology at the replication fork was not responsible for DNA entry (ARCHER and LANDMAN, 1969b).

There were a number of critical assumptions made in this paper that may be questioned and therefore make the data less unequivocal than suggested by the authors. Some of these are: 1. competent cells in a non-synchronized culture are replicating their genomes in a random manner; 2. all DNA is removed from the cell exterior upon protoplasting and DNAase treatment; and 3. the markers *trp* and *thy* are significantly removed from each other to produce the desired results (data of DUBNAU et al., 1967; suggest they are separated by only 3 % of the genome). In addition, as mentioned on page 72, there is some question as to whether or not amino acid-starved *B. subtilis* cells have aligned chromosomes. Needless to say, the relation between DNA synthesis and transformation is not clear and, undoubtedly will be the subject of additional study.

VII. A Model of Some of the Early Stages in Pneumococcal and *B. subtilis* Transformations

The pneumococcal transformation system appears to be a very efficient mechanism of genetic exchange. A model, based upon the work of TOMASZ and LACKS, is illustrated in Fig. 2. At some point in the growth of the culture a small segment of the total population begins to produce the competence activator. This activator is a surface constituent and also a diffusible substance that spreads rapidly throughout the culture and induces other cells to produce the activator protein. All cells in the population are capable of interacting with the activator. At the peak of competence this activator is found on the surface of all cells in the recipient culture and is involved in the initial interaction between cell and transforming DNA. Immediately upon binding, an exonuclease degrades one of the strands of the DNA duplex and passes the sister strand into the interior of the cell. This entry process takes place in a linear manner, from one end of the donor molecule to the other. This material very quickly escapes the action of competitive DNA and is integrated very soon after the initial interaction. A repressor then appears in the culture, inactivates exocellular activator and competence rapidly declines as the cell-associated activator decays.

Although such a model is also plausible for the *B. subtilis* transformation system, another model is also compatible with much of the accumulated data and is represented in Fig. 3. As a *B. subtilis* culture approaches the stationary phase of the growth cycle one subset of the total population becomes physio-

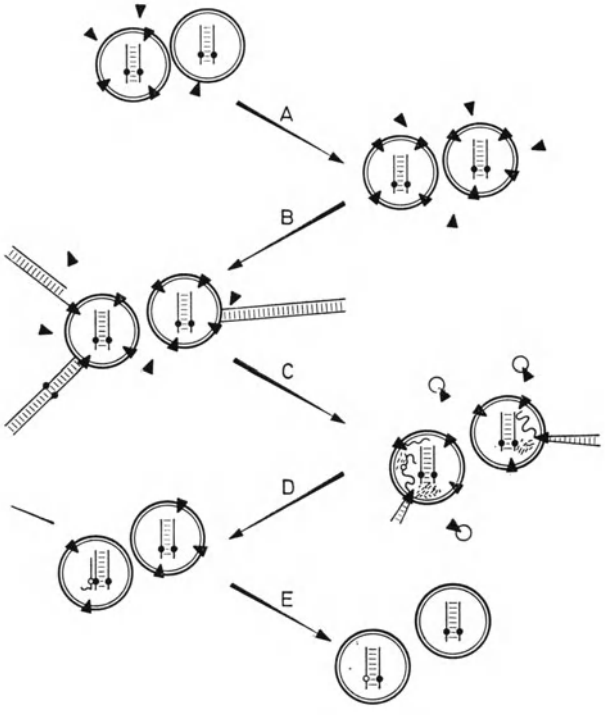

Fig. 2 A—E. A model for the regulation of competence in the pneumococcal transformation system. A Afew exceptional cells in a transformable culture begin to synthesize the protein competence activator. This cell product is a surface constituent and is also released into the excellular environment and interacts with other cells inducing these cells to produce the activator. Under optimal conditions all cells in a culture can respond to the activator stimulus. B In the presence of DNA, the competent cells bind the macromolecule (the interaction involving the cell-associated activator and an end of a DNA fragment) and rapidly begin to "pull in" the DNA to a DNAase resistant location in a linear manner. C The uptake of DNA involves an exonuclease which degrades one of the two strands while pulling the sister strand into the cell interior. This is the eclipse period and donor genetic activity can not be recovered because of its single-stranded nature. D The donor DNA strand searches for its homologous region in the cell interior. In the exocellular environment a competence repressor appears and inactivates free activator. At the same time the cell-associated activator begins to decay. E The cells are no longer competent and transformed cells are heterozygous

logically distinct. These cells are non-dividing, their biosynthetic capabilities are reduced and they have a distinct electrokinetic potential. This fraction of the population may be in such a physiological state that the unfavorable stationary phase growth conditions have suspended the cell division cycle at a critical point, perhaps in the process of cell division. In this state portions

of the plasma membrane are exposed, predominantly in the plane of cell division where the autolytic enzymes are preparing the cell wall for the addition of new sub-units. It is in this area that the transforming DNA is initially

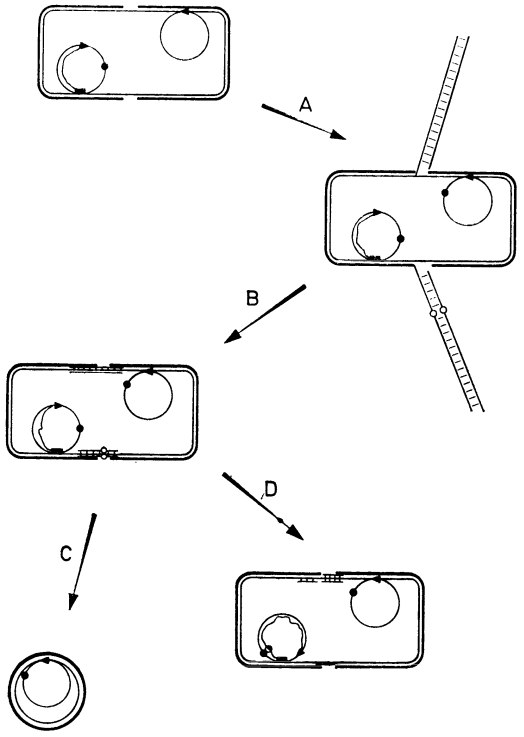

Fig. 3A—D. A model for the appearance of competence in a *Bacillus subtilis* culture. A As the culture enters the stationary phase of the growth cycle and conditions become unfavorable for growth, a segment of the population is caught between cell divisions. Since the cells are preparing for sporulation one genome is complete (the potential spore genome) while the other is partially replicated. Protein synthesis continues in the absence of significant nucleic acid synthesis, cell wall structure is weakened in the area of cell division and the charge distribution at the cell surface changes appreciably. B In the presence of DNA and Mg^{++} the cells rapidly bind the exocellular macromolecule at the exposed plasma membrane. The DNA fragments become DNAase resistant in a linear manner and become strongly complexed to the membrane. C If the recipient cell-DNA complex is left in the exhausted growth medium and spores are formed, no transformants are found (i.e., the potential transformant genome is not the potential spore genome). D If the cell-DNA complex is transferred to fresh media, the sporulation process is aborted, DNA synthesis resumes and penetration and/or entry can then occur. As time passes, hydrolysis of the membrane-associated DNA occurs, the strands become less firmly bound and can be replaced by subsequently added DNA in the displacement reaction. Cell division is also taking place at this time and the cells begin to become less competent

bound to the cell, the binding mediated by cationic bonds. The first stage of the interaction involves one end of a transforming DNA fragment and the cell membrane, the DNA being fixed to the membrane in a lengthwise manner. The binding may involve the negatively charged ribose phosphate

backbone, leaving the bases exposed, weakening the helical structure and conferring single-stranded characteristics upon the cell-associated molecule.

Located near the membrane binding sites is the cellular organelle responsible for the replication of the genome. Since the cells are in the act of dividing, the genomes (at least one genome per cell) are about one-half way through a round of replication and segments of the replicating material may be partially denatured and associated with the interior surface of the plasma membrane. If the transforming DNA-recipient cell complex is transferred to a medium more conducive to growth (i.e., transferred to a fresh medium or spread on an agar medium), DNA synthesis resumes, membrane transitions occur and the donor and recipient molecules interact. If the cells are not transferred to a fresh medium spores will be formed and, since sporulation only involves completed genomes, none of the spores will possess the transformed character [this has been shown to be the case by BOTT (personal communication)]. Hence, the genome that is partially replicated is the potential transformant genome and if allowed to remain in the exhausted growth medium the recombinant genome will be lost and the process of genetic exchange or the resulting increased mutation rate would have served no significant evolutionary purpose. Thus, transformation of vegetative cells in *B. subtilis* may actually be the result of unusual growth conditions created in the laboratory.

If this is correct, a question may be raised concerning the ultimate value of investigations into the nature of the competent state in *B. subtilis* transformation. This investigator feels quite strongly that such investigations are of great importance, for although a well integrated mechanistic model is intellectually pleasing and less ambiguous, it must be remembered that models are only simplified abstractions. If the knowledge gained from the study of competence is ever going to be used in genetic manipulations of higher organisms one is going to be dealing with cells possessing even more sophisticated uptake mechanism than those postulated for pneumococcal and *B. subtilis* transformation and any insights derived from a system such as that presented for the *B. subtilis* transformation system may be of great value in the recognition of evolutionary stages leading to highly evolved mechanisms of integration of exogenous DNA into a cell's genome.

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