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Molecular Biology of the Adeno-Associated Viruses[1]

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With 6 Figures

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I. Introduction

The adeno-associated viruses (AAV) are small, defective, DNA viruses which require co-infection with an adenovirus for productive infection (ATCHISON et al., 1965; HOGGAN et al., 1966; SMITH et al., 1966; PARKS et al., 1967). They have also been termed adenovirus-associated virus, adeno satellite virus, and adeno-associated virus satellite virus. AAV particles were first noted as smaller particles contaminating adenovirus preparations when the latter were observed in the electron microscope (ARCHETTI and BOCCIARELLI, 1963, 1964, 1965; BRANDON and MCLEAN, 1962; HOGGAN, 1965; HULL et al., 1965; MELNICK et al., 1965). Although initially considered to be cellular debris, or precursors or breakdown products of adenovirus, AAV was subsequently shown to be a separate virus which was structurally, immunologically, and genetically distinct from adenovirus (ATCHISON et al., 1965; HOGGAN et al., 1966; SMITH et al., 1966; PARKS et al., 1967). The defectiveness of AAV with the subsequent requirement of an adenovirus as a helper was also shown at that time by these groups. Initially four distinctive serotypes of AAV were identified, AAV 1–3 from humans and AAV 4 from monkeys (see HOGGAN, 1970).

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Subsequently, similar small viruses have been found associated with bovine, avian, and canine adenoviruses (Luchsinger et al., 1970; Dutta and Pomeroy, 1967; Yates et al., 1972; Domoto and Yanagawa, 1969). It would appear that any adenovirus can serve as a helper for AAV infection in any cell which is permissive for the adenovirus. For example, a chicken adenovirus will permit human AAV to productively infect either chick embryo fibroblasts or embryonated eggs (Blacklow et al., 1968).

Although AAV is ubiquitous in human populations which have been investigated with up to 50% of people being seropositive (Atchison et al., 1966; Blacklow et al., 1968), the virus has not been implicated as the causal agent in any human disease. Many of the facts concerning the biological and epidemiological properties of AAV as well as the various experimental techniques used in studying this virus have been extensively reviewed by Hoggan (1970). It is the aim of this review to emphasize recent findings concerning the structure and replication of this unusual virus.

II. Virus Structure

The AAV virion is icosahedral with a diameter which has been estimated to range from 18–28 nm (Atchison et al., 1965; Hoggan et al., 1966; Mayor et al., 1965; Archetti and Bocciarelli, 1964, 1965; Crawford et al., 1969). The number and arrangement of the capsomeres is uncertain. Estimates of the number have ranged from 12–20 (Mayor et al., 1965; Archetti and Bocciarelli, 1965), but one group reported that the capsomeres were not individually distinguishable as such (Smith et al., 1966). Chemical studies have revealed that the virion consists of DNA (20%) and protein (80%) (Parks et al., 1967). The relatively high DNA content gives the virion a density in CsCl ranging from 1.388–1.44 g/cm^3 which makes it readily separable from the helper adenovirus (density=1.35–1.36 g/cm^3) (Hoggan, 1971). The reported S value of 104 for AAV is consistent with the notion of a spherical virion of the size and composition described above (Crawford et al., 1969).

A. DNA

The DNA of any given AAV serotype will hybridize approximately 33% with the DNA of any of the other serotypes as judged by DNA : RNA hybridization assays using nitrocellulose filters (Rose et al., 1968). Further studies by Koczot (1970) using competitive hybridization have shown that the related sequences are the same for all four serotypes. More recent competitive DNA : DNA hybridization studies have indicated that the homology among the different serotypes is closer to 50% (Koczot, personal communication).

The structure of AAV DNA has proved to be unusual and complex. There was initial uncertainty as to whether the DNA was single- or double-stranded. Early studies on purified DNA seemed to indicate it was duplex in nature, but staining of viral preparations with acridine dyes seemed to indicate that the DNA within the virion might be single-stranded (Mayor and Melnick,

1966). Careful studies on extracted viral DNA by ROSE et al. (1966) and PARKS et al. (1967) proved that the purified DNA was indeed double-stranded. The composition of the DNA was determined to be 55% GC with G = C and A = T. The DNA had a sharp melting curve with a Tm (92° C in 0.15 M NaCl, 0.015 Na citrate) which was consistent with its base composition. Exposure to denaturing conditions produced an increase in the buoyant density of the DNA in CsCl and the DNA sedimented through linear sucrose gradients with an S value (15–16) consistent with a double-stranded molecule with a molecular weight of $3–3.6 \times 10^6$. Observations of the DNA in the electron microscope were also consistent with a linear duplex molecule of approximately 3.0×10^6 M.W. The simplest conclusion thus was that the DNA existed within the virion as a linear duplex. In retrospect the only incongruous fact was that the sedimentation profiles in sucrose gradients were indicative of some structural heterogeneity in that the profiles were broader than those of normal duplex DNAs and always had a skewed leading edge.

In 1969 CRAWFORD et al. published a study which was the impetus to our present better understanding of the structure of AAV DNA. These investigators carefully studied the physical properties of the intact virion using sedimentation velocity, density in CsCl, and electron microscopy measurements, and came to the conclusion that AAV virions were nearly the same size and chemical composition as the parvovirus, minute virus of mice and bacteriophage ϕX 174. This conclusion suggested a possible paradox. Assuming that the above data were correct, the molecular weight of $3.0–3.6 \times 10^6$ determined for purified AAV DNA was almost exactly twice as great as would be expected for a virion of this size and composition. CRAWFORD et al. suggested as an "unlikely" explanation for this seeming paradox the possibility that there were two types of AAV virions which contained complementary (plus and minus) single-stranded DNA molecules. Upon extraction of the DNA the complementary single polynucleotide chains from the different particles would base pair to form double-stranded DNA.

Data supporting this "unlikely" hypothesis were rapidly forthcoming. ROSE et al. (1969) directly demonstrated that the complementary strands in double-stranded AAV DNA originated from different virions. A population of AAV virions containing DNA in which bromodeoxyuridine (BUdR) had been substituted for thymidine was mixed with a preparation of AAV particles containing unsubstituted DNA. Purification of DNA from this mixed population gave rise to three types of duplex DNA molecules which banded at heavy-heavy, heavy-light, and light-light positions in a CsCl density gradient in a ratio of 1:2:1. Re-exposure to the conditions of DNA extraction of a mixture of double-stranded DNA isolated from pure heavy particles with double-stranded DNA from light particles resulted in only heavy-heavy and light-light peaks in CsCl. MAYOR et al. (1969) and BERNS and ROSE (1970) were able to demonstrate that AAV DNA was contained within virions in the single-stranded form by purifying DNA under conditions which did not permit annealing of the complementary strands but which would also not result in

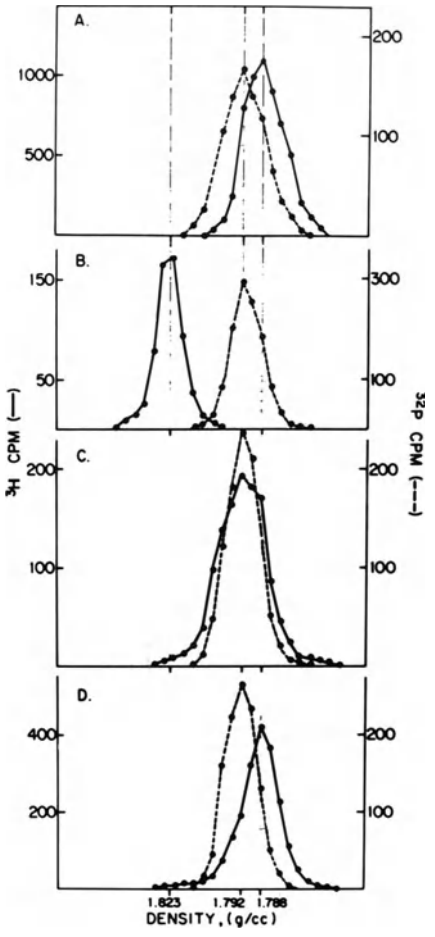

Fig. 1 A—D. Isopycenic CsCl centrifugation demonstrating the physical separation of the complementary strands of AAV DNA. Duplex ^{32}P BUdR-labeled AAV DNA was present in all gradients as a density reference. A Duplex ^{3}H BUdR-labeled AAV DNA. B Isolated heavy ^{3}H BUdR-labeled AAV single strands after self- annealing. C Isolated light ^{3}H BUdR-labeled AAV single strands after self-annealing. D The heavy and light ^{3}H BUdR single strands were mixed and then annealed. They now band at the initial density of the original duplex ^{3}H BUdR-labeled AAV DNA. Reprinted by permission of J. Virol. **5**, 693–699 (1970)

denaturation of double-stranded AAV DNA. BERNS and ROSE were also able to separate the complementary strands of AAV DNA by physical methods. Banding in CsCl of denatured AAV DNA in which BUdR had been substituted for thymidine led to the separation of two DNA species (Fig. 1) which would not self-anneal (as assayed by sedimentation behavior). Mixture of the two species prior to annealing led to the formation of duplex AAV DNA. ROSE and KOCZOT (1971) determined the base compositions of the separated strands. The overall base composition of duplex AAV DNA was A, 22.3%;T, 24.4%; G, 26.9%; C, 26.4%. The comparable figures for the strand which banded at

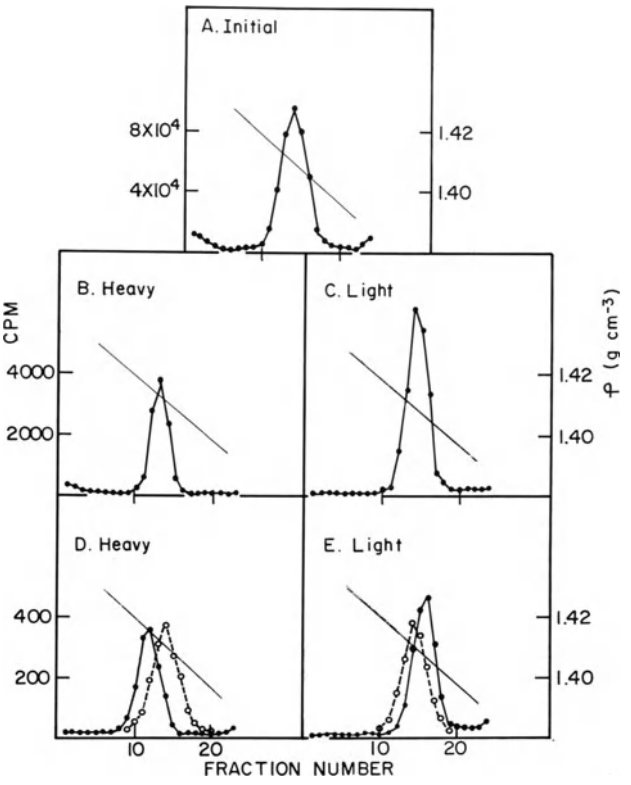

Fig. 2A—E. Isopycnic CsCl centrifugation demonstrating the separation of two classes of ^{32}P BUdR-labeled AAV virions with different densities. A Unfractionated virus. B Fractions 9–12 (heavy) from A were pooled and resedimented. C Fractions 16–19 from A (light) were pooled and resedimented. D Fractions 11 and 12 (heavy) from B were pooled, and a sample was resedimented in the presence of unfractionated ^{3}H BUdR-labeled AAV as a density marker. E Fractions 16 and 17 (light) from C were pooled, and a sample resedimented in the presence of unfractionated ^{3}H BUdR-labeled AAV as a density marker. Symbols: ^{32}P (●), ^{3}H (○). Reprinted by permission of J. Virol. 9, 394–396 (1972)

the heavy position in CsCl when labelled with BUdR were A, 20.5%; T, 26.5%; G, 26.7%; C, 26.3%; and for the light strand A, 25.2%; T, 21,7%; G, 26.6%; C, 26.5%. Thus it was clear that the heavy strands had a 22.3% greater T content which would cause these strands to band at a greater buoyant density when BUdR was substituted for thymidine. Taking advantage of this fact, BERNS and ADLER (1972) were able to separate the two types of AAV particles by labeling the DNA with BUdR (Fig. 2). Particles which banded at a heavier-than-average density in CsCl after several cycles of centrifugation did indeed contain the heavy complementary strand while those banding at a lighter density contained the light complementary strand. Thus it is now clear that the AAV virion contains a single-stranded DNA genome. For reasons that are not known both complementary DNA strands are encapsidated into separate particles in approximately equal numbers.

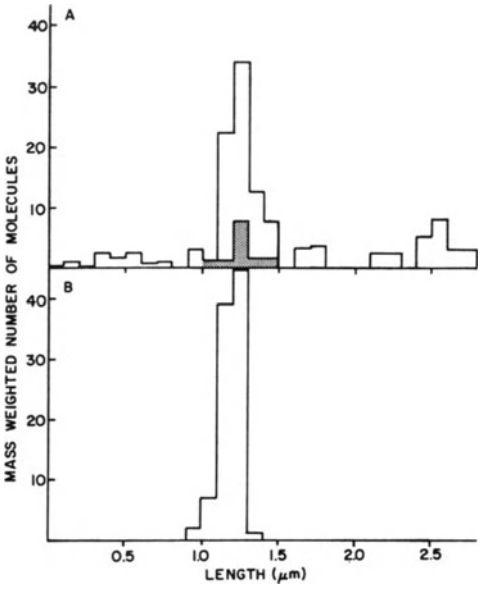

Fig. 3 A and B. The weight-average length distribution of duplex AAV DNA molecules observed in the electron microscope has been plotted. A An unfractionated preparation. Circular molecules are shaded. B Fractions representing the major peak in a neutral sucrose gradient were observed. Reprinted by permission of J. Molec. Biol. **79** (1973)

Although initial observations in the electron microscope were consistent with the notion that double-stranded AAV DNA was a linear molecule with a molecular weight of $3.0–3.6 \times 10^6$, as stated above (PARKS et al., 1967); it is now known that the complementary strands may form additional structures including duplex circles and oligomers (KOCZOT et al., 1973; GERRY et al., 1973). Studies of such duplex structures have led to the conclusion that the nucleotide sequence of purified AAV DNA is permuted in a highly restricted sense as detailed below. Recent work in our laboratory (GERRY et al., J. molec. Biol., 1973) has led to the discovery of at least two additional reproducible peaks in the leading edge of the duplex AAV DNA profile obtained by sedimentation though neutral sucrose gradients. Electron microscopy studies (Fig. 3) have indicated that the major peak in sucrose, which contained approximately one half the total DNA, corresponded to linear duplex molecules with a molecular weight of 2.8×10^6 when compared to T7 bacteriophage DNA. The two additional peaks observed in sucrose corresponded to duplex circular molecules of unit length and to linear duplex dimers. All duplex AAV DNA species observed were composed of unit length linear polynucleotide chains. Thus, in some way, duplex linear circles and linear dimers were held together by hydrogen-bonded overlapping regions. Linear monomers obtained from the major peak in sucrose did not form duplex circles of linear dimers when further exposed to annealing conditions. However, it was also possible to obtain duplex linear monomers by heating linear dimers at a temperature

10–20° C below the Tm of duplex AAV DNA. These duplex linear monomers did form duplex circular monomers and linear dimers when exposed to annealing conditions. Thus, annealing of intact linear AAV single polynucleotide chains led to the formation of two types of duplex linear monomers; (1) those without cohesive single-stranded termini, and (2) those with cohesive single-stranded termini.

The length and polarity of the hydrogen-bonded overlap regions of duplex circular monomers were investigated by limited digestion with 3′ and 5′ exonucleases. The results of these experiments indicated that one half the molecules with sticky ends had 5′ cohesive termini and the other half had 3′ cohesive termini (Table 1). In either case the sticky end represented less than 6% of the length of the genome. Because it was possible to demonstrate that linear AAV single polynucleotide chains were not randomly circularly permuted, it was concluded that AAV DNA contains a limited number of nucleotide sequence permutations, possibly only two, the start points of which occur within a limited region representing less than 6% of the length of the genome. Annealing complementary single strands containing a limited number of nucleotide sequence permutations of this type would lead to the formation of linear duplex monomers, some of which would have no cohesive termini and others of which would have either 3′ or 5′ cohesive termini (Fig. 4). The termini of AAV DNA were further characterized with regard to the presence of a natural terminal nucleotide sequence repetition. Duplex linear monomers from the major peak of a sucrose gradient did not cyclize (<1%) when annealed. After limited digestion (1%) with exonuclease III from *E. coli* up to 35% of the duplex linear monomers did cyclize when exposed to annealing conditions (Fig. 5). This was considered to be strong evidence for the existence

Table 1. Exonucleolytic digestion of duplex AAV circular Monomers

Enzyme	Extent of Digestion (%)	% Circles Remaining
3′-Exonuclease[a]	1	97
	1.6	87
	2.9	55
	5	47
	8	60
	16	61
	17	30
5′-Exonuclease	5	43
	8	47
	15	50
5′-Exonuclease + 3′-Exonuclease	7	7
	7	

[a] Composite of several experiments.

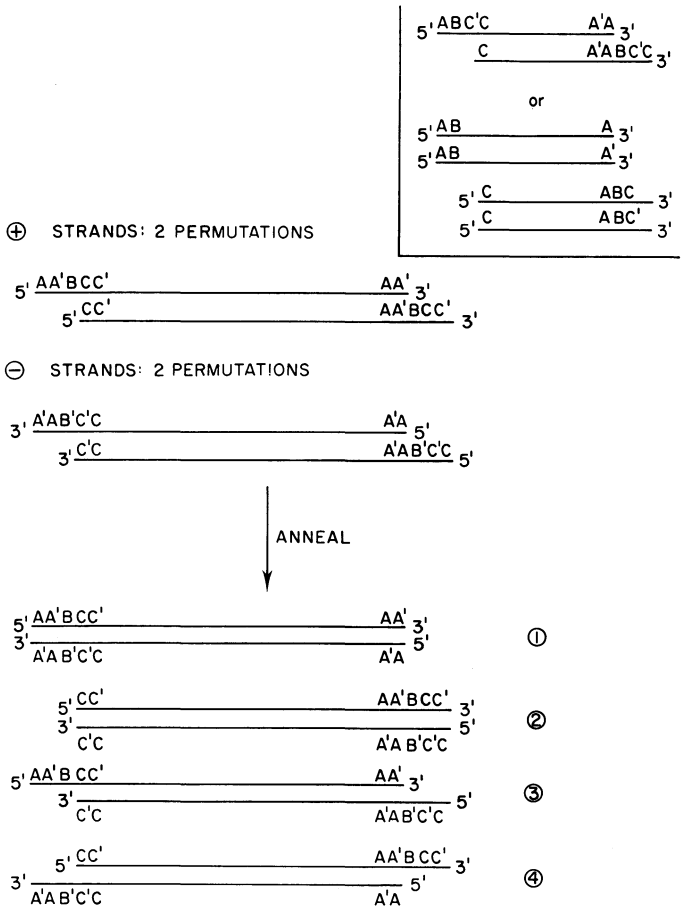

Fig. 4. A model of the nucleotide sequence arrangement contained within AAV DNA. Two nucleotide sequence permutations are illustrated. Plus and minus strands may anneal to form duplex linear monomers with (3 and 4) or without cohesive 3′ or 5′ termini (1 and 2). Duplex linear monomers with cohesive termini can then form duplex circular monomers or duplex linear oligomers. In the figure the terminal repetitions are depicted as symmetrical nucleotide sequences. In the inset two alternative types of terminal repetitions are illustrated; the first has the inverted repetition subterminal to the natural repetition, the second illustrates the possibility that a strand may have either an inverted or a natural terminal repetition

of a natural terminal repetition in AAV DNA of the type found in linear bacteriophage DNAs (Gerry et al., J. molec. Biol., 1973).

Additional study of the termini of AAV DNA by KOCZOT et al. (1973) has shown that AAV DNA also contains an inverted terminal nucleotide sequence repetition of the type found in adenovirus DNA (GARON et al., 1972; WOLFSON and DRESSLER, 1972). This conclusion was based upon the fact that up to 70% of separated plus or minus strands of AAV DNA formed single-stranded circles when annealed and these circles were converted to linear molecules by exonuclease III digestion. It was hypothesized that such single-

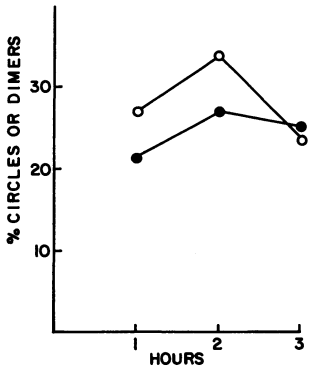

Fig. 5. Duplex AAV linear monomers which did not form circles or oligomers when exposed to annealing conditions (< 1 %) were digested to the extent of 1 % using exonuclease III and annealed in 2 × SSC (0.30 M NaCl, 0.03 M Na citrate) at either 45° C (●) or 55° C (○) for various periods of time. The percentage of circles and dimers formed is plotted. (No dimers were observed at 55° C)

stranded circles were held together by base-pairing of the termini of the single polynucleotide chains in the antiparallel configuration. If the base paired regions holding the single-stranded circles together were of sufficient length, it should have been possible to observe them under the electron microscope as a region of duplex DNA extending from the single-stranded circles. Although KOCZOT et al. (1973) were able to observe projections from the hydrogen-bonded single-stranded AAV circles, they were concerned that these might have represented artifacts. BERNS and KELLY (1974) were able to demonstrate projections which occurred at specific sites along the single-stranded circular molecules produced by annealing the isolated plus linear AAV single polynucleotide chains (Fig. 6). These projections corresponded to approximately 1.5% of the length of the DNA and thus offered a reasonable estimate of the length of the region of inverted terminal nucleotide sequence repetition. This length was quite close to the estimate of 1% of the length of the genome determined for the natural terminal repetition by GERRY et al. (J. molec. Biol., 1973).

There are three possible models to account for the data which indicate the existence of both inverted and natural terminal nucleotide sequence repetition in the population of purified AAV DNA molecules (Fig. 4). One possible structure would be that the terminal nucleotide sequence repetition is symmetrical. This possibility would be in accord with the fact that the lengths determined for both types of terminal repetition are similar. Two other alternatives are possible. The first is that the inverted and natural terminal repetitions occupy different positions along the genome. In that case the data of BERNS and KELLY would probably tend to overestimate the length of the inverted nucleotide sequence repetition if it were subterminal. Likewise the estimate of the length of the natural terminal repetition (1%) by GERRY et al. would be too great if the natural terminal repetition were subterminal. An unlikely third alter-

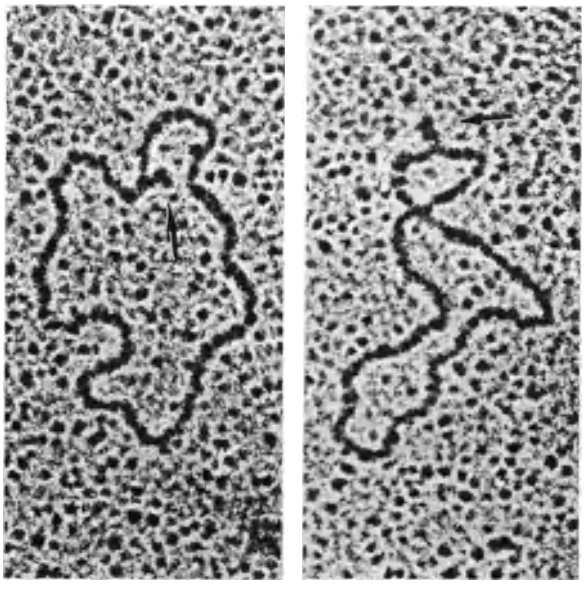

Fig. 6. Electron micrographs of hydrogen-bonded single-stranded circles formed when purified linear heavy AAV single polynucleotide chains are exposed to annealing conditions. The arrows point to the projections observed on such single-stranded circles

native consistent with available data would be that 70% of AAV single strands contain an inverted terminal repetition and 30% a natural terminal repetition. At this point it is not possible to distinguish among these possibilities.

The current model of the purified DNA is that it is a linear single polynucleotide chain containing a limited number of nucleotide sequence permutations, the start points of which occur within a region representing less than 6% of the genome, and also containing a terminal nucleotide sequence repetition (either inverted, natural, or both).

This model would tend to indicate that the DNA is linear within the virion.

B. Proteins

The structural proteins of AAV particles have been dissociated with sodium dodecyl sulfate (SDS) or urea and then separated by polyacrylamide gel electrophoresis (JOHNSON and HOGGAN, 1971; ROSE et al., 1971). Three proteins were identified with estimated molecular weights of 62–66000; 73–80000; and 87–92000, respectively. The smallest polypeptide represented about 80% of the total protein mass while the other two represented approximately 10% each. There was no correspondence in electrophoretic mobility with adenovirus structural proteins except that there was a partial overlap of the major AAV protein and the adenovirus fiber penton. The three AAV capsid proteins were present in all three human serotypes (AAV 1–3) and had similar mobilities with the exception that the major protein species of AAV 2 moved slightly faster than the comparable AAV 1 and 3 species. Very minor

components of both greater and lesser mobilities were noted but were not considered to be integral components of the capsid. There is evidence that some of the components of lesser mobility represent dimers and tetramers of the capsid proteins (JOHNSON and HOGGAN, personal communication). All the proteins contained equivalent amounts of arginine so that none appeared to represent a possible histone, although other basic amino acids were not measured (ROSE et al., 1971). The determined molecular weights for the AAV capsid proteins represented about twice the coding capacity expected for a single-stranded genome of molecular weight approximately 1.4×10^6. Several possible alternatives could account for this paradox: (1) one of the capsid proteins could be coded for by the host or by the helper adenovirus genome; (2) one large precursor polypeptide could be synthesized which could vary either at the point of initiation or of cleavage in subsequent processing; (3) some of the capsid proteins could be cleaved during virion purification.

Estimates for virion weight have ranged from $5.4–7.5 \times 10^6$ daltons. If the DNA represents 20% of the total mass, the total daltons of protein would be $5–6 \times 10^6$. The number of molecules of the major protein per virion has thus been estimated to range from 60–72. In a similar fashion the number of molecules of minor protein species per capsid would range from 5–8 for each of the two types.

AAV 1–4 are serologically distinct although AAV 2 and 3 cross-react (HOGGAN, 1970). Because the DNAS of the four serotypes have 30–50% of nucleotide sequences in common it might be expected that there would be some amino acid sequences of the structural proteins of the different serotypes which would be similar. Common amino acid sequences may not be exposed in the tertiary structure assumed by the capsid proteins. Support for this suggestion has been provided by the data of JOHNSON et al. (1972). These authors prepared antisera to the three structural proteins of AAV-3 after SDS treatment. Antisera to the SDS-polypeptides did not interact with intact virions, but the antisera raised against specific AAV-3 SDS-polypeptides did react with the analogous SDS-polypeptides of AAV-1 and AAV-2.

III. Virus Multiplication

It is not known whether one or both of the two types of AAV virions is individually capable of initiating a productive infection in the presence of adenovirus or whether both types of AAV particles are required. Titration studies of AAV infectivity in the presence of excess adenovirus have suggested single-hit kinetics for infection; this would imply that it is not essential for both types of co-infect a cell (BLACKLOW et al., 1967). Attempts to productively infect cells with physically separated particles (BERNS and ADLER, 1972) have been made but the data have not been of sufficient precision to resolve this problem (HOGGAN and BERNS, unpublished data). Purified AAV DNA has been shown to be infectious in the presence of helper adenovirus (HOGGAN et al., 1968). The infectivity of the DNA was increased by the addition of

DEAE-dextran (HOGGAN, personal communication). Because BERNS and ROSE (1970) have shown that it is possible to separate the complementary strands of AAV DNA when BUdR is substituted for thymidine and the BUdR-substitution does not markedly affect the infectivity (ROSE and HOGGAN, personal communications), it should be possible to test directly whether either strand is sufficient to initiate a productive infection.

A. Adsorption, Penetration, and Uncoating

The adsorption and penetration of AAV containing ^{3}H-thymidine-labeled DNA has been measured with KB cells in spinner culture (ROSE and KOCZOT, 1972; BERNS and ADLER, unpublished data). Adsorption reached a maximum and plateaued one to two hours after infection. Approximately 16–30% of the added viral label was irreversibly associated with the cells by this time. Depending on the method of cell fractionation, 25–45% of the bound radioactivity was eventually (by 20 hours) found in the nuclear fraction, the majority within two hours. Uncoating, as measured by sensitivity of the labeled DNA to DNase, took place in the nucleus. Maximum uncoating occurred by 16 hours post infection and represented approximately 85% of the total DNA (BERNS and ADLER, unpublished data).

AAV is usually recovered from infected cells using trypsin and deoxycholate. Sedimentation in CsCl leads to a major AAV band at a buoyant density of 1.40 g/cm^3 and a minor band at 1.467 g/cm^3. When sarkosyl was substituted for deoxycholate, the major AAV band in CsCl occurred at the same density as that of AAV purified in the usual manner. However, the amount of material in the denser minor band was increased. Virions in the minor band have been characterized as being smaller than AAV and probably have had some of the capsid stripped away (HOGGAN, 1971). After sarkosyl treatment even the AAV from the major normal density band in CsCl was unable to adsorb to KB cells (less than 10% of normal) implying that an intact capsid is required for adsorption (BERNS and ADLER, unpublished data).

Adsorption, penetration, and uncoating occurred to the same extent regardless of the presence or absence of helper adenovirus (ROSE and KOCZOT, 1972; BERNS and ADLER, unpublished data).

B. DNA Synthesis

AAV DNA synthesis has not been detected in the absence of helper virus infection. AAV-specific RNA synthesized *in vitro* has been used to detect AAV DNA sequences isolated from infected KB cells when both AAV and adenovirus were used to infect the cells (ROSE and KOCZOT, 1972). Under conditions of simultaneous infection of AAV and adenovirus, AAV DNA was first detectable at about 15 hours after infection which was the approximate time of onset of adenovirus DNA synthesis. If adenovirus infection preceded AAV infection by 10 hours, the onset of AAV DNA synthesis was 4–5 hours after AAV infection.

DNA replicative intermediates and modes of regulation have not been determined. Any model of the mechanism of AAV DNA replication would have to be able to account for the facts that the mature DNA is a linear single strand containing a limited number of nucleotide sequence permutations and both natural and inverted terminal nucleotide sequence repetitions. KOCZOT et al. (1973) have pointed out that a linear duplex AAV DNA molecule would have the same nucleotide sequence with the correct polarity at either end by virtue of the inverted terminal repetition and they have thus suggested this type of structure as a possible replicative intermediate. However, this type of intermediate *per se* would be inadequate if an RNA primer were involved in DNA synthesis, because there would be progressive loss of terminal nucleotide sequences with each round of replication as pointed out by WATSON (1972). Additionally, one would not expect the generation of the partially permuted nucleotide sequence reported for the mature DNA. If an initial linear duplex led to a concatenated duplex linear structure then both objections could be theoretically answered and the natural terminal repetition could play a role in the generation of such a concatemer (WATSON, 1972). Another possible model would involve a "rolling circle" mechanism of DNA replication (GILBERT and DRESSLER, 1968). A single-strand circle could be generated via the inverted terminal repetition and then sealed by means of a ligase present in the cell (SAMBROOK and SHATKIN, 1969). The circle could then act as a template for the generation of a single-stranded concatemer which could be sized and packaged by a variety of possible mechanisms.

C. RNA Synthesis

AAV-specific RNA synthesis does not precede AAV DNA synthesis (ROSE and KOCZOT, 1972). AAV RNA synthesis is first detectable approximately 2 hours after the onset of AAV DNA synthesis when AAV and adenovirus are used simultaneously to infect a cell. This is true even under conditions where adenovirus early messenger RNA synthesis is readily measurable. Thus the possibility exists that there is no class of AAV RNA synthesized prior to the onset of DNA replication. Again, if adenovirus infection preceded AAV infection by 10 hours, AAV-specific RNA could be detected starting at 5hours after AAV infection at the time of onset of AAV DNA synthesis (CARTER et al., 1973).

As stated previously, the complementary strands of AAV DNA have different thymidine contents (ROSE and KOCZOT, 1971). Therefore, when BUdR is substituted for thymidine the complementary strands have different boyant densities in neutral CsCl density gradients and may be separated by physical methods (BERNS and ROSE, 1970). AAV-specific RNA isolated from infected cells was able to hybridize only to the heavy BUdR-substituted DNA strand and had an average base composition which was identical to that of the light strand (low thymidine-content) (ROSE and KOCZOT, 1971). Thus, the authors concluded, that, within the limits of detectability, AAV RNA was transcribed

only from the complementary AAV DNA strand with higher thymidine content.

AAV-specific RNA has been isolated using extraction techniques designed to minimize breakage. Under those conditions the RNA had a mean molecular weight of 7.5×10^5 based on sedimentation through dimethyl sulfoxide gradients (Carter and Rose, 1972). This would correspond to 40–50% of the sequences in a single strand of AAV DNA. Using pulse-chase experiments, the authors noted no post-transcriptional cleavage. If the whole AAV genome were being transcribed, the size of the RNA isolated would imply that only two species of RNA molecules need be transcribed. In fact the AAV messenger may be a single molecule. Attempts to measure the fraction of total AAV DNA nucleotide sequence contained in AAV-specific RNA by means of DNA : RNA hybridization in solution have shown that the RNA only complemented approximately 35% of the DNA (Carter et al., 1972). These experiments were performed under conditions where both DNA strands were present but at a very low concentration so that DNA : DNA annealing was minimal (only 9% by the end of the experiment). By means of single strand-specific nucleases the authors directly determined the actual extent of base pairing. Because transcription had previously been shown to be from only one DNA strand, it was concluded that approximately 70% of the nucleotide sequences of this strand were represented in the *in vivo* AAV-specific RNA. If this data is accurate, the disparity between the coding capacity of the AAV genome and that necessary for the AAV capsid proteins mentioned previously would be increased.

D. Protein Synthesis

AAV protein synthesis (using antisera raised against SDS-polypeptides) has been detected approximately 2–4 hours after the onset of AAV-specific DNA fand RNA synthesis (Johnson et al., 1972). The antisera against the proteins from SDS-disrupted virions stained infected cells about two hours earlier than antiserum against intact virus, as determined by means of an indirect immunofluorescence assay. All three SDS virion polypeptides were detectable at nearly the same time during the course of infection, but the patterns of distribution in the infected cells were distinctive for antisera to each of the three different types of SDS polypeptides. The authors have termed the SDS-polypeptides VP-1 through 3 in order of increasing molecular weight (VP-1 is the major capsid protein). Anti-VP-1 stained the cytoplasm diffusely by 14 hours after infection; by 18 hours intranuclear areas were also stained. VP-2 antiserum stained discrete intranuclear areas and VP-3 antiserum diffusely stained the entire nucleus. Intact virion antiserum has been found to stain only the nucleus (Blacklow et al., 1967). The authors (Johnson et al., 1972) conclude that at least the major capsid protein appears to be synthesized in the cytoplasm, but that the virion is assembled in the nucleus. They suggested that possibly all virus proteins are synthesized in the cyto-

plasm, but that the concentration of the minor capsid proteins may have been too low to detect.

E. Defectiveness

As already indicated adsorption, penetration, and uncoating of AAV do not require helper virus and purified DNA is infectious but requires helper. However, in the absence of helper no DNA or RNA synthesis is detected. Therefore, a helper function(s) appears to be required to initiate AAV-specific nucleic acid synthesis. Co-infection with herpes simplex virus type 1 (HSV-1) also serves to allow AAV DNA or RNA synthesis (BOUCHER et al., 1971; ROSE and KOCZOT, 1972). The extent of AAV nucleic acid synthesis appeared to be comparable with either HSV or adenovirus as the helper. AAV DNA synthesized in the presence of HSV has been reported to be infectious (BOUCHER et al., 1971). Additionally, AAV antigens in cells co-infected with HSV are detectable by means of a fluorescent antibody staining technique (ATCHISON, 1970; BLACKLOW et al., 1970; BLACKLOW et al., 1971; JOHNSON et al., 1972). However, no infectious particles can be recovered with HSV as the helper. Interestingly, antisera to both AAV-specific SDS-polypeptides and virion protein stain the infected cells.

A possible conclusion is that adenovirus co-infection supplies a specific function which is required for virion assembly. Alternatively, however, HSV co-infection might inhibit a step in AAV assembly. There may be several functions between the initiation of AAV nucleic acid synthesis and virion assembly which require a helper virus supplied function. A recent paper by CARTER et al. (1973) presents evidence that separate helper functions may be required for both AAV DNA and RNA synthesis. In a simultaneous infection with AAV and adenovirus there is a lag of 2–3 hours between the onset of AAV DNA synthesis and then RNA synthesis. As mentioned, AAV DNA synthesis is initiated at the time of onset of adenovirus DNA synthesis and AAV RNA synthesis begins when late adenovirus RNA synthesis is initiated. If cells are pre-infected with adenovirus and AAV not added until late adenovirus messenger RNA synthesis has started, the lag between the initiation of AAV DNA and that of AAV RNA synthesis is greatly decreased so that it essentially disappears. Additional helper functions might well be required for translation of AAV messenger as well. The distinct possibility exists that the AAV genome codes only for the virion structural proteins, if, indeed, its coding capacity proves to be sufficient for that. Therefore, it may well be that all functions involving regulation of the replication of its genome and synthesis of its structural proteins may be supplied to AAV by the helper adenovirus. In this case it could be considered as the animal virus equivalent of the defective satellite virus of tobacco necrosis virus, the RNA genome of which has been shown to code only for the virion structural protein (CLARK et al., 1965).

F. AAV as a Provirus

As stated, AAV infection in the absence of helper virus is abortive. In nature, AAV has been found to be commonly associated with adenovirus in-

fection. Additionally, AAV seems to be able to establish a provirus state in cells in nature and in the laboratory. HOGGAN (1970) found that primary human embryonic kidney cells and African green monkey-kidney cells would release infectious AAV when exposed to purified adenovirus and were immunologically positive with regard to antisera. These cells were immunologically negative for AAV as well as producing no infectious particles prior to exposure to adenovirus. Approximately 1–2% of the human cells and up to 20% and greater of the monkey cells from various sources were positive when tested with helper virus in this way.

Although a previous report by MAYOR et al. (1967) indicated that the AAV genome did not replicate in BSC-1 (monkey) cells in the absence of helper virus, HOGGAN has been able to achieve an AAV provirus state in the laboratory, by exposing human Detroit 6 cells to AAV of any of the three human serotypes in the absence of helper. Such abortively infected cultures remain positive for the ability to release AAV when exposed to helper virus for over 100 passages (10^{100} dilution) although not every cell is still positive after the 100 passages (HOGGAN et al., 1972). Thus, AAV seems to behave analogously in biological terms to the papova viruses in that an abortive infection can lead to a provirus state from which infectious virus can often be rescued when the conditions again become permissive for the virus (*e.g.*, cell fusion with permissive cells for the papova viruses and infection with helper virus to rescue AAV). This does not seem analogous to a carrier culture infection because of the fact that AAV is strictly defective in the absence of a helper and there has been no evidence for the presence of a helper in these cultures. It is possible that the Detroit 6 cells contain a partial HSV genome which could function as a partial helper to promote AAV DNA replication, but PAGANO (personal communication) has not been able to detect any HSV nucleotide sequences by means of DNA renaturation-kinetics studies.

Attempts are under way to quantitate the number of any AAV nucleotide sequences which may be present in the DNA of cryptically infected Detroit 6 cells, using the method of annealing developed by BRITTEN and KOHNE (1968) and used by GELB et al. (1970) to detect simian virus 40 nucleotide sequences in SV 40-transformed cells. The assay measures the ability of DNA from appropriate Detroit 6 cell cultures to increase the rate of annealing of a given concentration of denatured AAV DNA. Results of preliminary experiments have been positive and indicate the presence of approximately one genome equivalent of AAV DNA per cryptically infected Detroit 6 cell (PINKERTON, BERNS, and HOGGAN, unpublished data). The manner of potential association of an AAV genome with that of the cell has not yet been investigated.

G. AAV Inhibition of Helper Virus

Co-infection by AAV and adenovirus reduces the yield of adenovirus. It is not certain whether inhibition results from the failure of some co-infected cells to produce any infectious adenovirus or whether all such co-infected cells

produce reduced amounts of adenovirus. Evidence has been presented to support both possibilities and the discrepancy is not yet resolved (HOGGAN et al., 1966; CASTO et al., 1967a, b; PARKS et al., 1968). As stated above, adenovirus infection may precede infection with AAV by 10–12 hours without affecting AAV replication except that the AAV eclipse period is shortened. Adenovirus DNA synthesis begins 10–12 hours after adenovirus infection. Infection after this time does not decrease adenovirus production (BLACKLOW et al., 1967). Thus one possibility is that AAV and adenovirus compete for a common function required for DNA synthesis, although quantitative data with regard to this point have not been reported.

AAV has also been reported to inhibit adenovirus oncogenicity (KIRCHSTEIN et al., 1968; GILDEN et al., 1968b; MAYOR et al., 1973). Adenovirus infection of neonatal Syrian hamsters leads to the formation of tumors. Co-infection of hamsters with AAV and adenovirus has been reported to reduce the frequency of tumors and to delay the detectable appearance of those which do occur. Tumor morphology was unaltered. The AAV present during the co-infection had to be viable to have this inhibitory effect (GILDEN et al., 1968a and b). A more recent study has found that pre-infection with AAV one day prior to adenovirus infection was more efficacious in preventing the tumors (MAYOR et al., 1973). Whether this timing effect and the overall ability of AAV to reduce adenovirus oncogenicity were related to the temporally dependent ability of AAV to inhibit adenovirus multiplication in tissue culture is unknown. The ability of AAV to inhibit adenovirus-induced oncogenicity was virus-specific. The inoculation of newborn hamsters with AAV did not prevent the formation of simian virus 40-induced tumors. Parvoviruses have been reported to cross the placenta to infect the fetus in experimental hamsters (KILHAM and MARGOLIS, 1969). Therefore, inoculation of pregnant hamsters with AAV to prevent adenovirus-induced tumors in the newborn was attempted, but was unsuccessful (MAYOR et al., 1973). If anything, the ability of adenovirus infection to induce tumours in female offspring was considerably increased.

IV. Concluding Remarks

The major specific advances in our knowledge of the molecular biology of AAV over the last four years have included the following: (1) the discovery that there are two types of virions containing complementary single polydeoxyribonucleotide genomes; (2) a detailed knowlege of the fine structure of the genome, including the concept of a limited number of nucleotide sequence permutations the start points of which occur close together within a restricted segment of the genome, and the determination that AAV DNA contains both natural and inverted terminal nucleotide sequence repetitions; (3) the findings that all messenger RNA is transcribed from one viral DNA strand and that there is no detectable RNA synthesis prior to the onset of DNA replication; (4) the fact that HSV will serve as a partial helper for the synthesis of AAV-specific DNA, RNA, and protein although assembly does not occur; (5) ex-

tension of the finding that AAV can cryptically infect cells both *in vivo* and *in vitro*.

Major distinct problems for experimental study in the near future include: (1) the question of whether both types of virions are capable of or necessary for productive infection; (2) a mechanism of DNA replication which will account for the fine structure of the DNA and the observation that approximately equal numbers of plus and minus strands are separately encapsidated; (3) determination of the portion of the amino acid sequence of the structural proteins which can be or are coded for by the viral genome; (4) the manner of association of the viral genome with latently infected cells and (5) further studies on the defectiveness of AAV.

AAV presents an excellent opportunity to study a defective virus-helper relationship. By the same token AAV infection minus the helper represents an abortive Infection in the same cell line which is the normal host in the presence of the helper. As seems to be the case in abortive infections by other DNA viruses which replicate in the nucleus, an AAV infection in the absence of helper (*i.e.*, abortive infection) leads to an apparently stable association of the viral genome with the host cell, which can be studied uniquely in the normally permissive host cell.

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Precommitment in the Immune System

IVAN LEFKOVITS*

With 5 Figures

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Abbreviations

B lymphocyte (or B cell), bone marrow-derived lymphocyte
BRC, burro red cells
BSA, bovine serum albumin
ChRC, chicken red cells
DNP, dinitrophenyl
Fab-anti MIg, monovalent Fab fragments of rabbit antimouse-Ig antibody
FγG, fowl gamma globulin
Fl, fluorescein (isothiocyanate)
G anti RIg, goat anti-rabbit-Ig antibody
GRC, goat red cells
HRC, horse red cells
HSA, human serum albumin
Hy, hemocyanin
Ig, immunoglobulin
KLH, keyhole limpet hemocyanin
LN, lymph node

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LPS, lipopolysaccharide
NIP, 4-hydroxy-5-iodo-3-nitrophenacetyl
N_2Phlac, azophenyl-β-lactoside
OA, ovalbumin
PFC, plaque-forming cells
(Phe,G)-Pro-L, poly-l-(Phe,Glu)-poly-l-Pro-poly-l-Lvs
POL, polymerized flagellin
PVP, polyvinylpyrrolidone
R anti-POL, rabbit-anti-POL antibody
R anti-MIg, rabbit anti-mouse-Ig antibody
Rd, tetramethylrhodamine (isothiocyanate)
RFC, rosette-forming cells
Sa, Salmonella adelaide (heat-killed)
SRC, sheep red cells
T lymphocyte (or T cell), thymus-derived lymphocyte
TdR, thymidine
TDL, thoracic duct lymphocytes
TGAL, poly-l-(Tyr,Glu)-poly-dl-Ala-poly-l-Lys
(T,G)-Pro-L, poly-l-(Tyr,Glu)-poly-l-Pro-poly-l-Lys
TNP, trinitrophenyl
T $\times$ BM mice, adult thymectomized, irradiated mice protected with bone marrow cells

I. Introduction

The present review attempts to analyze the available experimental evidence for the immunological commitment of the antigen-sensitive precursors of B lymphocytes.

The precommitment hypothesis postulates that the precursor of an antibody-forming cell is already committed to produce antibody of a single specificity prior to contact with antigen. Basic to the precommitment hypothesis are the following assumptions:

a) The organism as a whole possesses a large repertoire of precommitted precursor cells, and for a particular antigenic stimulus only a distinctive subset of cells is capable of responding.

b) The responding subset is a small fraction of the whole population of precursor cells, and upon antigenic stimulation it is amplified by a sequence of rapid mitotic divisions.

c) Each cell of the responding subset produces antibody of one molecular species only (*V* region), and the progeny of each cell is producing antibody of the same specificity as the ancestor cell (except rare mutations).

d) The conversion of a cell from the uncommitted to the committed state is an antigen-independent process; uncommitted cells cannot be triggered by antigen.

e) Information on the three-dimensional structure of the antigenic determinant is not required intracellularly at any stage of antibody synthesis or folding.

The precommitment hypothesis is considered throughout this review in its strict sense, thus as precommitment to synthesis of a single species of antibody molecules. It will be referred to as *strict precommitment.* Where it would be experimentally undistinguishable from precommitment to form a small number of different specificities, it will be referred to as *quasi precommitment.*

In obtaining experimental evidence for the precommitment hypothesis, the basic problem is to distinguish whether a cell, at the time when it is exposed to antigen, is yet uncommitted or already committed.

The term "*uncommitted cell*" is ambiguous. For opponents of the precommitment hypothesis it may mean a pluripotential or totipotential cell which may gain its antibody-synthesizing capacity as a consequence of contact with antigen. Proponents of the precommitment hypothesis accept that *immature* uncommitted cells do exist, but they postulate that uncommitted cells cannot be triggered by antigen.

This review is concerned with the production of antibodies and hence with the behaviour of B lymphocytes. Experiments on non-immunized animals or their cells are preferentially considered. Reactions not directly connnected with antibody production, such as graft-versus-host reactions, mixed-lymphocyte reactions, or the problem of histocompatibility antigens will not be analyzed. The switch from one class of immunoglobulins to another, allelic exclusion and the problem of tolerance will not be considered either.

The experimental evidence for the single specificity of the receptors carried by a lymphocyte will be reviewed. It will be shown that the frequency of precursors of antibody-forming cells for any given epitope is low, and that these specific precursors can be eliminated or separated from a population of lymphocytes.

The problem of precommitment lies at the centre of present immunological thought. The clonal selection theory (JERNE, 1955; BURNET, 1957) which has served as the theoretical framework of immunology over the past 20 years postulates that precommitted cells interact with antigen and are thereby triggered to form a clone of antibody-forming cells. If the precommitment hypothesis were proved to be false, immunology would have to begin the search for new basic tenets.

II. Receptors

Precursors of antibody-forming cells are small non-dividing B lymphocytes resting in the G_0 phase of the mitotic cycle. They synthesize very little, if any, DNA, but they do synthesize immunoglobulin molecules which become located on cell surface where they serve as receptor molecules for antigen recognition (GREAVES and HOGG, 1971). RABELLINO et al. (1970) have estimated that 50000 to 150000 immunoglobulin molecules are distributed on the cell membrane of B lymphocytes. It has been recently shown that the lymphocyte membrane behaves as a two-dimensional fluid and that the receptors and other surface molecules are free to move about in the plane of the membrane (TAYLOR et al., 1971). Binding of divalent antibodies directed against determinants of surface immunoglobulin as well as binding of multivalent antigens induces a dramatic redistribution of the membrane immunoglobulin molecules (TAYLOR et al., 1971; LOOR et al., 1972; ROELANTS et al., 1973; ASHMAN 1973). These first aggregate into patches which move and become localized over one pole

of the cell, finally coalescing to form a "cap". Univalent Fab and monosubstituted hapten-carrier conjugates (DNP_1-BSA) fail to induce the immunoglobulin redistribution and cap formation.

It has been suggested (GREAVES, 1972; RAFF and DE PETRIS, 1973) that multipoint binding with subsequent cap formation is a crucial step in triggering the lymphocyte to transform into blast cells, divide, and initiate the antibody response. In this view, the binding of antigen to a receptor would not be sufficient to trigger the cell, but binding followed by aggregation of cross-linked receptors into a "cap" would serve as a signal for cell activation (GREAVES, 1972; RAFF and DE PETRIS, 1973).

It has been shown that different surface molecules cap independently (TAYLOR et al., 1971; GREAVES, 1972; KOURILSKY et al., 1972). If cap formation would serve as a triggering signal, it follows that several stimulants may evoke the same triggering effect via different cross-linking and capping. Thus the act of triggering itself cannot signal *what kind* of antibody should be synthesized by the cell, but rather that the antibody which the cell is able to produce *should be* synthesized. The cell must not have "wrong" receptors, to avoid unwanted stimulation. This can be achieved only if the immunoglobulin receptor has the same specificity (*V* region) as the antibody ultimately produced by the cell.

This bears directly on the precommitment hypothesis, which predicts not only that a cell has to possess receptors of the same specificity as the antibody produced, but that no immunoglobulin receptors of other specificity should be present on the cell surface. RAFF et al. (1973) designed an experiment to test whether or not this is true. Because of the fundamental importance of this experiment for the subject of this review, I am going to describe the experiment and discuss its significance in some detail.

The experimental approach of RAFF et al. (1973) is summarized in Fig. 1. Spleen cells from non-primed mice (a) were incubated with antigen (POL) under conditions known to induce optimal capping (b). The cells were washed and transferred to non-capping conditions (NaN_3, cold) (c), and then incubated with rhodamine-coupled rabbit anti-POL (R anti-POL~Rd) (d) to label the antigen (POL)-binding cells. Finally, the cells were incubated with fluorescein-coupled rabbit anti-mouse immunoglobulin (R anti-M-Ig~Fl) (e) to label all immunoglobulin receptors. POL-binding cells were identified by scanning for rhodamine staining, then examined for anti-immunoglobulin fluorescein labeling to check whether on the POL-capped cells some immunoglobulin was left behind on the remainder of the cell surface.

Out of 440000 cells, 17 were "capped" rhodamine-stained-POL-binding cells (Table 1). The fluorescein labeling coincided with the rhodamine labeling in 14 cells out of 17. In these cases, no fluorescence was detectable outside the cap. In the rest (3 out of 17) the fluorescein staining was most intense at the rhodamine cap but also could be seen on the rest of the cell surface. Further experiments showed that non-capped immunoglobulin on such cells had anti-POL specificity. This was determined by a double treatment with POL, first

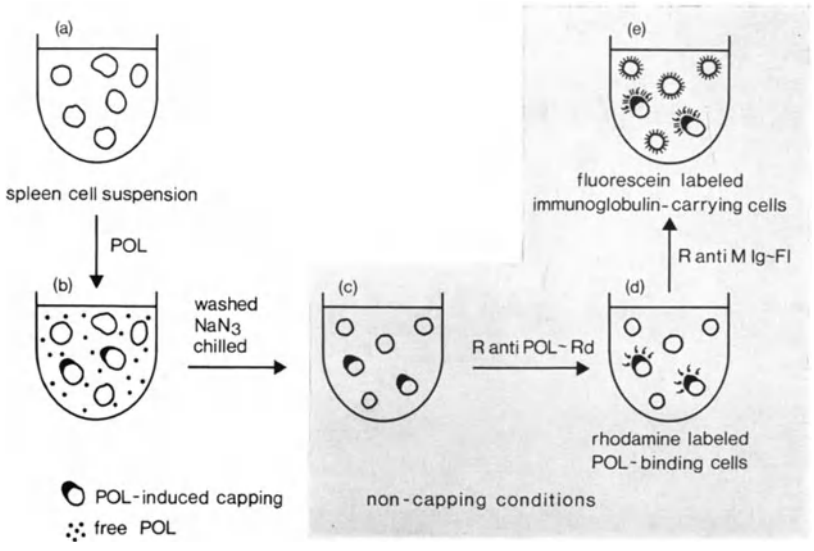

Fig. 1. Design of an experiment to show that individual B cells display only one type of receptor

under capping conditions, second under non-capping conditions. Rhodamine and fluorescein anti-POL staining revealed that in those POL-binding cells, where not all immunoglobulin molecules moved to the cap, the noncapped immunoglobulin was specific for POL.

The experiment has shown that POL was able to cap all of the immunoglobulin detectable by fluorescence on most POL-binding cells. The validity of this conclusion depends on the available evidence that when POL-bound receptors are redistributed into a cap, other molecules, in general, are not trapped and capped non-specifically in the process. This is probably not the case, since there is increasing evidence that different surface molecules move and cap independently. For example, immunoglobulin has been shown to move independently of histocompatibility antigens (Taylor et al., 1971; Preudhomme et al., 1972). Moreover, structurally similar molecules (like allelic products of histocompatibility antigens) cap separately (Neauport-Sautes et al., 1973; Bernoco et al., 1972). The strongest argument, that receptors would be redistributed independently if they were heterogenous, is derived, however, from experiments of Pernis and co-workers (personal communication) who demonstrated that immunoglobulins of different classes present on the same cell show independent cap formation.

It is possible to argue, that at the moment when the experiment was initiated, all cells had a heterogeneous set of receptors and that the antigen-induced capping disbalanced the ratio of receptors. This argument is, however, incompatible with data on the turnover of surface immunoglobulin (Melchers et al., 1973). While the shedding of irrelevant receptors is conceivable, the resynthesis of new receptors can be excluded, because it was shown that the

Table 1. Experimental evidence for all receptors on an individual B cell having the same specificity. (Compiled from RAFF et al., 1973)

POL[a] 26 μg/ml	R-anti-[b] POL Rd	R-anti-[c] M Ig Fl	Additional treatment or pretreatment	Capped rhodamine staining (POL-binding cells)	Fluorescein labeling coinciding with capped rhodamine staining
−	+	+	−	0/200000	−
+	+	+	−	17/440000	14/17
+	+	−	Fab anti MIg Fl[aa]	12/200000	11/12
+	+	−	Pretreatment with R-anti MIg[bb]	0/200000	−

[a] POL, polymerized flagellin.
[b] R-anti POL~Rd, rhodamine-coupled rabbit anti-POL antibody.
[c] R-anti MIg~Fl, fluorescein-coupled purified rabbit antimouse immunoglobulin antibody.
[aa] Fab-anti MIg~Fl, fluorescein coupled monovalent Fab fragments of rabbit antimouse-Ig antibody.
[bb] R-anti MIg, rabbit antimouse-Ig antibody.

cap formation can occur in absence of protein synthesis (RAFF and DE PETRIS, 1973).

The authors have furthermore examined the sensitivity of the assay. They found that if fluorescent antisera were diluted eightfold, with "bleached" fluorescent antisera, the percentage of fluorescent cells remained unchanged. The sensitivity of detection can be further increased with an additional layer of G anti-R-Ig~Fl, but minute amounts of immunoglobulin (below 3%), would however escape detection.

RAFF, FELDMAN and DE PETRIS conclude that individual B cells display receptors of a single antibody specificity. Do their experiments show this unequivocally? If the threshold of detection of immunoglobulin molecules on the cell surface is 3% as shown by the fluorescent method, 1500–4500 immunoglobulin molecules would remain undetected on the surface of the cell. If receptors of a few thousand *different* specificities would be present on the cell, the number for each specificity would be negligible. Even if these receptors were all of *one* specificity (e.g. product of the other allele), their number would be still so small that it is doubtful whether their presence on the cell surface would be functionally meaningful.

Note that RAFF et al. (1973) used "capping" merely as a technique for the visualization of receptors. The validity of their results are in no way dependent on the interpretation of the function of cap formation.

Hence it can be concluded that the above experiments strongly support the precommitment hypothesis. It is not possible to distinguish between strict precommitment and quasi precommitment (see Introduction) because the method is insufficiently sensitive.

III. Frequency of Precursor Cells

The lymphoid system of a mouse consists of about 6×10^8 lymphocytes (MAKINODAN et al., 1962) from which approximately 3×10^8 are mature immunocompetent lymphocytes, and one third of the latter are B lymphocytes (SPRENT, 1973). If the cells were uncommitted, potentially each of the B cells would be able to make antibody to the presented antigen. If, on the other hand, B cells were precommitted, only a small fraction of cells could respond to a given antigenic determinant and the repertoire of the rest of the B lymphocyte population should cover the rest of the antigenic spectrum.

Thus, if the precommitment hypothesis is valid, the frequency of B cells potentially capable of responding to any foreign antigenic determinant should be low. The actual frequency depends first on the repertoire of variable regions of immunoglobulin that an organism can make at a given point in time, and second, on the number of different antibody molecules which combine more or less well with a given epitope. If we assume that an animal is able to make 10^6–10^7 different antibody molecules (JERNE, 1955, 1967, 1972) and that 10–100 different antibody molecules can combine with a given epitope, then the average frequency of B precursor cells specific to one epitope would be 10^{-4}–10^{-6}. This would mean 100 to 10000 specific B precursor cells per mouse. Note that, although low frequencies of specific B cells are a logical requirement for precommitment, the experimental finding of low frequencies does not prove precommitment. What are the alternative explanations for low frequencies? One could postulate that there is a low but finite probability for any cell to be stimulated by any antigen; the frequency of cells apparently specific for a given antigen would correspond to the probability of successful stimulation. We will return to this point in Chapter IV.

There have been several attempts to estimate the frequencies of cells specific to complex antigens or to single epitopes. Many of the experiments were performed before T and B cell cooperation was known, thus making interpretation difficult. Many of the experiments have been, however, repeated under more defined conditions.

A. Splenic Foci Technique

The splenic foci technique was developed independently in two laboratories (KENNEDY et al., 1965; PLAYFAIR et al., 1965), with the same goal: to obtain an assay for the precursors of antibody-forming cells. In principle, a small inoculum of donor spleen cells and antigen (red cells) is injected into lethally irradiated syngeneic mice. After 5 or more days the host spleens are cut into many slices, each of which is further cut into separate pieces. These are placed on petri dishes containing a layer of agar with embedded red cells. Antibody is allowed to diffuse out and complement-dependent lysis is observed around some pieces. In this way, it is possible to construct a three-dimensional map of foci of antibody-producing cells.

The method was, however, developed at a time before T and B cell interaction was established. In order to interpret the data of KENNEDY et al. (1965) and PLAYFAIR et al. (1965), we have to take into account the contribution of cell-cell interaction. Both groups were using for transfer spleen cells from normal non-immunized mice. It has been shown (PLAYFAIR et al., 1965; KENNEDY et al., 1965; MILLER et al., 1967; GREGORY and LAJTHA, 1968) that the number of hemolytic foci in a recipient spleen is directly proportional to the number of cells injected, suggesting that only one kind of cell was limiting. KENNEDY et al. (1965) and PLAYFAIR et al. (1965) interpreted a focus as a single clone of antibody-forming cells. Furthermore, CELADA and WIGZELL (1966) have found that the assortment of antibody activity for two antigens is independent, and KLINMAN (1969) demonstrated that the antibody produced by the foci is often homogeneous.

On the other hand, the work of VANN and CAMPBELL (1970) and LUZZATI et al. (1970) showed that in many foci more than one B precursor cell and probably a single T cell participated in antibody formation. In one case (VANN and CAMPBELL, 1970) an allogeneic mixture of donor cells was used, and prior to PFC assay one or the other cell population was killed by specific antisera (anti H-2) and complement. It was shown that many foci contain both H-2 types of PFC. LUZZATI et al. (1970) demonstrated that a single focus can synthesize antibodies which migrate as more than one discrete electrophoretic band, suggesting that there is more than one B precursor cell per focus. CAMPBELL (1971) designed an experiment to show that T cell foci can be obtained. Irradiated recipient mice were injected with a small inoculum of spleen cells as a sole source of T cells, as well as with a large excess of bone-marrow cells. Partially cross-reacting red cells (SRC and GRC) were used as antigen and it was found that almost all foci contained both anti-SRC and anti-GRC PFC.

In Table 2 there is presented a summary of estimates of precursor cells obtained using the splenic foci technique. Unfortunately, in most cases it is not possible to determine strictly whether T cells or B cells are limiting. Thus, it is not clear in many cases what precursor cell frequency has been calculated. Nevertheless, in cases where T cells are limiting, the frequency of B cells must be *greater than* values given in the table.

The frequencies given in the table were not corrected for the homing efficiency, that is for the fraction of injected immunocompetent cells that become lodged in the spleen. The homing efficiency has been estimated by using a double transfer method and was found to be 4% by PLAYFAIR et al. (1965), 10% by BOSMA et al. (1968) and MILLER et al. (1967), and 15% by KENNEDY et al. (1966). Thus the "true" frequencies might be 7–25 fold higher than that observed. For these reasons the precursor cell frequency estimated by the splenic foci technique may be considered to be a minimum estimate. For all immunogenic epitopes of SRC for example, the minimum frequency of precursor cells has been estimated to be 0.4×10^{-6}–2×10^{-6}.

Table 2. Frequencies of precursor cells estimated by splenic-foci technique

Donor spleen cells	Antigen	Recipient	Transferred to culture	Time of assay	Frequency	Reference
Unprimed	SRC	LAF_1 1260r	—	8 days	2×10^{-6}	PLAYFAIR et al. (1965)
Unprimed	SRC	C57Bl 900r	—	6–10 days	2×10^{-6}	KENNEDY et al. (1965, 1966)
Unprimed	SRC	LAF_1 750r	—	6–8 days	0.4×10^{-6}	KIND and CAMPBELL (1968)
Unprimed	SRC	CBA 900r	—	5–10 days	0.45×10^{-6}	CUNNINGHAM (1969a, b)
Primed DNP-Hy[a]	DNP-Hy	BALB/c 650r	after 24 hrs + DNP-Hy	6–16 days	0.7×10^{-7}	KLINMAN (1969)
Primed SRC	SRC	CWB 550r	after 8 days	24 hrs[b]	2.4×10^{-6}	LUZZATI et al. (1970)
Unprimed	SRC	$C3AF_1$	—	7 days	10^{-6} [c]	GREGORY (1971)
Primed DNP-Hy	—	BALB/c 650r	after 24 hrs + DNP-Hy	8–30 days	1.4×10^{-6}	KLINMAN and ASCHINAZI (1971)

[a] DNP-Hy (DNP-Hemocyanin).
[b] Assayed 24 hrs after transfer to culture (i.e. on 9th day after injecting the cells to recipient mouse).
[c] Frequency has been calculated by the present author.

B. Antigen-Binding Cells

Since B lymphocytes have receptors on their surface, they can selectively bind antigen. Bound antigen can be visualized by means of radioautographic techniques, rosette formation, enzymatic reactions and fluorescent techniques. In this section, I intend to collect some estimates on the frequency of antigen-binding cells from normal non-immunized animals. Because the estimation of the frequencies by these techniques is not based on analyzing the capacity of cells to produce an antibody response, special attention must be paid to the specificity of the binding and its relevance to the antibody response.

NAOR and SULITZEANU (1967) determined the fraction of normal mouse spleen cells which is capable of binding a given antigen. After treatment of cells with ^{125}I-BSA, autoradiography revealed that some 2% of cells become lightly labeled and about one cell per 1000 showed quite heavy labeling. Labeled cells included large, medium and small lymphocytes and macrophages, but the heavily labeled cells were mainly small lymphocytes. This line of investigation has been taken up by several laboratories (ADA and BYRT, 1969; MANDEL et al., 1970; DWYER and MACKAY, 1970; HUMPHREY and KELLER, 1970), and results similar to those of NAOR and SULITZEAU were obtained.

It was noted that the fraction of labeled cells was largely dependent on the concentration of labeled antigen in the reaction mixture. BYRT and ADA (1969) observed that a 100-fold increase of flagellin concentration resulted in an almost linear increase of labeled cells. Similar observations were made with hemocyanin (discussed by ADA, 1970). Furthermore, an increase in exposure time resulted in an increase in the number of labeled cells observed. This is related to a further parameter, the number of grains overlaying the cells in the radioautograph: at a low antigen concentration (5 ng/ml), the majority of hemocyanin-binding cells (ADA, 1970) has 12–50 grains (32 out of 2×10^5 scanned cells) and few had more (6 out of 2×10^5). The observations on concentration dependence differ from those recently made by DIENER and PAETKAU (1972). These authors used tritiated rather than iodinated antigen, and they found that the fraction of antigen-binding cells was independent of the antigen concentration over the range 50 ng/ml–20 μg/ml. DIENER and PAETKAU attributed this discrepancy to differences in the labeling technique. While preparation of ^{125}I tracers involves chemical oxidation procedures, tritium labeling is achieved by biosynthetic means and the label constitutes an integral part of the antigen molecule. Furthermore, the short range of tritium β particles facilitates discrimination between labeled cells and artefacts.

None of the above experiments demonstrated whether the binding is achieved by B or T cells. DWYER et al. (1971) performed antigen binding experiments using nude mice, and results similar to those with normal mice were obtained.

The specificity of binding (NAOR and SULITZEANU, 1967) was demonstrated by inhibition of binding with an excess of non-iodinated BSA. While a 1000-fold excess of BSA inhibited the binding of iodinated BSA, a 100000-fold excess of HSA did not.

SERCARZ et al. (1971) used a different approach to estimate the frequency of antigen-binding cells. They incubated cells with β-galactosidase, then washed the unbound enzyme away, distributed the cells in microdroplets (0.2 μl) in the presence of a substrate, and checked which droplets became stained by the color product of the enzymatic reaction. The frequency of β-galactosidase-binding cells obtained with this method is very high: 7.2×10^{-3} was reported for spleen cells. This was still exceeded by 2.2% of β-galactosidase-binding cells in bone marrow. This is strongly contrasted by the finding of MELCHERS (personal communication) who measured the frequency of cells which have surface immunoglobulin capable of activating a mutant of β-galactosidase. The observed frequency was about 5×10^{-7}. MELCHERS presumably measures the frequencies of cells specific for a single epitope. There is no satisfactory explanation for the very high frequency obtained by SERCARZ and co-workers, though it must be kept in mind that antigen binding is not equivalent to responsiveness since the latter may require binding above a certain affinity threshold. The binding seems to be specific, it is inhibited by anti-immunoglobulin and the number of antigen-binding cells is related to antigenic experience.

Table 3. Frequency of antigen-binding spleen cells from non-immunized mice

Antigen	Conc. (ng/ml)	Specific activity (μCi/μg)	Frequency	Reference
^{125}I-BSA	1.2–20	10–100	5×10^{-4}	NAOR and SULITZEANU (1969)
^{125}I-hemocyanin *M. squinado*	400	450	1.4×10^{-3}	HUMPHREY and KELLER (1970)
^{125}I-hemocyanin *J. ialandii*	100	40	1×10^{-3} [a]	DWYER et al. (1971)
	300	20	0.9×10^{-3}	BYRT and ADA (1969)
^{125}I-TGAL	300	1400	2.8×10^{-3}	HUMPHREY and KELLER (1970)
	9	4	1.8×10^{-3} [a]	DWYER et al. (1971)
^{125}I-flagellin	500	60	4×10^{-4} [a]	DWYER et al. (1971)
	300	30–40	1×10^{-4}	BYRT and ADA (1969)
^{125}I-polymerized flagellin	500	70	2×10^{-4}	ADA et al. (1970)
^{3}H-polymerized flagellin	50–20000	0.15	$1.4–2.6 \times 10^{-5}$ [b]	DIENER and PAETKAU (1972)
polymerized flagellin	26000	—	3.8×10^{-5} [c]	RAFF et al. (1973)
SRC			1.1×10^{-3} [aa]	BACH et al. (1971)
			$4–9 \times 10^{-4}$ [aa]	DECREUSEFOND et al. (1970)
			7×10^{-4} [aa]	GREAVES and HOGG (1971)
			2.7×10^{-3} [aa]	HASKILL and AXELRAD (1971)
β-galactosidase	50000	—	7.2×10^{-3} [bb]	SERCARZ et al. (1971)

[a] Nude mice.
[b] Mesenteric lymph node cells instead of spleen cells.
[c] Staining with rhodamine-coupled rabbit anti-POL.
[aa] Rosette-forming cells.
[bb] β-galactosidase assay: 5-bromo-4-chloroindol-3-yl-β-D-galactosidase gives rise to a precipitable blue product.

The further technique by which the frequency of antigen-binding cells can be measured is the rosette technique. The rosette formation was described by NOTA et al. (1964) and ZAALBERG (1964) who observed that when SRC were mixed with mouse spleen cells, some nucleated cells were surrounded by agglutinated SRC reminiscent of a "rosette" shape. About one rosette in 1000 cells was observed. Rosettes were shown to be specific and not due to cytophilic antibody; two distinguishable types of erythrocytes formed homogeneous but not "mixed" rosettes (BIOZZI et al., 1966; LASKOV, 1968). It is not clear what proportion of rosette-forming cells are B cells and T cells. The above experiments are different from the recently described phenomenon in humans where the majority of T cells formed rosettes with SRC (BRAIN et al., 1970; BRAIN and MARSTON, 1973).

In Table 3 are given the frequencies of antigen-binding spleen cells from non-immunized mice as measured by several authors. It is evident that the

estimates differ considerably among themselves depending on the antigen and the method which was used. In many, but not all cases, primarily B cells were detected by the antigen-binding. Concentrations of the antigen used for binding as well as the specific activity (where it applies) are indicated. It is interesting to note that more spleen cells bind hemocyanin than flagellin. This is, however, not a reflection of the relative immunogenicity, since flagellin is more immunogenic than hemocyanin (ADA, 1970). The lowest estimates are those of DIENER and PAETKAU (1972) and RAFF et al. (1973).

Do these techniques, or at least some of them, detect precursors of antibody-forming cells? The affirmative answer which is often given by several arguments (see suicide technique, next chapter) merely says that among antigen-binding cells there are also precursor cells. But what fraction of antigen-binding cells are precursor cells is not answered at all.

There are certainly artefacts which simulate antigen-binding cells. ADA (1970) discusses the fact that damaged cells, macrophages and in particular isolated nuclei may take up labeled antigen. Phagocytosis of the antigen by macrophages can be prevented by the presence of sodium azide. Furthermore, it is now generally accepted that for successful triggering of a cell, the specific binding of antigen is a necessary but not sufficient condition. Whatever the signal for the immune stimulus (receptor redistribution or any other event), it is reasonable to assume that affinity, concentration, valency, solubility and probably other parameters of the antigen as well, can be decisive for triggering.

From the concentration-dependence, dependence on exposure time, and grain distribution, it seems that, at any given concentration, antigen is able to bind to a certain array of cells possessing receptors of certain affinities. Thus the antigen-binding cells probably comprise a subgroup of cells whose receptors will fit with different degrees of complementarity to the given antigen. There will be low numbers of cells with essentially perfect complementarity and increasing numbers of cells with receptors of lower and lower degrees of complementarity.

In conclusion, the frequencies of antigen-binding cells reported in this section can be considered as upper limits for precursor estimates. The number of cells physiologically significant as precursor cells in a given antibody response is unknown.

C. Cell Transfer Limiting Dilution Experiments

Limiting dilution analysis is an all-or-none type of assay, conducted within a range of lymphoid cell dose where there is a measurable probability that a sample will not contain a species necessary for a response (GROVES et al., 1969).

The cell transfer limiting dilution experiments were initiated when T and B cell cooperation was not yet discovered, but in most of the experiments care was taken to ensure that only one kind of cell was limiting (BROWN et al., 1966; SHEARER et al., 1968; BOSMA and WEILER, 1970). In recent years the method has been adapted to estimate the frequencies separately for B cells

(CUDKOWICZ et al., 1969; MILLER and CUDKOWICZ, 1970) and for T cells (SHEARER et al., 1969; SHEARER and CUDKOWICZ, 1969). It has been used to compare the frequencies in low and high responder strains (MOZES et al., 1970) and to study the frequencies for two distinct regions of a single antigen (SHEARER et al., 1971).

In a typical experiment of this type, CUDKOWICZ et al. (1970) injected (i.v.) graded numbers of syngeneic bone marrow cells (ranging from 0.37 to 6×10^5

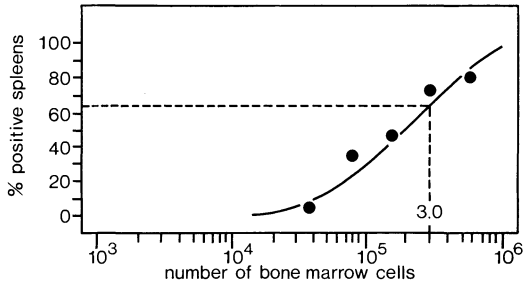

Fig. 2. Estimation of frequency of B precursor cells specific for SRC (redrawn from CUDKOWICZ et al., 1970). Symbols indicate observed percentages and the curve expected percentages according to the Poisson distribution. About 3×10^5 cells correspond to 63% positive spleens ($f = 3.3 \times 10^{-6}$)

cells) into groups of lethally irradiated mice along with a large excess (5×10^7) of syngeneic thymus cells. The next day 5×10^8 SRC were given and individual spleens were assayed for their content of PFC at the time of peak response, i.e. 9–10 days after cell transfer. The results of such an experiment are shown in Fig. 2. The number of transferred bone marrow cells that produces 63% positive spleens (dotted line) contains, on the average, one precursor cell detectable by the assay. In this case, there is one precursor cell in 3×10^5 bone marrow cells and the frequency is thus 3.3×10^{-6}. The full line is the expected Poisson distribution. The points lie very close to the expected line. Occasionally, the data deviate from the theoretical Poisson curve in which case the frequency of precursor cells cannot be calculated.

There is an alternative approach to ensure that B cells are titrated. Using a thymus independent antigen (lipopolysaccharide), MÖLLER and MICHAEL (1971) estimated the frequency of precursor cells in an experiment in which a graded number of spleen cells (10^4–8×10^6) was injected into irradiated mice together with the antigen. The observed fractions of non-responding mice in a semi-log plot fit well to a straight line (Fig. 3). The calculated frequency was 10^{-5}. However, lipopolysaccharide is a mitogen (ANDERSSON et al., 1972), and might have nonspecifically stimulated the immune system.

In Table 4 frequencies of precursor cells from non-immunized mice are compiled (see also Fig. 3). Some authors used spleen cells for limiting dilutions, others bone marrow cells in combination with a constant number of thymus cells. The latter, which guarantees that B cells are titrated, was used by CUDKOWICZ et al. (1969, 1970) and MILLER and CUDKOWICZ (1970). Further-

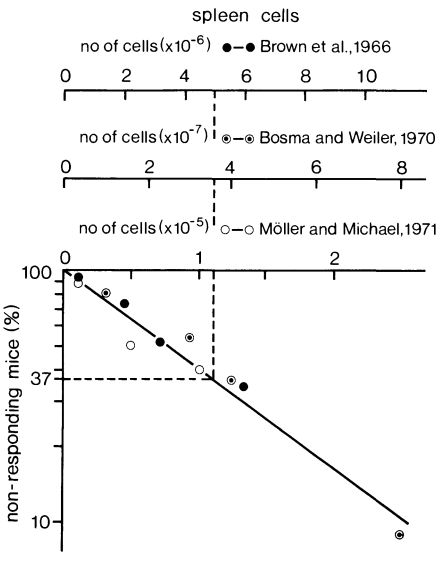

Fig. 3. Estimation of frequencies of precursor cells specific for rat red cells (BROWN et al., 1966), poly-ala-BSA (BOSMA and WEILER, 1970) and LPS (MÖLLER and MICHAEL, 1971). In all three instances, spleen cells from non-immunized mice were used

more, in the experiment of MÖLLER and MICHAEL (1971) a thymus-independent antigen was used.

Homing efficiency has already been discussed in the section on the splenic foci technique. The estimates for the fraction of specific cells which lodged in the spleen was in the range of 4–15% (see Chapter A). In experiments on transfer of an anti-DNP clone, ASKONAS et al. (1972) estimated the effective homing of B precursor cells in the spleen to be around 1%.

The estimates for precursor cells specific for red cell determinants were in the range of $0.2–3.3 \times 10^{-6}$, for synthetic polypeptide antigens of limited heterogeneity $0.3–3 \times 10^{-7}$, and for lipopolysaccharide 1×10^{-5}. These estimates are not corrected for the effective homing efficiency. For similar reasons to those mentioned in the section on the splenic foci technique, the above frequencies may be considered to be a minimum estimate.

D. Microculture Experiments

The development of culture systems for obtaining *in vitro* antibody response (MISHELL and DUTTON, 1967; MARBROOK, 1967) made it possible to culture small numbers of spleen cells so that a limiting number of precursor cells would be present.

OSOBA (1969a, b) used the MARBROOK system to culture varying numbers of normal mouse spleen cells ($0.25–4 \times 10^6$) mixed with irradiated spleen cells (to maintain a constant cell density) in the presence of SRC and/or ChRC. He observed that a certain fraction of cultures responded while others remained negative.

Table 4. Frequencies of precursor cells estimated by cell-transfer limiting dilutions

Cells from non-immunized donors		Antigen	Assay	Frequency of precursors	Reference
Spleen	$BC3F_1$	Rat RC	titer[a]	0.2×10^{-6}	BROWN et al. (1966)
Bone marrow + thymus	$BC3F_1$	Sheep RC	PFC	—[b]	CUDKOWICZ et al. (1969)
Bone marrow + thymus	$BC3F_1$	Sheep RC	PFC	—[b]	MILLER and CUDKOWICZ (1970)
Bone marrow + thymus	BDF_1	Sheep RC	PFC	3.3×10^{-6}	CUDKOWICZ et al. (1970)
Spleen	$BC3F_1$	poly-ala-BSA	PFC	0.03×10^{-6}	BOSMA and WEILER (1970)
Spleen	SJL	(T, G)-Pro-L	titer	0.14×10^{-6}	MOZES et al. (1970)
	DBA/1			0.03×10^{-6}	
Spleen	SJL	(Phe, G)-Pro-L	titer (anti Phe, G)	0.05×10^{-6}	SHEARER et al. (1971)
	DBA/1			0.12×10^{-6}	
	SJL		titer (anti Pro, L)	0.3×10^{-6}	
	DBA/1			0.03×10^{-6}	
Spleen	CBA	LPS	PFC	0.1×10^{-6}	MÖLLER and MICHAEL (1971)

[a] Titer = antibody level in the serum as criterium for responding mice.
[b] Frequency not computed because the data did not fit Poisson distribution.

In our laboratory (LEFKOVITS, 1972) a microculture method was developed which made it feasible to culture and assay many small aliquots (10 µl) of lymphoid cells. The samples of the culture fluid or cells are handled by a "replicator" (LEFKOVITS and KAMBER, 1972) which can remove samples from 60 wells of a tissue culture tray and release them on an assay plate which contains agar with embedded red cells. Complement-dependent zones of lysis mark the cultures which have produced antibody (Fig. 4).

With an input of 2×10^5 spleen cells per microculture in 12 experiments (about 2600 microcultures) 12–75% non-responding microcultures were obtained (LEFKOVITS, 1972). In these experiments, spleen cells from ordinary mice were used. Hence, it could not be shown unequivocally that any non-responding culture was lacking only one type of cell—the B precursor cell.

To estimate the frequency of B precursor cells with confidence, it was necessary to manipulate separately the cells involved, so as to ensure that only the B precursor cells were limiting. QUINTÁNS and LEFKOVITS (1973) described an experiment where a limiting dilution analysis of B cells was performed under conditions where adequate numbers of T cells in all microcultures were guaranteed. The following strategy was used to ensure that non-responding wells would correspond to those microcultures which lack only the B precursor cell specific to SRC:

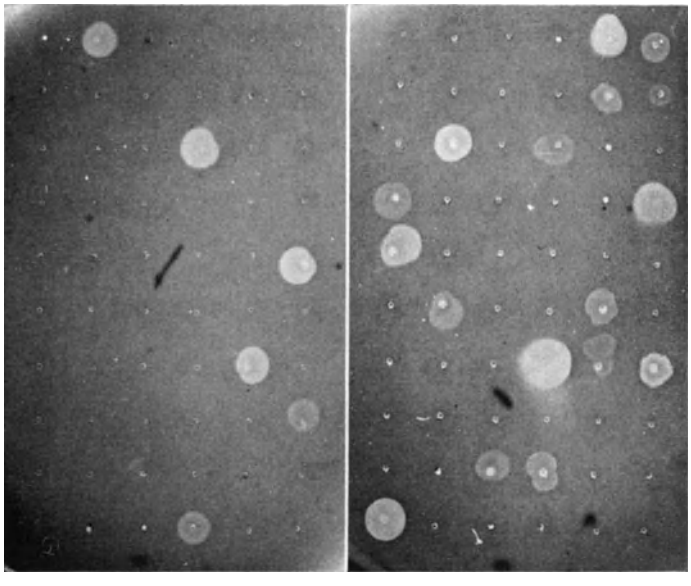

Fig. 4. Hemolytic spot test for antibody response in microcutlures. Complement-dependent zones of lysis mark the cultures which have produced antibody

a) Spleen cells from nude mice were titrated over the range $0.2–1.8 \times 10^5$ nude spleen cells per microculture.

b) Functional excess of T cells was ensured by means of allogeneic complementation. The number of allogeneic spleen cells was kept constant, 2×10^4 cells per microculture.

c) Constant cell density, 2×10^5 cells per microculture was maintained by compensating the titrated active nude spleen cells with irradiated nude spleen cells. Since macrophages are thought to be radioresistant (ROSEMAN, 1969; TALMAGE et al., 1971; COSENZA et al., 1971), the constant cell density ensured a constant number of macrophages.

The results of a limiting dilution experiment are shown in Fig. 5. The microcultures were incubated for 4 days in the presence of SRC and then assayed by the spot test. The fraction of non-responding microcultures decreased exponentially as the number of active B cells was increased. The linearity of the plot ("single-hit" curve) confirms that only a single cell type is being diluted out. Interpolating in Fig. 5, about 4.7×10^4 active B cells would yield 37% negative microcultures. Therefore, the frequency of precursor cells specific for SRC was 2.1×10^{-5}.

If microculture studies on the frequency of precursor cells are compared with those using the antigen-binding method, splenic-foci technique or *in vivo* dilution method, the following picture emerges:

The antigen-binding method is not based on analyzing the capacity of cells to produce antibody. It is not known what fraction of antigen-binding cells is capable of giving an antibody response. Thus, the frequency of antigen-

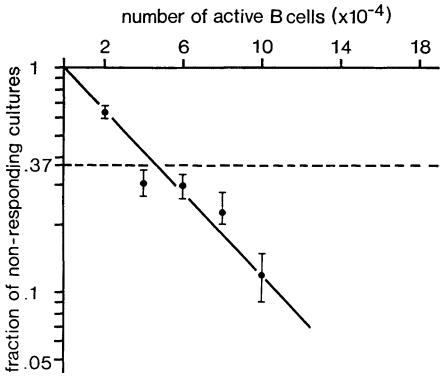

Fig. 5. Titration of B cells in microcultures. (From QUINTÁNS and LEFKOVITS, 1973.) All microcultures contained a constant number of allogeneic cells (2×10^4 per microculture). Irradiated and non-irradiated (active) nude spleen cells were mixed in different rations to give 1.8×10^5 total nude spleen cells per well. Abscissa indicates the input of active nude spleen cells per microculture. Each point is based on duplicate spot tests from 5 trays (300 microcultures). About 4.7×10^4 active B cells correspond to 37% negative microcultures ($f = 2.1 \times 10^{-5}$)

binding cells is probably the upper limit for specific precursor cell frequency. The remaining techniques, the splenic foci technique, cell transfer limiting dilution technique, microculture technique, all depend upon obtaining a complete antibody response. For this reason they yield minimum estimates. The microculture technique does not suffer from one inherent limitation of the two *in vivo* techniques—the problem of homing efficiency. As expected, the microculture technique gives higher values than the *in-vivo* techniques.

IV. Specific Elimination of Precursor Cells

In the previous chapter it was shown that the frequencies of precursor cells for antibody-producing cells are low. The most straightforward interpretation is, of course, that a small fraction of the cells responds to a given antigen because of the commitment of only a small fraction of the cells to formation of antibody to that antigen. However, this is not the only possible interpretation. The alternative hypothesis is that any lymphocyte is potentially able to respond to any antigen, but the probability of successful triggering is low. If this were the case, any attempt to remove the specific cells would fail, since the essence of this hypothesis is that there are no specific cells.

Conversely, if one were able to separate from a cell population a minor segment with antibody specificity against a given antigenic determinant and show that reactivity to this determinant (1) was lacking in the remaining population, and (2) was present in the removed or retained "minor segment" (if not destroyed), this would be strong evidence in favour of the precommitment hypothesis. It would not distinguish, however, between *strict precommitment* and *quasi precommitment* (see Introduction).

A. Physical Separation

There are several methods described in the literature which enable antigen-specific physical separation of immunocompetent cells: antigen-coated columns, derivatized nylon fibers, gradient techniques and electronic cell sorters. These methods will now be considered:

Columns. Three kinds of functionally different columns for cell separation have been described in the literature: (a) normal glass bead columns, on which lymphocytes, macrophages and damaged cells can be separated (RABINOWITZ, 1964; PLOTZ and TALAL, 1967; SHORTMAN, 1966), (b) antibody-coated columns, on which cells with certain surface antigens can be selectively retained (WIGZELL et al., 1972; GOLSTEIN et al., 1972; RUBIN and WIGZELL, 1973), and (c) antigen-coated columns, on which cells specific to homologous antigen can be selectively retained (WIGZELL and ANDERSSON, 1969, and others in this section).

Antigen-coated columns consist of glass or plastic beads to which the antigen is physically adsorbed or covalently coupled. The beads have an average diameter of 200–250 μm (WIGZELL and ANDERSSON, 1969) and the coating is achieved by incubation of the beads with the antigen (serum albumin). The uptake of the antigen by plastic beads (polymethylmeta-acrylic beads) was shown to be approximately 25 times higher (10000 molecules per μ^2) than by the glass beads. Such a density of antigen molecules would enable a simultaneous contact of 10^2–10^4 bead-attached albumin molecules with a single cell (WIGZELL and ANDERSSON, 1969). The beads are packed into columns, washed free of unattached antigen and used in the cold for cell separation. The cells are allowed to pass into the column (10^8 cells or more), whereupon elution is carried out using cold medium. If covalent coupling is performed, CNBr-activated Sepharose beads of a diameter 800–1500 μm (RUBIN and WIGZELL, 1972) are reacted with the antigen.

Spleen cells from two groups of mice, one immunized with HSA and the other with OA, were mixed and passed on HSA- or OA-coated columns, in the presence or absence of free antigen (WIGZELL and ANDERSSON, 1969). The authors found that cells specific to homologous antigen were retained, while cells specific to unrelated antigen passed through. The selective retention was blocked if free antigen was present in the medium during filtration. This was found to be the case both for their contents of PFC against various albumin-coated erythrocytes and for the memory cells.

The column technique has been applied to lymphocytes from non-immunized mice as well (WIGZELL and MÄKELÄ, 1970). A mixture of normal lymph node, spleen and bone-marrow cells was passed through an OA-coated column. Column passed cells as well as control cells were transferred to lethally irradiated mice (10^7 cells per mouse) and followed by immunization with OA and BSA. The antibody content in the sera was detected by the FARR assay. There was no response to OA, while the response to BSA was normal. Control cell suspensions which were not subjected to the column passage resulted in a good response to both OA and BSA (Table 5).

Table 5. Specific separation of precursor cells on antigen-coated columns

Cells	Column	Irradiated recipients injected with	Antibody response to		Reference
			relevant antigen	unrelated antigen	
			anti-OA[a]	anti-BSA[a]	
Mixture of lymph node, spleen and bone-marrow cells from non-immunized mice	ovalbumin polymethyl-metaacrylic plastic beads	10^7 column passed cells + OA + BSA	all negative	−1.029	WIGZELL and MÄKELÄ (1970)
		10^7 control cells + OA + BSA	−0.478	−1.075	
			anti-PVP[b]	anti-SA[b]	
Spleen cells from non-immunized mice	polyvinyl-pyrrolidone glass beads	2×10^7 column passed cells + PVP + SA	1/7	7/7	WIGZELL (1970) WIGZELL et al. (1971)
		2×10^7 control cells + PVP + SA	7/7	7/7	
			anti-N_2Phlac[c]		
Spleen cells from non-immunized mice	azophenyl-β-lactoside polyacryl-amide beads	6×10^3 eluted cells[aa] + KLH-N_2Phlac	62 IgM 2040 IgG		HENRY et al. (1972)
		2×10^7 control cells + KLH-N_2Phlac	22 IgM 3154 IgG		

[a] Anti-OA and anti-BSA = $\log_{10}$ units of the titer by Farr test; 4–8 mice in each group.
[b] Anti-PVP and anti-SA = number of responding animals/total number of mice.
[c] Anti-N_2Phlac = IgM and IgG PFC response per recipient spleen.
[aa] The cells were admixed to 2×10^7 irradiated thymus cells as "diluent cells".

The experments described above did not distinguish whether the eliminated cells were of thymic or bone marrow origin or both. WIGZELL (1970) coated a column with a thymus-independent antigen polyvinylpyrrolidone (PVP), and passed normal spleen cells through this column. The non-retained spleen cells as well as control spleen cells were injected into irradiated recipients together with PVP and an unrelated antigen (*Salmonella adelaide* heat-killed [SA]). In a group of seven mice receiving cells passed through the column only one mouse responded to PVP, while all responded to SA. Control cells elicited in another group of mice a good response for both PVP and SA. It can be thus concluded that antigen-coated columns are capable of retaining specific precursors of antibody-forming cells.

In all instances, however, only the passed and control (non-passed) cells were used. The specific elution of retained cells by adding free antigen to the medium in the column was not successful. It was suggested (WIGZELL and

MÄKELÄ, 1970) that the actual retention of the specific cells occurs in a two-step fashion, the first one involving combination between receptors and bead-attached antigen, and then retention at the bead surface by non-immunological forces.

In the above experiments it was not possible to show that the retained cells were those cells which were postulated to be missing in the cell suspension which had been through the columns. That is, the cells may not have been eliminated but rather rendered tolerant. An experiment of HENRY et al. (1972) indicated that this was not the case. Spleen cells from normal mice were passed through an azophenyl-β-lactoside-coated column, and the retained cells were eluted off of the columns by free lactoside hapten. Apparently, while elution in the case of glass-bead columns and polymethylmeta-acrylic bead columns is unsuccessful, it may be carried out with polyacrylamide beads. As few as 6000 eluted cells were injected into irradiated recipient mice (admixed with 2×10^7 irradiated thymus cells as "diluent cells") together with the antigen and it was observed that the antibody response to azophenyl-β-lactoside in most of the animals was as good as in animals which received 3000 times higher numbers of unseparated cells. The response with few retained-eluted cells is evidence for a real separation, and against induction of tolerance.

The results obtained by Wigzell's group and those of HENRY et al. (1972) are shown in Table 5. In all three experiments, cells from normal non-immunized mice were used, and the capacity to elicit a primary antibody response was assayed. In Wigzell's experiments the emphasis was on the inability of column-passed cells to lead to a specific antibody response, while in the experiments of HENRY et al. the function of the eluted cells was demonstrated.

A new approach in recovering the retained cells, now in development, is based on the principle of an enzymatically digestible matrix (SCHLOSSMAN and HUDSON, 1973; PHILLIPS and ROITT, 1973). The cells isolated from these digestible columns are fully functional on adoptive transfer (HUDSON and HAMILTON, 1973, in prep.).

Nylon fibers. EDELMAN et al. (1971) employed hapten-derivatized fibers to separate antigen-binding cells specific for various haptens. The nylon fiber is strung in a polyethylene frame and is submerged in a cell suspension. Cells specific to the antigen which is coupled to the fiber can be removed from the general population, and characterized with respect to the specificity, number, affinity, as well as other parameters. The cell attachment, arrangement on the fiber and the binding pattern, can be directly observed under the microscope.

RUTISHAUSER et al. (1972) demonstrated that the binding of cells to antigen fibers is inhibited by soluble antigen. KIEFER (1973) studied the conditions of release of cells from the fibers and he reported that cells bound at 4° C for more than twelve hours can be released quantitatively from the fiber at temperatures above 22° C within two hours in a fully viable state. KIEFER suggests that capping of lymphocytes is involved in the release of cells from the fibers. Functional studies on the ability of the retained and depleted cells

Table 6. Primary anti-SRC and anti-ChRC response with lymphocytes depleted of SRC or ChRC rosettes[a]

Depletion of RFC[b]	Spleen cells	Antibody response anti-SRC	anti-ChRC	Reference
		No. of PFC per 10^6 cells[c]		
Velocity sedimentation	normal cells	109	104	Osoba (1970)
	cells depleted of SRC-RFC	26	151	
	cells depleted of ChRC-RFC	82	7	
		Haemagglutinin titers ($\log_2$)[aa]		
Ficoll gradient	normal cells	6.5	5.3	Bach et al. (1971)
	cells depleted of SRC-RFC	2.9	5.2	
	cells depleted of ChRC-RFC	5.6	2.1	

[a] SRC—sheep red cells; ChRC—chicken red cells.
[b] RFC—rosette-forming cells(s).
[c] In Marbrook cultures.
[aa] Recipient mice treated with cyclophosphamide.

from non-immunized animals to respond by antibody production have not yet been reported.

Velocity Sedimentation and Ficoll Gradients. Rosettes are considerably larger and heavier than cells which have not bound erythrocytes, and can be separated from them by velocity sedimentation (Miller and Phillips, 1969, 1970; Osoba, 1970; Brody, 1970; Edwards et al., 1970) or by passage through a Ficoll gradient (Bach et al., 1971; Gorczynski et al., 1971). Osoba (1970) mixed normal spleen cells either with sheep red cells (SRC) or chicken red cells (ChRC) and dispensed the mixture in the sedimentation vessel. A nonlinear gradient of 15–30% fetal calf serum was loaded under the cell suspension. The cells were allowed to sediment for several hours, in cold. The fractions of spleen cells largely depleted of either SRC rosettes or ChRC rosettes were cultured in the presence of SRC or ChRC for 4 days. The results (Table 6) show that the antibody response to the red cells with which rosettes were formed initially, was diminished, while the response to other red cell types was not affected.

Bach et al. (1971) performed a similar experiment, but used a Ficoll gradient and determined the antibody response *in vivo*. Normal spleen cells have been mixed with SRC or ChRC and placed on a Ficoll-Triosil gradient. Lymphocytes remained on the top and red cells and rosettes sedimented through the gradient. The spleen cells were thus specifically depleted of rosettes and then these spleen cells were injected together with ChRC and SRC to cyclophosphamide-treated mice, and hemagglutinins and RFC were determined seven days later. Passage through the gradient induced a specific unresponsiveness to the type of red cells used for rosette formation (Table 6). Unresponsiveness was not induced if a cell suspension without admixture of red cells was placed on the gradient.

In both kinds of experiments, a clear depletion of specific cells was demonstrated, but it did not distinguish between T and B cells. By GORCZYNSKI et al. (1971) it was shown that the rosette-enriched population was functional.

Cell sorter. Cells labeled with fluorescein-conjugated antibody can be separated using a fluorometric preparative apparatus ("cell sorter") (HULLETT et al., 1969). The cell suspension is forced under pressure through a micronozzle which is vibrated, breaking the jet into 40000 droplets per sec. The droplets pass through a laser beam and if a fluorescent cell is present in a droplet, it will be charged and in an electrostatic field deflected into one container, while uncharged droplets are collected in another container.

JULIUS et al. (1972) have described experiments in which cells from KLH-primed mice were stained with fluorescein-conjugated KLH and separated with the sorter. Marked enrichment of stained cells was obtained in deflected fractions.

In one experiment, where 1.2% fluorescent cells were present in the unseparated cell suspension, the separation procedure enriched in the deflected fraction the percentage of fluorescent cells to 70% and depleted in undeflected fraction to $<0.2\%$. The adoptive transfer experiment showed that 3×10^6 unseparated cells gave an anti-KLH titer $4.6 (\log_2)$, 3×10^6 undeflected fraction gave a barely detectable response, titer $1 (\log_2)$, and 5×10^4 deflected cells mixed with 90×10^6 thymus cells yield an anti-KLH-titer 2.3 (thymus cells alone gave no response).

The great advantage of the cell-sorter separation technique is that both enriched and depleted fractions can be used for functional studies. When the separation methodology is refined further, it should be possible to use cells from unprimed animals in order to investigate the primary response.

B. Specific Lethal Radioactive Antigens

It has been shown in the previous chapter that if normal unprimed lymphocytes are incubated with an antigen, a small fraction of cells will bind the antigen (NAOR and SULITZEANU, 1967; SULITZEANU and NAOR, 1969). Such an antigen-binding can be fatal for a cell if the antigen is labeled with ^{125}I of high specific activity. Radiation from the antigen inactivates or eliminates the antigen-binding cell. Hence, the binding of the antigen becomes a "suicidal" act.

HUMPHREY and KELLER (1970) found that the synthetic antigen TGAL, extensively iodinated to very high specific activity (1400 μCi μg^{-1}) was unable to elicit an antibody response in mice, whereas TGAL substituted with non-radioactive iodine could do so.

ADA and BYRT (1969) exposed normal mouse spleen cells to ^{125}I-polymerized flagellin, unbound antigen was removed and the cells were incubated in cold to allow radiation from the iodine to damage selectively cells which had bound the antigen. The cell suspension was injected to syngeneic irradiated mice together with ^{127}I-polymerized flagellin (non-radioactive) and with poly-

Table 7. Killing of B-precursor cells by specific lethal radioactive antigens (suicide experiment). From BASTEN et al. (1971)

Pretreatment of spleen cells from T × BM[a] mice	Lethally irradiated recipient mice injected with T × BM cells and with	Anti-FγG[b] response (PFC/spleen)	Anti-HRC[c] response (PFC/spleen)
None	FγG + HRC	30	—
^{127}I-FγG[aa]	thymus cells + FγG + HRC	3620	45110
^{125}I-FγG[aa]	thymus cells + FγG + HRC	130	38400

[a] T × BM mice: adult thymectomized irradiated mice protected with bone marrow cells.
[b] FγG: fowl gamma globulin.
[c] HRC: horse red cells.
[aa] ^{127}I-FγG non-radioactive iodinated FγG; ^{125}I-FγG radioactive iodinated FγG.

merized flagellin from a different strain. The recipient mice responded normally to the second antigen but failed to respond to the antigen used in the initial cell treatment.

The above work did not determine whether B or T cells were killed by "suicide". ROELANTS and ASKONAS (1971) have shown that carrier-reactive T cells can be killed by the radioactive antigen. UNANUE (1971) demonstrated that bone-marrow cells can be killed by "suicide". BASTEN et al. (1971) performed an experiment using non-immunized T × BM mice (adult thymectomized irradiated mice protected with bone-marrow cells) as a source of B cells. The spleen cells (containing no T cells) were incubated in the cold either with radioactive (150 μCi μg^{-1}) iodinated fowl gamma globulin (^{125}I FγG) or as a control of "sham" killing with non-radioactive iodinated fowl gamma globulin (^{127}I FγG). The excess of iodinated FγG was washed out and 5×10^7 of treated or untreated T × BM spleen cells were injected with 10^8 thymus cells and FγG and HRC into lethally irradiated mice. Both antigens were injected again on day 9 and the assay of indirect PFC were performed on day 15. The experiment (Table 7) clearly shows that B cells specific to one antigen were killed, whereas B cells with specificity to unrelated antigen were not influenced.

C. Hot Pulse Experiments

It has been shown in the previous section that a cell can be killed by binding an antigen which is highly radioactive. If a proliferating, and thus DNA-synthesizing cell were incubated with tritiated thymidine of very high specific activity, then the cell might incorporate such a quantity of radioactivity that the cell would be inactivated. DUTTON and MISHELL (1967) designed an experiment where they utilized this strategy to damage a small pool of cells which was induced by antigen to proliferate. To restrict the damage to the chosen segment of the population, they limited the presence of the tritiated thymidine to that period of time when it was expected that this segment of the population would proliferate. The "hot pulse" experiment

Table 8. The effect of a hot pulse on the simultaneous response of two non-cross-reacting antigens. (From DUTTON and MISHELL, 1967)

Treatment of the cultures (1.5×10^7 spleen cells from normal mice per ml)			Antibody response (PFC/10^6)			
			Anti-SRC		Anti-BRC	
Day 0	Day 1	Day 2	Day 4	Day 5	Day 4	Day 5
—	—	resuspended in	32	4	0	2
BRC[a]	SRC[b]	medium contain-	103	445	88	187
BRC	SRC + hot TdR[c]	ing an excess	73	460	0	8
BRC	SRC + hot TdR + cold TdR[aa] (excess)	cold TdR[aa]	150	960	97	330

[a] BRC = burro red cells.
[b] SRC = sheep red cells.
[c] Hot TdR = 10 μC of tritiated thymidine in 0.16 μg of thymidine per 1 ml culture (15 C/mM).
[aa] Cold TdR = 100 μg non-radioactive thymidine.

utilizes the fact that if antigen is added to the cultures, the cells specific to that antigen do not proliferate for the first 24 hours. Thus, if an antibody response is initiated to a given antigen, and after an interval of 24 hours a second antigen is added, it should be possible to injure with a hot pulse the cells involved in the response to the first antigen, while leaving undamaged those cells specific to the second antigen.

Burro red cells (BRC) were added to spleen cell suspensions from normal mice at time 0. Sheep red cells (SRC) and a hot pulse (10 μC of tritiated thymidine in 0.16 μg thymidine per 1 ml culture) were added 24 hours later. Thus, in the same culture, the response to BRC was pulsed at 24–48 hours after addition of BRC, while the response to SRC was pulsed at 0–24 hours after addition of SRC. It can be seen in Table 8 that the response to BRC is completely inhibited, while the response to SRC is normal.

This study did not, however, determine which of the two specific cell types (B or T, both required for the antibody response against red cell antigens) was eliminated by the hot pulse. This question was recently answered independently by TROWBRIDGE (1972) and KETTMAN et al. (1973). Both used a hapten-carrier system in which spleen cells of mice recently primed with SRC were placed in culture with NIP-SRC (TROWBRIDGE, 1972) or TNP-SRC (KETTMAN et al., 1973) and given a hot pulse. Because the precursors to anti-SRC were activated by *in vivo* priming, and precursors to anti-hapten only by the antigen added *in vitro*, a differential action of the hot pulse was expected. Indeed, the anti-hapten response (Table 9) was unaffected by the hot pulse (0–24 hour period), while the anti-SRC response was greatly reduced. If T cells had been dividing, then both the anti-hapten and anti-SRC response would have been depressed by the hot pulse. Since only the anti-SRC response was depressed, the helper T cell survives the hot pulse. Hence, it is possible to conclude that antigen-specific B cells can be eliminated by a hot pulse.

Table 9. The effect of a hot pulse on the anti-hapten and anti-carrier response with carrier-primed cells

Treatment of the cultures[a]			Antibody response (PFC/10^6)		Reference
Day 0	Day 1	Day 2	Day 4	Day 5	
			Anti-NIP	Anti-SRC	
SRC	—	—	39	2976	TROWBRIDGE (1972)
NIP-SRC	—	—	769	3141	
NIP-SRC + hot TdR[c]	removal of pulse[b]	—	593	119	
NIP-SRC	hot TdR	removal of pulse[b]	7	33	
			Anti-TNP	Anti-SRC	
TNP-SRC + hot TdR	TNP-SRC + cold TdR[aa] (excess)	—	720	1168	KETTMAN et al. (1973)
TNP-SRC + hot TdR + cold TdR (excess)	TNP-SRC	—	777	6601	

[a] Trowbridge used 1.5×10^7 spleen cells/ml from mice primed with SRC 4 days earlier. Kettman et al. used 1.2×10^7 spleen cells/ml, priming 3 days prior to culture.
[b] Trowbridge removed the pulse by washing the cells, Kettman et al. by chasing with excess of cold thymidine.
[c] Hot TdR = 10 μC of tritiated thymidine/per 1 ml culture (15 C/mM).
[aa] Cold TdR = 100 μg non-radioactive thymidine.

D. Antigen-Induced Depletion of Precursor Cells

It is very much tempting to discuss in this section the problem of immunological tolerance. The question of tolerance, however, would be relevant only if it were possible to show that tolerance, or a certain form of it, involves elimination of a specific cell. There is, to my knowledge, no experiment which would show this. If a tolerogenic dose of antigen causes unresponsiveness to the tolerizing antigen and does not affect responsiveness to an unrelated one, this by no means distinguishes whether a unipotential cell was eliminated or the corresponding specificity in pluripotential cell was "locked".

Nevertheless, experiments have been reported which do show antigen-induced depletion of specific precursor cells. These experiments are related to the selective removal of cells from the recirculating lymphocyte pool. Thoracic duct lymphocytes (TDL) from mice (SPRENT et al., 1971) and rats (ROWLEY et al., 1972) recently primed with heterologous erythrocytes were found to be specifically unresponsive to the primary antigen on adoptive transfer; unresponsiveness was shown to involve both T and B lymphocytes. These findings were interpreted in terms of an antigen-induced selective removal of reactive lymphocytes from the recirculating lymphocyte pool to organs such as the spleen, i.e. regions where high concentrations of antigen would be expected

Table 10. Antibody response of irradiated mice injected with spleen cells, TDL[a] or lymph node cells from recently primed mice

Cells transferred to irradiated mice[b]	PFC per spleen (7S)[aa]			
	at 7 days (SRC+HRC[bb] at day 0)		at 14 days (SRC+HRC at day 7)	
	anti-SRC	anti-HRC	anti-SRC	anti-HRC
Spleen cells from SRC primed mice	110	113660	70450	15400
Spleen cells from HRC primed mice	67180	20	65090	36460
TDL from SRC primed mice	0	32970	0	2890
TDL from HRC primed mice	102180	0	43920	0
LN[c] cells from SRC primed mice	0	7830	0	7150
LN cells from HRC primed mice	21050	0	42930	0

[a] TDL = thoracic duct lymphocytes.
[b] Cell recipients were thymectomized before irradiation to prevent de novo formation of T cells from stem cells in donor cell inocula; recipients of TDL or lymph node cells were given a small number of bone marrow cells on day +4 to prevent the mice from dying.
[c] LN = mesenteric lymph node.
[aa] 19S PFC gave similar results.
[bb] SRC, HRC = sheep and horse red cells.

to trap the responding cells. SPRENT and MILLER (1974) recently tested a corollary to this interpretation, namely that if i.v. injection of antigen does indeed lead to a specific recruitment of lymphocytes to the spleen, then spleen cells, in contrast to TDL or lymph node cells should be unimparied in their capacity to respond to such antigens on transfer. The authors used the following experimental design to test this prediction:

a) Prospective donors of TDL or mesenteric lymph node cells or spleen cells were primed with either SRC or HRC for 24 hours and transferred to irradiated mice.

b) The recipient mice were injected with a mixture of SRC and HRC, either at the time of transfer or 1 week later.

c) PFC responses to SRC and HRC in spleens of recipient mice were measured one week after challenge with antigen.

As can be seen from Table 10, when the irradiated recipients were challenged with antigen 1 week after cell transfer and antibody production was measured at 2 weeks, only the recipients of spleen cells produced antibody to the priming antigen. In other experiments the authors observed that the antigens used for priming localized in the spleen but not in the lymph nodes. The results were thus compatible with the idea of mobilization of specific precursor cells from the lymph nodes into the antigen-rich environment of the spleen. The failure of recently primed spleen cells to respond to the priming antigen within the first week after transfer was considered to be related to the transformed state of the specific cells in the spleen at that time (SPRENT and MILLER, 1973).

V. Arguments against the Procommitment Hypothesis

In spite of the overwhelming body of evidence in favour of the precommitment hypothesis we have to be aware of several experimental findings which are (or seem to be) incompatible with the precommitment hypothesis.

Before turning to the subject, it has to be restated that the precommitment in this review is meant in the strict sense: when a precursor of an antibody-forming cell meets an antigen for the first time, the cell is already committed to formation of antibody of a *single* specificity. Thus, among arguments against the precommitment hypothesis are not only experiments demonstrating that cells are non-committed, but also findings of oligopotentiality.

A. Antigenic Competition

If two antigens are used to immunize the same animal, it is sometimes observed that there is a decreased response to one or both antigens as compared to the response when each antigen is injected separately. In such cases, the antigens are said to "compete" with each other (for review see ADLER, 1964 and TAUSSIG, 1973). If precursor cells were non-committed, such antigenic competition might be expected. Let us examine, however, a few consequences of assuming that antigenic competition results from competition for a B precursor cell:

a) A competition for a common non-committed precursor cell should be more pronounced when the two competing antigens would be administered simultaneously, than if there would be a time interval between the two antigens injected.

b) In an adoptive transfer system, the competition for a common non-committed precursor cell should be more pronounced using lower numbers of injected cells than with higher numbers.

c) If cells removed from an animal immunized with one antigen are exposed to a second antigen either *in vitro* or in adoptive transfer system, a diminished response to the second antigen should be seen.

As to the first point, it has been shown by ADLER (1964), RADOVICH and TALMAGE (1967), EIDINGER et al. (1968) and MÖLLER and SJÖBERG (1971) that this is not the case; on the contrary, a time interval of several days between the two injections is more effective.

The second prediction was tested by RADOVICH and TALMAGE (1967). Lethally irradiated mice received transfers of 10^7 or 5×10^7 normal spleen cells; on the same day as transfer, horse red cells were also injected and sheep red cells were given four days later. The results of the experiment showed, that contrary to what would be expected if the cells were uncommitted, the competition was more pronounced at the higher cell concentration.

The third point was tested by several groups. WATERSTON (1970) reported that prior *in vivo* exposure to pig red cells caused an increase rather than a decrease in the *in vitro* response to SRC. Furthermore, MÖLLER and SJÖBERG (1971) have shown that when HRC-primed mice (which would have shown a decreased response *in vivo* to SRC) are sacrificed and their spleens injected

together with SRC into irradiated recipient mice, no sign of antigenic competition was observed. The findings of WATERSTON (1970) and MÖLLER and SJÖBERG (1971) strongly suggest that the milieu of the precursor cells rather than the precursor cells themselves or their number is affected during antigenic competition.

The greatest obstacle to taking antigenic competition as a serious argument against the precommitment hypothesis is that it is irregular. There are many cases in which an *increased* response is obtained, when the same animal is immunized with two antigens. Here, one speaks of an "adjuvant effect" (DRESSER et al., 1970) or antigenic promotion (WU and CINADER, 1971). I consider the above arguments as an indication that the antigenic competition, whatever complex events it may include, is not competition for a B precursor cell.

B. The Bussard Phenomenon

The magnitude of the antibody response is usually not considered to be relevant for the precommitment hypothesis. If, however, a large number of antibody-forming cells were to appear early in the antibody response in the absence of cell division, then a considerable fraction of cells must have participated in the initiation of the antibody response. This might be incompatible with the precommitment hypothesis.

A series of papers (BUSSARD and LURIE, 1967; NOSSAL et al., 1970; BUSSARD et al., 1970; BORIS et al., 1970) deals with the *in vitro* antibody response to SRC by peritoneal cells. Peritoneal cells from non-immunized mice yield a large number of anti-SRC antibody-forming cells within 24 hours of culture. The cells were cultured in a carboxymethylcellulose-containing medium in presence of SRC. Peritoneal cells from retired breeders (3rd or 4th pregnancy) formed plaques more extensively and rapidly, yielding peak PFC numbers twenty fold higher than those found with young male mice. The highest figure reported in the series of papers mentioned is about 60 PFC/10^3 peritoneal cells from retired breeder mice. These cells became plaque-formers without cell division. A frequency of "precursors" of 6×10^{-2} is clearly incompatible with the precommitment hypothesis.

Only peritoneal cells, and not spleen cells or lymph-node cells, contain the unusual large number of cells which in short term cultures can be stimulated to produce anti-SRC antibody. Furthermore, peritoneal cells react so strongly only with sheep red cells; there are essentially *no* cells capable of reacting to horse red cells (BORIS et al., 1970). There are no reports concerning other antigens. While the authors present convincing evidence that the observed plaques result from antibody formation, the lack of generality precludes acceptance of these results as strong arguments against the precommitment hypothesis.

C. RNA Transformation

FISHMAN and ADLER (1963) extracted RNA from macrophages which had been incubated previously with bacteriophage and they demonstrated that

this extract was able to stimulate synthesis of phage-neutralizing antibody in normal lymph node cells. ASKONAS and RHODES (1965) have shown that such RNA extracts contain antigen and suggested that macrophages processed the antigen and converted it to a highly immunogenic form. ADLER et al. (1966), however, extended these studies and found that phage-neutralizing antibody (induced by the extract) carried an allotypic marker from the donor of the RNA. This implied that information had been transferred by the RNA.

A number of experiments have been reported by BELL and DRAY (1969, 1970, 1971 a, b, 1972, 1973) in which the authors describe conversion of non-immune rabbit spleen cells by RNA from immunized rabbit to produce IgM and IgG of donor light chain allotype. Furthermore, spleen cells from non-immunized rabbits were converted to antibody-forming cells by incubation with RNA extracts of lymph node cells obtained from rabbits immunized with SRC. If the RNA was extracted from lymph nodes of rabbits immunized 5 days previously, IgM anti-SRC antibody-forming cells were produced, while if the donor of RNA was immunized 18 to 24 days previously, IgG anti-SRC antibody-forming cells were detected in the converted spleen cells.

Although the RNA transformation experiments are often considered to be arguments against the precommitment hypothesis, I believe that in fact they are irrelevant to the subject. First of all, it is not surprising that a cell equipped with a functional protein synthetic machinery would be able to translate RNA to protein if the former could enter the cell intact. It has recently been shown that RNA, injected into amphibian eggs, will be readily translated into myeloma polypeptide chain (STEVENS and WILLIAMSON, 1972). I suggest the RNA transformation experiments should be disregarded in the present context as they probably do not reflect normal *in vivo* processes.

D. Small Animals

It has been shown (for review see DU PASQUIER, 1973) in studies with tadpoles, that the first antibody response could be detected in spleens containing 10^4 cells. A small larvae of *Xenopus laevis* at 4 weeks of larval life may have about half a million lymphocytes, from which (if thymus cells, immature cells and T cells are subtracted) not more than 100000 lymphocytes might be presursors of antibody-forming cells. Are these 100000 cells able to cope with the whole spectrum of immunogenic determinants which an animal of this size may encounter?

The precommitment hypothesis entails the intrinsic assumption that the repertoire of the antibody-forming capacity in any individual cannot be larger than the number of lymphocytes the individual possesses. If a tadpole with a very small number of lymphocytes is able to respond by antibody production to any tested antigen, the following possibilities are implied:

a) The cells are precommitted, and the "repertoire" (although smaller than in a large animal) is capable of discriminating any antigen.

b) The cells are precommitted, and each animal possesses at any given time only a fraction of the "repertoire" of a large animal, and is able to produce well-fitting antibody only to a limited segment of the antigenic spectrum, while for the rest of the antigens less well-fitting antibodies would be produced.

c) The cells are precommitted, but due to the rapid lymphocyte turnover, the antigen selects from far more cells than are present at any one time.

d) The cells are uncommitted and the antibody response might be initiated in any cell upon an antigenic stimulus.

There are various observations which are relevant to this issue. Amphibian larvae are able to mount a measurable antibody response to a large number of antigens (heterologous red cells, serum proteins of fishes and mammals, TMV, bacteriophages, Salmonella, Streptococcus, DNP, TNP, TGAL etc.) (COOPER et al., 1964, 1968; DU PASQUIER, 1967, 1969, 1970; HAIMOVICH and DU PASQUIER, 1973; CHING and WEDGWOOD, 1967). The larval stage differs from species to species; in *Xenopus laevis* metamorphosis occurs at about 2 months of larval life, while in *Alytes obstetricans* it may occur after 2 years. Irrespective of these differences the larvae at the age of 4–5 weeks have about half a million lymphocytes. There is no strict correlation between the stage of development and the size of the spleen (DU PASQUIER, 1973). At an early stage, when a spleen contains about a thousand cells, the frequency of rosette-forming cells to human red cells is about 10^{-2}; this frequency decreases to 10^{-3} with increasing spleen size. Antibody response to heterologous red cells was regularly detected in animals the spleens of which had more than 10^4 cells. The magnitude of the response is, however, low (rarely more than 10 PFC/10^5 spleen cells) (DU PASQUIER, 1970). Tadpoles of *Rana catesbiana* (at a stage when they possess about two million lymphocytes) are able to produce anti-DNP and anti-TNP antibody of the same spectrum of cross-reactivity and the same affinity as is found in several mammalian species (HAIMOVICH and DU PASQUIER, 1973).

When it is stated that larvae are able to produce antibody to a large number of antigens, it has to be stressed that most of the experiments were dealing with "large" larvae, possessing many millions of lymphocytes. A set of interesting experiments by HAIMOVICH and DU PASQUIER (1973) on cross-reactivity and affinity was performed with tadpoles which had about two million lymphocytes. The only kind of experiments where the number of cells was rigorously controlled were experiments on rosette-forming cells. Although it is unquestionable that tadpoles which had at least 10^4 cells in their spleens gave regularly an antibody response to red cells, specific cells may have been recruited from a much larger pool of lymphocytes than 10^4 cells.

Thus, I suggest that the evidence on the antibody response in small animals is as yet inconclusive. Nevertheless, the issue discussed above is probably the most significant impediment for accepting the precommitment hypothesis.

VI. Conclusions

I have defined the precommitment hypothesis as follows: when a precursor of an antibody-forming cell meets an antigen for the first time, the cell is already committed to formation of antibody of a *single* specificity.

The review was concerned with the behavior of B lymphocytes. Experiments on non-immunized animals or their cells were considered in particular.

The experimental evidence in favour of the precommitment hypothesis was based on the following findings:

a) Individual B cells display only one type of receptor, homogeneous in respect to specificity. This was demonstrated by antigen-induced redistribution of receptors (cap formation) and subsequent examination for presence of immunoglobulin molecules of irrelevant specificity outside the cap. It was found that the antigen (POL) was able to cap all of the immunoglobulin detectable by fluorescence on most POL-binding cells. Minute amounts of immunoglobulin (below 3%) would escape detection.

b) The fraction of cells specific for a given antigen is low. The frequency of specific precursor cells was estimated by several methods for a number of different antigens. One of the methods, the antigen-binding technique, is not based on analyzing the capacity of cells to produce an antibody response, and the estimates of frequencies obtained by this method were considered as upper limits of frequencies. The size of the subset which is physiologically significant as precursor cells in the antibody response is not known. The estimated frequency for flagellin was about 10^{-4}, for hemocyanin about 10^{-3} and for sheep red cells about 10^{-3}. All other methods for frequency estimation involved tests for the capacity of precursor cells to produce antibody. This was measured either by the splenic foci technique or by cell-transfer limiting dilution experiments, as well as by the *in-vitro* microculture technique. The estimates for precursor cells specific for red-cell determinants by the cell-transfer method were in the range of $0.2–3.3 \times 10^{-6}$, for synthetic polypeptide antigens of limited heterogeneity $0.3–3 \times 10^{-7}$ and for lipopolysaccharide 1×10^{-5}. These estimates are not corrected for the effective homing efficiency. In the microculture experiments the frequency of precursor cells specific for SRC was estimated as 2.1×10^{-5}

c) It was possible to remove from a cell population cells specific for a given antigen. It was shown that reactivity to the chosen antigen was lacking in the remaining population, and was present in the removed or separated subset in some appropriately designed experiments. Methods used to achieve the above were (1) physical separation (antigen-coated columns, derivatized nylon fibers, gradient techniques and electronic cell sorter); (2) suicide experiments, (3) hot pulse experiments and finally antigen-induced depletion of specific precursor cells. By all the methods, depletion to the relevant antigen and indifference to an irrelevant one were shown. In few cases (polyacrylamide columns, rosettes) the eliminated cells were shown to be fully functional in

the specific antibody reponse and a considerable enrichment of precursor cells was demonstrated.

The reported studies have supported the precommitment hypothesis at least in respect to B lymphocytes. No attempt has been made in this review to analyze the evidence for precommitment of T lymphocytes. There are many experiments showing that different subpopulations of T cells respond to different antigens (SALMON et al., 1971; ZOSCHKE and BACH, 1971) and a thorough analysis of this evidence may well show that the precommitment hypothesis is as valid for T cells as for B cells.

Although all experiments are fully compatible with the idea of *strict precommitment* (e.g. precommitment to *single* specificity) this was not proven. Some methods by their nature cannot prove *strict precommitment*, and others are not sufficiently sensitive. Extreme restriction of the immunologic specificity and potentiality of a given cell was however proven beyond any doubt.

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DNA-Directed Enzyme Synthesis *in vitro*

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With 15 Figures

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Introduction

During the last few years, the method of DNA directed enzyme synthesis *in vitro* has become a powerful tool in the study of gene expression.

While earlier polynucleotide directed synthesis of polypeptides in cell-free systems served as the key for the elucidation of the genetic code and the basic mechanism of protein synthesis (WOESE, 1967; LUCAS-LENARD and LIPMANN, 1971), DNA directed enzyme synthesis is now the key to the mechanism and the regulation of specific transcription and translation. Before this approach had been worked out, transcription could be studied only by the gross incorporation of nucleotide label into acid-insoluble material (ZILLIG et al., 1966; review by SETHI, 1971) and by hybridization techniques (HALL and SPIEGELMAN, 1961; GILLESPIE, 1968). However, little information on the fidelity or specificity of transcription could be obtained by these methods. The direct test for both fidelity and specificity became available with the successful DNA directed synthesis of enzymes *in vitro*. The synthesis of active enzymes is an excellent indicator for the proper recognition of transcription signals such as the signals for initiation and termination, and for the fidelity of chain elongation.

Enzyme synthesis *in vitro* supplements genetic analyses by providing information on the map positions of promotors and genes. And above all, the coupled transcription-translation systems have opened new approaches into the regulation of protein synthesis such as the understanding of the control of the lactose operon and of catabolite repression. Enzyme synthesis *in vitro*

has promoted the study of phage control mechanisms. Initial *in vitro* work on various *E. coli* operons and even eukaryotic regulation looks promising.

The advantage of *in vitro* enzyme synthesis in these studies is clearly due to the intrinsic properties of such *in vitro* systems: The removal of the cell wall and membrane barrier from the protein synthesis machinery allows the thorough analysis of the reaction, of the requirements of the reaction and of optimal ion conditions. Molecules that would not pass the membrane such as proteins, RNA or DNA can be added or removed. Equally advantageous are the possible exchange of factors from different organisms, the free access of inhibitors, nucleoside triphosphates and other substrates.

The potentials of cell-free systems for various applications were recognized early, and, therefore, attempts to develop cell-free systems date back to the early sixties (MATTHAEI and NIRENBERG, 1961; WOOD and BERG, 1962; DOERFLER et al., 1962). It was only in 1966 that a specific protein was formed *in vitro:* the bacteriophage T4 DNA directed synthesis of specific T4 early antigens (TRAUB et al., 1966). The synthesis of another specific peptide, the α-fragment peptide of β-galactosidase, was detected by enzymatic complementation (DE VRIES and ZUBAY, 1967). Briefly thereafter, the DNA directed synthesis of complete active enzymes was achieved (SCHWEIGER, 1967; SCHWEIGER et al., cited by SALSER et al., 1967; SCHWEIGER, 1968; LEDERMAN and ZUBAY, 1968; GOLD and SCHWEIGER, 1969; former attempts to synthesize enzymes *in vitro* have been reviewed by NISMAN and PELMONT, 1964). Cell-free enzyme synthesis was developed thereafter to a standard procedure and is used in many laboratories.

This review will summarize data and ideas that have, in any way, been collected and promoted by cell-free enzyme synthesis (Chapter I). The second part will deal in some detail with the characteristics of cell-free enzyme synthesis and the principles of available systems (Chapter II). We feel that these details will make the potential of the method apparent.

[Detailed descriptions of methods have been published elsewhere (ZUBAY et al., 1970; GOLD and SCHWEIGER, 1971; HERRLICH and SCHWEIGER, 1974). We should like to draw the reader's attention to the following review articles on related subjects: LUCAS-LENARD and LIPMANN, 1971; HAYASHI, 1971; CALENDAR, 1970; ZUBAY and CHAMBERS, 1971; LOSICK, 1972; STUDIER, 1972; LIPMANN, 1973.]

I. Enzyme Synthesis *in vitro* as a Tool for the Study of Regulation of Gene Expression

A. Bacteriophage Regulation

One of the most interesting problems of protein biosynthesis concerns the mechanism by which a virus gains control of the synthetic apparatus of its host and governs the synthesis of its own products. The control mechanisms of protein synthesis differ from one bacteriophage to the other. A whole

spectrum of control mechanisms are being detected through the use of phage. Three groups of phage have been examined with the methods of DNA dependent cell-free protein synthesis: Bacteriophage T7 and T3 are the smallest among those examined. T3 and T7 induce their own RNA polymerase. The T even phage T4 and T2 are particularly interesting because of the close relationship: replication–transcription. The study of $\phi 80$ and λ has just begun to reveal the mechanisms involved in lysogeny and appears rewarding since the collection of genetic data of phage λ is rich.

1. Regulation of Gene Expression after Infection with Bacteriophage T3 and T7

a) The Genome

T3 and T7 are members of a group of related bacteriophages (HAUSMANN et al., 1962; ADAMS and WADE, 1954; CUZIN, 1965). The similarities between T3 and T7 in morphology, antigenic properties, size of DNA and life cycle are balanced by various differences mainly in genetic details, the most important one being the gene for S-adenosylmethionine hydrolase which is present only in T3 (HAUSMANN, 1967). Enzyme synthesis *in vitro* has been the major tool that has led to many of the developmental control mechanisms of these phage. Because more work has been done on T7, T3 will be referred to only when differences between both phage systems have been found. Most comparative examinations suggest similarity.

T7 DNA with the molecular weight 25×10^6 (STUDIER, 1965) is double-stranded, linear, not permuted (RITCHIE et al., 1967) and, theoretically, would have coding capacity for about 30 proteins. The isolation and examination of a large number of amber mutants (800) served to define 19 genes (HAUSMANN and GOMEZ, 1967; STUDIER, 1969; STUDIER, 1972). The genes were numbered from "left" to "right" (Fig. 1). The complementation groups 1–6 are deficient in DNA synthesis, while groups 7–19 are concerned with structural proteins and with maturation. Enough unassigned DNA remains, particularly at the left side of the genome to suggest that this DNA codes for non-essential cistrons or for repetitive cistrons of similar function.

Some of these genes have now been detected and characterized, either by cell-free enzyme synthesis (HERRLICH and SCHWEIGER, 1970; SCHWEIGER et al., 1971; SCHERZINGER et al., 1972a) (A), by measurement of UV sensitivity (SCHERZINGER et al., 1972a; HERRLICH et al., 1974) (B) or by the isolation of deletion mutants (STUDIER, 1972; STUDIER, 1973) (C).

(A) By kinetic measurements of messenger and enzyme synthesis *in vitro* one can determine the location of a gene. In T7, the mapping of genes by *in vitro* synthesis of their products is greatly facilitated because *E. coli* RNA polymerase starts from one promoter region only (see Fig. 1). This promotor was found to be between 0.5 and 1.5% (of total genome) from the left end of the DNA (DAVIS and HYMAN, 1970; DUNN and STUDIER, 1973). The promoter may be complex with several polymerase molecules binding (SCHÄFER

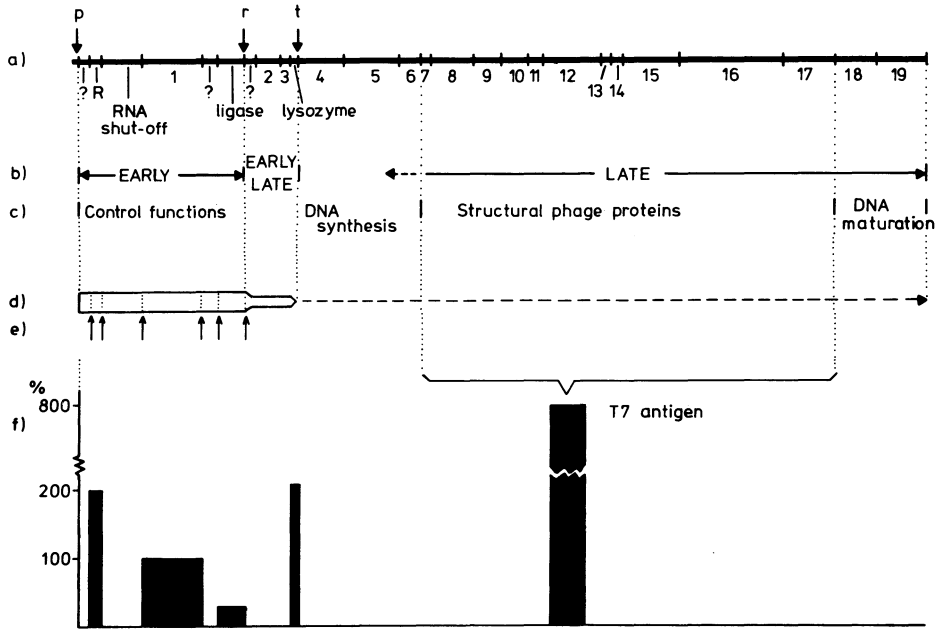

Fig. 1a—f. The T7 genome. a Genetic and physical map. b Time of gene expression. c Gene functions. d Relative transcription by *E. coli* RNA polymerase, thickness of arrow related to transcription frequency. e Posttranscriptional cleavage by RNase III. f Relative transcription frequency by T7 phage RNA polymerase *in vitro*. The height of the columns indicates transcription frequency in percent of the template activity obtained by *E. coli* RNA polymerase. (The data are taken from Table 1 of ref. SCHERZINGER et al., 1972b.) p = Promotor used by *E. coli* RNA polymerase. r = Reduction signal, site which causes a decrease of transcription by *E. coli* RNA polymerase. The signal can be deleted (STUDIER, 1972). The host polymerase then reads through. t = Termination signal. t is recognized *in vitro* by *E. coli* RNA polymerase (MILLETTE et al., 1970). Evidence is not sufficient to decide whether t is located between the lysozyme gene and gene 4 or beyond gene 4. t is probably also recognized *in vivo* (SCHERZINGER et al., 1972a). The relative sizes of the genes 1, 2, 3, 4–19 correspond to published data (STUDIER, 1972). Size and positions of the other genes were determined as described in Fig. 2. R = Repressor gene which is responsible for the shut-off of host translation

et al., 1973) or with more than one independent chain initiation site (DUNN and STUDIER, 1973; CHAMBERLIN and RING, 1972), but its position is sufficiently defined for kinetic measurements. The various lag periods for enzyme synthesis *in vitro* could be attributed to lag periods of transcription (SCHWEIGER et al., 1971; SCHERZINGER et al., 1972a). From the unique promoter region, the genes are transcribed in a sequential order—the time of appearance of messenger and enzyme respectively are correlated with the distance between the promoter and the right end of each gene. The characteristic time course of appearance of those genes that are transcribed by *E. coli* RNA polymerase, is a function of the order of genes (the completely asymmetric transcription from the H strand of phage T7 was first shown by SUMMERS and SZYBALSKI, 1968).

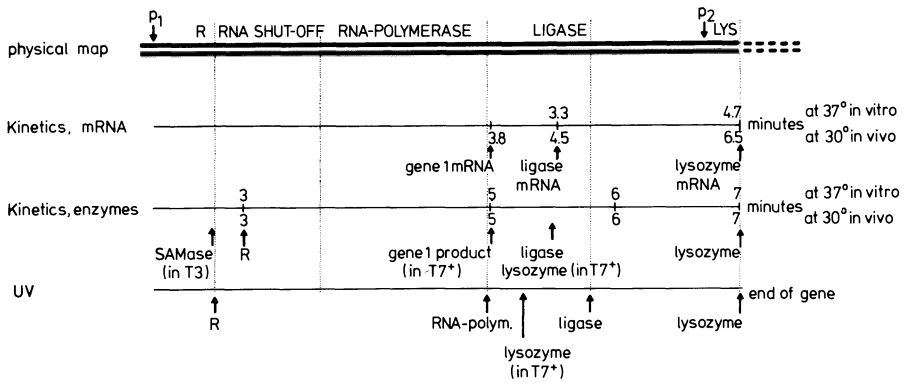

Fig. 2. Physical map of the left-most part of the T7 genome. The data were obtained by measuring kinetics of mRNA and enzyme appearance, both *in vivo* and *in vitro*, and by determining the UV sensitivity of gene expression after infection of *E. coli* B_{s-1} with irradiated T7. T7 gene 1 mutants were used in all *in vivo* experiments except where indicated. The physical map was compared with the map obtained from genetic crossing of available mutants. p_1 = Promoter used by *E. coli* RNA polymerase. p_2 = one of the promoters used by T7 RNA polymerase (gene 1 product). p_2 was mapped by calculating the difference between UV sensitivities and kinetics of appearance of gene 1 product and lysozyme in cells infected with T7 wild type. R = Repressor of host translation. There is a time loss at the start of the kinetic mesurement which may be due to delay by adsorption of phage and by initiation of RNA synthesis. UV data and kinetics determine the promoter-distal ends of genes, not the gene sizes themselves. The gene for RNA shut-off is to the left of gene 1. It does not necessarily fill the space between R and gene 1

By *in vitro* synthesis, the genes for lysozyme and ligase have been mapped (HERRLICH and SCHWEIGER, 1970; SCHERZINGER et al., 1972a).

As will be described later, the kinetics of appearance of enzymes *in vitro* correspond well to the kinetics *in vivo* (Fig. 2). Together with other methods, *in vivo* kinetics helped to map various other genes in the left-side section of T7 DNA: the genes for the translational-repressor, for the RNA shut-off function, the protein phosphokinase and a phospholipid synthesizing activity (SCHWEIGER et al., 1972; HERRLICH et al., 1973 and 1974; RAHMSDORF et al., 1973 and 1974).

(B) The sensitivity of enzyme synthesis to UV irradiation *in vivo* or *in vitro* is another method for physical mapping of genes. UV irradiation introduces transcriptional blocks into the DNA (SAUERBIER et al., 1970) and the UV sensitivity of a gene is proportional to the length of DNA between the promoter and the end of this gene (Fig. 2 and HERRLICH et al., 1971a; HERRLICH et al., 1974). The location of genes determined by UV-sensitivity measurements and by cell-free synthesis of enzymes correspond well and both results were confirmed by genetic mapping (SCHERZINGER et al., 1972a; HERRLICH et al., 1974).

(C) By mapping deletion mutants, a third method became available in the search for the missing T7 genes. Based on the selection method of PARKINSON and HUSKEY (1971) deletion mutants of T7 were isolated in two locations of

the genome: at about 17% (of total genome, counting from left to right) and in the region adjacent to gene 1 (below 8%; STUDIER, 1972; STUDIER, 1973). The accurate mapping of these deletions by electron microscopic length determinations of heteroduplex DNA and comparison with point and amber mutations yields another link between physical and genetic map (SIMON and STUDIER, 1973).

By cell-free enzyme synthesis, the UV irradiation technique, and deletion mapping, most of the genes that were missing could be identified (Fig. 2). Between the promoter and gene 1 the gene for the following functions have been located: alteration of phospholipid metabolism (HERRLICH et al., 1973), the shut-off of host macromolecular synthesis (SCHWEIGER et al., 1972; STUDIER, 1972; BRUNOVSKIS and SUMMERS, 1972; HERRLICH et al., 1974; STUDIER, 1973) and a protein phosphokinase (RAHMSDORF et al., 1973 and 1974). Gene 1 (RNA polymerase) is followed on the right by the gene for DNA ligase (SCHERZINGER et al., 1972a; STUDIER, 1972), gene 2, 3 (endonuclease) (STUDIER, 1969) and lysozyme (SCHWEIGER et al., 1971) (Fig. 1).

b) In vitro and in vivo Transcription of T7 DNA

Bacteriophage T7 depends initially on the transcriptional machinery of the host. Transcription of T7 DNA *in vitro* should, therefore, imitate the first steps of infection.

In vitro transcription of T7 DNA by purified *E. coli* RNA polymerase in the absence of additional factors yields a specific RNA product (MILLETTE et al., 1970; confirmed by MAITRA et al., 1970, and DUNN and STUDIER, 1973). The majority of this RNA has an approximate molecular weight of 2.4×10^6 daltons; a small amount of 3.4×10^6 dalton species is also synthesized (MILLETTE et al., 1970). End labeling experiments (KAMEN, 1969; LEPPLA et al., 1968) indicate that the 2.4×10^6 dalton species is terminated uniquely with U (MILLETTE et al., 1970) and the 3′-terminal decanucleotide has been identified (MILLETTE et al., 1970). Thus, *in vitro*, *E. coli* RNA polymerase transcribes this RNA species from one promoter and terminates at a distinct point without the need for any additional factors.

The T7 RNA synthesized *in vitro* was shown by *in vitro* enzyme synthesis to be polycistronic and carrying the information for at least RNA polymerase (gene 1), ligase and lysozyme (MILLETTE et al., 1970; SCHWEIGER et al., 1971; SCHERZINGER et al., 1972a). If one assumes that the *in vitro* RNA is initiated at the natural promoter, the 2.4×10^6 dalton RNA would be complementary to about 20% of the T7 length and include the genes up to the end of DNA ligase, the 3.4×10^6 dalton RNA would be complementary to about 30% of the T7 length and would include the lysozyme gene. Since the 2.4×10^6 dalton and the 3.4×10^6 dalton species are synthesized *in vitro* in a ratio of about 4:1 (data compiled from Fig. 4 in MILLETTE et al., 1970, and from Table 1 in SCHWEIGER et al., 1971), it appears that about 80% termination occurs at 20% of the genome and 20% termination at about 30% of the genome.

Experiments using a coupled transcription-translation system from uninfected *E. coli* instead of a pure transcription system, led to the same conclusion. Under conditions which did not allow the activation of phage RNA polymerase, *E. coli* RNA polymerase transcribed the lysozyme gene (HERRLICH and SCHWEIGER, 1970), but apparently terminated RNA synthesis thereafter because no late proteins were detected (unpublished).

These *in vitro* data reflect the transcription pattern *in vivo*. The region up to 20% of the genome is transcribed efficiently by host RNA polymerase, and, with reduced rate, RNA is synthesized up to a termination signal at about 30%.

The following findings led to this conclusion:

In cells infected with gene 1 mutants, where no active phage RNA polymerase is induced, the left-most 20% of the T7 genome are efficiently expressed (STUDIER and MAIZEL, 1969; SCHERZINGER et al., 1972a; STUDIER, 1972). Gene 1 mutants also induce lysozyme synthesis indicating that the region between the first termination signal at 20% and the second termination signal at 30% (early-late region) is transcribed (Fig. 3; HERRLICH et al., 1971a). The efficiency of transcription of this region appears to vary from one T7 strain to the other. Some gene 1 mutants induce lysozyme synthesis with high efficiency whereas other gene 1 mutants induce less lysozyme synthesis (Fig. 3). The T7 induced protein kinase (RAHMSDORF et al., 1974)—as one of the factors—influences the extent of early-late transcription (RAHMSDORF et al., 1974 in prep.).

Using T7 am 342 as the parental strain, a deletion of the first termination signal was isolated (STUDIER, 1972). Then, *E. coli* RNA polymerase appears to transcribe through to the right end of the T7 genome. The efficiency of transcription of late genes, however, is low (Fig. 1 and 3 in STUDIER, 1972). In addition, most of the read-through RNA in this deletion mutant seems to end at about 30% (SIMON and STUDIER, 1973).

The existence of a transcription unit reaching from the unique promoter region to 30% of genome is also indicated by the kinetics of messenger RNA and enzyme synthesis of the various genes in that region (see page 64 and Fig. 2), and is indicated by the determination of UV-sensitivity of enzyme synthesis, both *in vitro* and *in vivo* (HERRLICH and SCHWEIGER, 1971b; HAUSMANN and HÄRLE, 1971; HERRLICH et al., 1974). The genes at the left side of T7 DNA show the same UV-sensitivity gradient *in vitro* and *in vivo*.

When RNA is isolated from infected cells, however, such a polycistronic messenger is not found (SUMMERS, 1969; SIEGEL and SUMMERS, 1970). Instead discrete species of smaller sizes are found. A posttranscriptional modification mechanism *in vivo* has been proposed which would allow the production of smaller species (MILLETTE et al., 1970). Very recent experiments have produced evidence that such posttranscriptional cleavage indeed exists (DUNN and STUDIER, 1973a). RNase III from uninfected cells cleaves the "*in vitro* RNA" into just those 5 main RNA species which occur *in vivo* after T7 gene 1

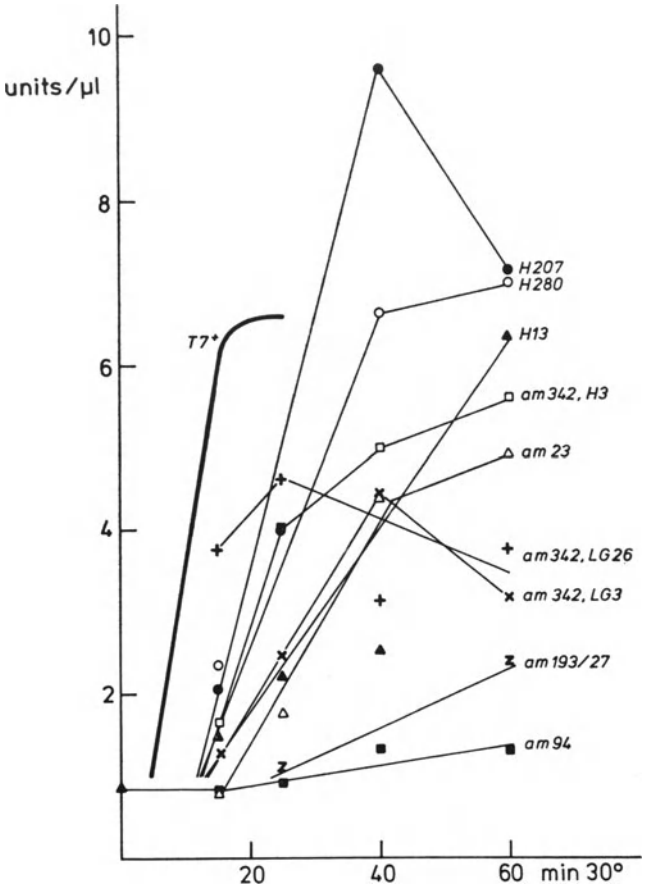

Fig. 3. Lysozyme synthesis in *E. coli* B_{s-1} infected with T7+ and various T7 gene 1 mutants. *E. coli* B_{s-1} were grown at 30° in M9 (0.5% glucose, 10^{-4} M $MgSO_5$) to $O.D._{600} = 0.5$. 10 ml aliquots were infected with phage at a multiplicity of infection of 10. The surviving cells were reduced to 0.5–2% at 3 minutes after infection. At 8 minutes after infection, enough T7 antiserum was added to reduce the nonadsorbed phage to 10^{-6}. 2 ml aliquots were collected on ice and 50 µg/ml chloramphenicol. The cells were resuspended in 100 µl TMA buffer containing 100 µg/ml bovine serum albumin and treated by freeze-thawing (5 times). The resulting extracts were used for the determination of lysozyme activity. The mutants H207, H280, H13 were obtained from Dr. HAUSMANN'S collection. T7+, am 342-H3, am 342-LG26, am 342-LG3, am 23, am 193-27, am 94 have been isolated by Dr. STUDIER. (For literature see HAUSMANN and GOMEZ, 1967; STUDIER, 1972; STUDIER, 1969). Dr. HAUSMANN'S mutants were found to be protein kinase negative

infection (DUNN and STUDIER, 1973b). Processing of the polycistronic mRNA is required for translation (HERCULES et al., 1974).

A completely different pattern of initiation and termination *in vitro* has been reported upon using dinucleotides as primers under particular reaction conditions (MINKLEY and PRIBNOW, 1973). Five initiation and five termination sites in the early region of T7 DNA have been postulated.

Phage RNA polymerase transcribes with high efficiency the genes from 30% to the end of the T7 genome (Fig. 2) (see also page 70; STUDIER and MAIZEL,

1969; SUMMERS and SIEGEL, 1970; SCHERZINGER et al., 1972b). In addition, it reads the region between 20 and 30% of T7 genome, which is also copied by host RNA polymerase (HERRLICH et al., 1971a). As shown by *in vitro* enzyme synthesis, phage RNA polymerase can even transcribe genes of the region between 0 and 20% of the genome, the region which is transcribed most efficiently by *E. coli* RNA polymerase (SCHERZINGER et al., 1972b; DUNN et al., 1972).

The transcription pattern of T7 has led to the current nomenclature for T7 genes: The region of the T7 genome between the promoter for *E. coli* RNA polymerase and the first termination signal is called "early" or "control region", the stretch between the two termination signals is called "early-late" and the remaining 70% of genome are designated "late". Previous nomenclature based on the time of appearance of gene products (ligase, an early gene product appears together with most late proteins) or on the transcribing enzyme (SUMMERS and SIEGEL, 1970), is no longer valid. The nomenclature may, however, be based on the efficiency of transcription by *E. coli* RNA polymerase: The control region is transcribed by host polymerase with highest efficiency, the efficiency of transcription is lower in the early-late region and very low in the late region.

c) Control of Development

The conversion of macromolecular syntheses of the host to phage specificity is a complicated but systematic process:

α) In the first phase of phage development, various phage proteins, so-called early proteins, are produced according to a well-defined time schedule.

β) Soon after appearance of the first phage proteins, host protein and RNA synthesis ceases.

γ) In the late phase of the infectious cycle, the bulk of the phage proteins (late proteins) are synthesized.

δ) Accumulation of early enzymes is stopped during this later phase of virus development.

Ad α) Early proteins. Early proteins are synthesized *in vitro* according to a characteristic time course (Fig. 2 and SCHWEIGER et al., 1971; SCHERZINGER et al., 1972a). At 37°, the first protein is completed at 2.5–3 minutes: for instance the enzyme S-adenosylmethioninehydrolase under the direction of T3 DNA. Protein phosphokinase is completed at 4 minutes, lysozyme at 7–8 minutes. The appearance of enzymes *in vivo* is faster but the sequence and relative time schedule was shown to be strikingly similar (HERRLICH et al., 1971a; HAUSMANN and HÄRLE, 1971; SCHERZINGER et al., 1972a; SCHWEIGER et al., 1972; STUDIER, 1972).

The analysis *in vitro* demonstrated the synthesis of a polycistronic messenger RNA (see page 65). Later such a transcription unit was also shown *in vivo* (page 66). As a consequence, one has to expect the existence of a common promoter which was indeed shown (DAVIS and HYMAN, 1970; GOMEZ and

LANG, 1973). Thus, *E. coli* RNA polymerase transcribes continuously from the promoter at about 1% to a strong termination signal beyond the lysozyme gene. The map positions of the genes dictate the time sequence of their expression (sequential transcription).

Ad β) Shut-off of host. Host enzyme induction is inhibited by phage T7 (BRUNOVSKIS and SUMMERS, 1971; SCHWEIGER et al., 1972). Concomitantly with the formation of the first T7 proteins, host enzyme induction is prevented (SCHWEIGER et al., 1972) and, a brief period later, RNA synthesis is also shut off (unpublished; STUDIER, personal communication; BRUNOVSKIS and SUMMERS, 1972; HERRLICH et al., 1973 and 1974). The inhibition of host enzyme induction is caused by a phage gene product since infection by T7 phage whose genome has been inactivated by UV irradiation, does not prevent β-galactosidase induction (SCHWEIGER et al., 1972). However, it is prevented by infection with T7 gene 1 mutants which induce proteins only via the host RNA polymerase (BRUNOVSKIS and SUMMERS, 1971; SCHWEIGER et al., 1972). The gene or the genes responsible must therefore be early genes.

By cell-free enzyme synthesis, the host shut-off was analysed and two mechanisms distinguished: Shut-off of host translation and inhibition of host messenger RNA synthesis.

Cell-free systems prepared from cells at various times after infection with T7, show at 2.5 to 3 minutes after infection a lessened capacity to synthesize β-galactosidase (SCHWEIGER et al., 1972; HERRLICH et al., 1974) or other non-T7 enzymes such as the T3-specific SAMase. The synthesis of the T7 enzymes: DNA ligase or lysozyme is not affected. Extracts from infected cells repress the SAMase synthesis in cell-free systems from uninfected cells and this response served as a test system for the purification of the factor (HERRLICH et al., 1974). The factor is a protein and acts on the level of translation as shown by the observation that mRNA dependent SAMase synthesis is inhibited as well as is DNA directed synthesis. The protein is named "T7 translational repressor". Only when the translational repressor is present at the beginning of the *in vitro* synthesis, is repression complete, and detailed kinetic analysis indicated that the translational repressor inhibits initiation of translation. In the purification procedure most of the translational repressor activity sediments with the ribosomes and is removed together with the initiation factors from the ribosomes by high salt treatment (1 M NH_4Cl) (HERRLICH et al., 1974). The map position of the translational repressor gene has been determined. The gene is an early gene, since it is transcribed by host RNA polymerase. Its appearance very early during phage development (at 2.5 to 3 minutes at 30°, gene 1 product appears at 4.5–5 minutes) indicates that the gene maps to the left of gene 1. More precise experiments using UV sensitivity measurements, confirmed this location (HERRLICH et al., 1974). While these experiments indicate a specific shut-off mechanism by blocking the initiation of translation, other authors reported a non-specific loss of initiation function (LEDER et al., 1972).

Inhibition of host messenger RNA synthesis was determined using ^{14}C-uridine pulses. The rate of RNA synthesis in the presence of nalidixic acid

is decreased after infection with T7 gene 1 mutants (gene 1 mutants are unable to induce the larger part of T7 specific RNA synthesis (see below), and the reduction in host RNA synthesis thus becomes visible). Again by the UV irradiation technique, the location of the gene responsible for shut-off of host RNA synthesis was determined. The gene is proximal to gene 1 (it seems to end at about 5%) (HERRLICH et al., 1974). This gene seems to be involved in the exclusion of coinfecting T3 phage as well (mutual exclusion, HAUSMANN et al., 1961) since exclusion is inactivated with a similar UV dosage dependence (unpublished results together with BEIER and HAUSMANN). A deletion mutant of T7, mapping to the left of gene 1, was also found to be deficient in the shut-off mechanism for host RNA synthesis (STUDIER, personal communication; BRUNOVSKIS and SUMMERS, 1972). But screening of several deletion mutants between 2.5 and 7% revealed that those which affected two proteins (of the genes that end at 3.2% and at 8%) were not defective in the shut-off of host RNA synthesis (SIMON and STUDIER, 1973). This would offer an alternative explanation for the fact that the UV sensitivity method mapped the right end of the shut-off gene at 5% instead of 8%. More genetic functions have to be known before the genetic details of this region will be fully understood[1]

The functions discussed so far, influence the macromolecular syntheses of the host. These functions, however, are not responsible for killing of the host. The UV sensitivity of the killing function is still much lower than the UV sensitivity of the two genes discussed. Our preliminary experiments suggest that the killing is correlated with changes in the cell membrane (see page72).

Ad γ) Phage RNA polymerase. Gene 1 product is essential for the expression of most T3 and T7 genes (pleiotropic protein; HAUSMANN and GOMEZ, 1967; STUDIER and MAIZEL, 1969). It acts on the transcriptional level (SIEGEL and SUMMERS, 1970) and was identified as a phage-specific RNA polymerase (CHAMBERLIN et al., 1970).

Phage RNA polymerase has various properties different from those of host RNA polymerase: Both enzymes, T3 and T7 polymerase, seem to contain only one single polypeptide chain (m.w. for T7 100000 to 105000; CHAMBERLIN et al., 1970; T3: 90000; our own unpublished data) and the template specificity is remarkable: T7 RNA polymerase will only transcribe T7 or T3 DNA, but not at all T2 DNA, *E. coli* DNA, λ DNA, SP50 DNA, and several others tested. It uses, with low efficiency, salmon sperm DNA and M13 DNA as templates (CHAMBERLIN and RING, 1973a). Among synthetic polynucleotides, only $d(G)_n.d(C)_n$ or $d(I)_n.d(C)_n$ were good templates (CHAMBERLIN and RING, 1973a). The enzyme may recognize dC rich sequences that were found in T7 DNA (SUMMERS and SZYBALSKI, 1968). Like many other phage proteins T7 RNA polymerase works only at low ionic strength (CHAMBERLIN et al., 1970; SCHERZINGER et al., 1972b; CHAMBERLIN and RING, 1973b; see also review by HERRLICH et al., 1973). The transcription rate appears to be faster than that of *E. coli* RNA polymerase (CHAMBERLIN and RING, 1973a; DUNN et al., 1972).

1 Recent evidence demonstrates the involvement of at least two proteins in the shut-off of host RNA synthesis (PONTA et al., 1974; RAHMSDORF et al., 1974 in prep.; and page 71).

On T7 DNA, T7 RNA polymerase transcribes the "late" genes both *in vivo* and *in vitro* (STUDIER and MAIZEL, 1969; SUMMERS and SIEGEL, 1970; SCHERZINGER et al., 1972b). Preliminary evidence suggests that the late genes are transcribed in 3–4 polycistronic units (SAUERBIER and BRÄUTIGAM, personal communication; SCHWEIGER and HERRLICH, unpublished; CALLAHAN and LEDER, 1972). Although *E. coli* RNA polymerase may read through to the right end of T7 DNA (GOFF and MINKLEY, 1970; DAVIS and HYMAN, 1970), phage RNA polymerase magnifies the transcription of the "late" genes.

As mentioned above, it has recently been demonstrated in a cell-free system that T7 phage RNA polymerase also transcribes the ligase gene, the lysozyme gene and its own gene: gene 1 as well (SCHERZINGER et al., 1972b) and that T3 phage RNA polymerase transcribes the corresponding T3 genes and the SAMase gene (SCHERZINGER et al., 1972b; DUNN et al., 1972). That transcription *in vivo* is also overlapping, both polymerases transcribing the same part of the genome, has as yet been shown only for the lysozyme gene (HERRLICH et al., 1971a).

Ad δ) Inhibition of Host RNA Polymerase-Dependent T7 Transcription. The synthesis of some gene products of the control region ceases in the late phase of development (SCHWEIGER et al., 1972). This negative control consists of two mechanisms: One involving T7 protein kinase (RAHMSDORF et al., 1974 in prep.) and the T7 transcriptional inhibitor (PONTA et al., 1974). To analyze the controls, mutants are used which are deleted in the kinase gene. In this condition, much more ligase synthesis is induced in the non-permissive host by mutants in gene 1 than by T7 wild type (SCHERZINGER et al., 1972a). This difference is a matter of transcription since more ligase RNA is made after infection with a gene 1 mutant than after $T7^+$ infection (SCHWEIGER et al., 1972). Gene 1 RNA shows a similar behaviour. This was shown by extracting the RNA from non-permissive cells infected with a gene 1 mutant (kinase-negative) and using this RNA as template in a cell-free system which included suppressor tRNA. The kinetics showed that normally the synthesis of ligase RNA is stopped after the appearance of late proteins, but mutants unable to induce phage polymerase (and late proteins) stop the synthesis later, resulting in an overproduction. This delay of cessation of ligase mRNA synthesis was postulated to be due to a delay in the production of an inhibitor of RNA synthesis or a delayed accumulation of sufficient amounts of inhibitor (SCHWEIGER et al., 1972). The inhibitor has been purified (PONTA et al., 1974). The reverse, a study of kinase in the absence of transcriptional inhibitor has not yet been completed.

d) Other Regulation

The kinetics of early T7 messenger RNA synthesis *in vivo* discussed above also suggested some form of translational control. Ligase messenger RNA is not produced beyond 7 minutes (or 12 minutes in gene 1 mutants, 30°) and translatable ligase messenger decays with a half life of 2.75 minutes (or 4.5 minutes in gene 1 mutants). However, the translation of ligase message *in vivo* stops at times where still high levels of messenger could be isolated (Fig. 4 in ref.

SCHWEIGER et al., 1972). And although lysozyme messenger shows almost no decay at all, *in vivo* lysozyme synthesis stops. Both, translational control of available messenger RNA and a differential change in the messenger decay rates seem to be involved. This control seems different from that responsible for the mechanism of shut-off of host translation.

There is other circumstantial evidence that during T7 development translational control and a change in messenger decay rates may occur:

α) Previously it has been reported that T7 cannot grow on certain F^+ strains (MORRISON and MALAMY, 1971) and that this may be due to a defect in translation. *In vitro* translation of T7 mRNA by ribosomes from male cells seemed to suggest a lack of initiation (CALLAHAN and LEDER, 1972). F^+ cells infected with T7, lose protein with the antigenic properties of IF3 (SCHEPS et al., 1972). Also other phages of this group like T3 and ϕII are supposed to be F^- specific (CUZIN, 1965; SCHELL et al., 1963).

β) After T7 infection, ^{32}P-phosphate label appears in proteins that copurify with ribosomal proteins through the usual ribosome washing procedures and two-dimensional gel electrophoresis of ribosomal proteins (RAHMSDORF et al., 1974).

γ) A specific regulation by messenger decay rates is supported by the following observations: Reports on the life time of messenger RNA after T3 and T7 infection are somewhat conflicting. T7 RNA was found to be much more stable [20–30 minutes halflife (SUMMERS and SIEGEL, 1970a)]; whereas others found only slightly more stability for T7 RNA than either for total RNA in uninfected cells or tryptophan operon mRNA in both uninfected and infected cells (MARRS and YANOFSKY, 1971). Both results suggest messenger specificity. There may be a difference in the decay rate not only between host mRNA and T7 mRNA but also among individual species of T7 mRNA which would offer an explanation for the different levels of translatable ligase and lysozyme messenger described above (SCHWEIGER et al., 1972). On top of this, we have not been able to observe polarity after T7 infection also suggesting that the RNA does not break down: 10 different amber mutations in gene 1 did not change the rate of synthesis of ligase (unpublished). These results all suggest a change in mRNA stability after T7 infection.

Since *in vitro* protein synthesis is very sensitive to ionic strength and magnesium concentrations (see Chapter II), it is reasonable to assume that this is also relevant for the synthesis of proteins *in vivo*. In fact we have recently observed that during T7 infection changes occur in the cellular envelope (HERRLICH et al., 1973). The phospholipid pattern is different from that of uninfected cells and T7 even induces a phospholipid synthesizing activity detectable in cell extracts, which incorporates ^{32}P from γ-labeled ATP into phosphatidic acid, phosphatidylethanolamine and phosphatidylglycerol.

Not only is host RNA and protein synthesis inhibited during phage infection, but host DNA synthesis is also reduced (unpublished, in collaboration with WALTER MESSER) and host DNA is broken down according to an elaborate scheme (LABAW, 1953; SADOWSKI and KERR, 1970; KERR and SADOWSKI, 1972; CENTER 1972; SADOWSKI, 1971).

The host cell provides part of the protein synthesis and replication machinery for the phage. That the host cell provides even specific factors and that this aspect of host-virus relationship is still not understood is illustrated by a few observations which will be mentioned only briefly.

T7 codes for a "conditional-lethal" gene, the SS^+ function (for suicide in shigella) which leads to abortive infection in a shigella sonnei strain missing the sin gene (for suicide inhibition) while allowing growth in *E. coli* (sin^+) (HAUSMANN, 1973; HAUSMANN, 1968). SS^+ is dominant over SS^-. SS^- mutants of T7 grow on either host. The mechanism affects DNA synthesis, but is not known in detail. The abortive infection kills the host.

Several *E. coli* mutants have been isolated which permit only abortive development of T3 or T7. *E. coli* E8 seems to lack a factor participating in phage DNA maturation (HAUSMANN and HÄRLE, 1971). *E. coli* mutants deficient in DNA ligase or carrying an altered DNA ligase do not support the growth of T7 ligase deletion mutants, and another *E. coli* mutant, BR 3, supports T7 wild type growth at 37° but not at 25° and does not develop T7 deletion mutants at either temperature no matter where the deletion is located (SIMON and STUDIER, 1973). The need for specific host factors is also stressed by the interference of the sex factor with T7 infection (see page 72).

Conclusion. Several control mechanisms are involved in the conversion of host macromolecular synthesis into T7 (T3) phage specificity. The time schedule of phage protein synthesis in the early phase is dictated by the sequential arrangement of the genes on the genome. Host protein synthesis is redirected into phage protein synthesis by a set of control genes. Gene 1 product, the phage RNA polymerase, takes over transcription of the phage genome. Another gene codes for a specific repressor of host translation, several controls including a T7 protein kinase and a transcriptional inhibitor stop transcription by *E. coli* RNA polymerase. Host DNA, finally, is broken down by phage nucleases, the nucleotides being used for phage DNA synthesis. In addition, the infectious process itself and possibly a phage gene product seem to affect the cell metabolism by membrane changes. The cooperation of all these control mechanisms leads to efficient phage progeny production.

2. Studies on the Development of Bacteriophage T4[2]

After infection of *E. coli* by bacteriophage T4 many changes occur in the pattern of protein synthesis. The production of host proteins is terminated (MONOD and WOLLMAN, 1947; FRENCH and SIMINOVITCH, 1955; DUCKWORTH and BESSMAN, 1965; NOMURA et al., 1966; KAEMPFER and MAGASANIK, 1967; KAEMPFER and SARKAR, 1967; TERZI, 1967; HATTMAN and HOFSCHNEIDER, 1967; LEVINTHAL et al., 1967; DUCKWORTH, 1970; ENNIS, 1970) and various groups of phage proteins are synthesized in a consecutive sequence (LEVIN-

2 Since the literature on bacteriophage T4 has become very voluminous, the discussion will be restricted to areas where the technique of *in vitro* protein synthesis has been utilized.

THAL et al., 1967). Because T4 is so much larger than T7, and T4 DNA codes for a large number of proteins (approximately 200; molecular weight of DNA $= 1.3 \times 10^8$; KOZLOFF, 1960; MATHEWS, 1971), the classification of the proteins into groups on the basis of time of appearance has been more or less arbitrary. Additional criteria for classifying T4 proteins have been sought with the hope of elucidating the regulatory mechanisms of this bacteriophage.

a) Prereplicative Phage Protein Synthesis

Unlike in T7, DNA replication divides the development of T4 into two distinct periods: a prereplicative and a postreplicative period. Late proteins are not synthesized efficiently without replication (EPSTEIN et al., 1963).

During the prereplicative period of phage development *in vivo*, three groups of phage proteins have been distinguished (LEVINTHAL et al., 1967; HOSODA and LEVINTHAL, 1968; BOLLE et al., 1968a; GRASSO and BUCHANAN, 1969; SALSER et al., 1970): "immediate early", "delayed early" and "quasi late". The synthesis of the "quasi late" proteins was supposed to begin before replication and to continue into the post-replicative period, while the synthesis of the other two groups ceases after DNA replication begins (SALSER et al., 1970). "Immediate early" proteins were distinguished from the other two groups by infecting cells in the presence of chloramphenicol. Under these conditions, only "immediate early" RNA can be detected (BOLLE et al., 1968).

The DNA dependent *in vitro* synthesis of T4 phage enzymes allowed a more careful analysis of the regulation of T4 protein synthesis. The formation in a system from uninfected *E. coli* of the T4 enzymes: α- and β-glucosyltransferase (GOLD and SCHWEIGER, 1969a), dCMP deaminase (SCHWEIGER and GOLD, 1970), and lysozyme (SCHWEIGER and GOLD, 1969a) indicate that host RNA polymerase is able to transcribe these genes. The glucosyltransferase and dCMP deaminase are considered to be "delayed early" enzymes, lysozyme is a "quasi late" enzyme. If *E. coli* RNA polymerase can initiate transcription only at "immediate early" promoters (promoters in front of "immediate early" genes), it follows that either "delayed early" genes (α-, β-glucosyltransferase and dCMP deaminase) plus "quasi late" genes (lysozyme) can be transcribed starting from these promoters and these genes are located adjacent to "immediate early" genes, or that an activation mechanism for the recognition of new promoters can be induced *in vitro*.

Several lines of evidence suggest strongly that the order of genes is indeed the following:

promoter — immediate early — delayed early — quasi late and that they can be transcribed *in vitro* as polycistronic transcriptional units.

α) Assuming that the rates of transcription and translation are constant in the DNA dependent *in vitro* system, the time period between the start of incubation and the appearance of an enzyme reflects the distance of the corresponding gene from the promoter from which it is transcribed. Kinetic measurements show that the lag periods for the synthesis of the enzymes

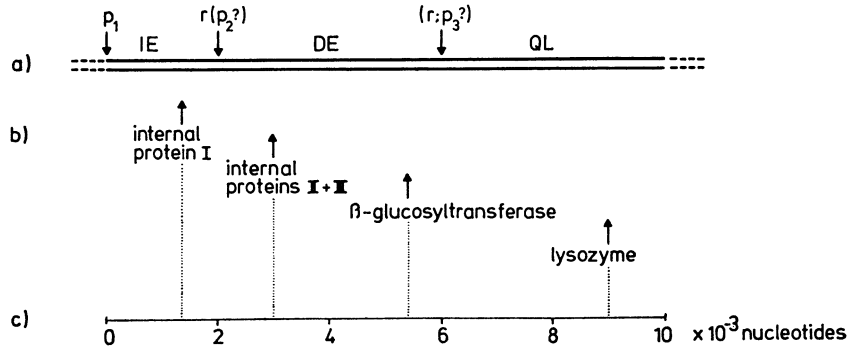

Fig. 4a—c. Order of genes in bacteriophage T4. a Section of the genetic map. IE = "Immediate early" genes, DE = "Delayed early" genes and QL = "quasi late" genes follow each other. p_1 = "Immediate early" promoter. r = Reduction signal, suggested because there is less DE mRNA in the absence of translation. A T4 function may help to overcome reduction. p_2 and p_3 are hypothetical new promoters (for discussion see text). b Approximate locations of genes as calculated from kinetic data (GOLD and SCHWEIGER, 1970; BLACK and GOLD, 1971). An average transcription rate of 28 nucleotides per second (at 37° C *in vitro*, at 30° C *in vivo*) was used for the calculation. The genes are not located in one single transcriptional unit. The arrows indicate end of message in corresponding units. c Nucleotide length of messenger RNA started at "immediate early" promoters

mentioned above are fairly long: 5–6 minutes at 37° for β-glucosyltransferase and 10 minutes at 37° for lysozyme (GOLD and SCHWEIGER, 1970; and Table 1). At an average rate of translation of 5 amino acids per second the gene for β-glucosyltransferase would be 1800 amino acids or 5400 nucleotides of single stranded DNA from the promoter. The corresponding distance for lysozyme would be 9000 nucleotides. The RNA transcribed to the end of the lysozyme gene would be 2.7×10^6 daltons. There would be enough space in such a transcriptional unit to accommodate at least three genes (Fig. 4).

β) A requirement of this model is that the transcription of all three classes of phage genes is initiated at the same time. This was shown by experiments in which further initiation of transcription was inhibited a short time after the beginning of the *in vitro* reaction (GOLD and SCHWEIGER, 1970).

γ) A correlation between the lengths of the lag periods in the *in vitro* syntheses and the distances of the genes from their promoters, has been obtained by shearing T4 DNA and using the DNA fragments as templates. Lysozyme synthesis, which has the longest lag period of all proteins tested is most sensitive to DNA fragmentation; the synthesis of β-glucosyltransferase is distinctly less sensitive. Internal proteins of T4 which belong to the "immediate early" class, are even less sensitive. DNA fragments of 3×10^6 daltons are as effective as templates for the synthesis of internal proteins as is native T4 DNA, while the synthesis of β-glucosyltransferase is reduced to 34% and that of lysozyme to 9% (BRODY et al., 1971; MILANESI et al., 1970).

We can safely say that *in vitro* T4 RNA synthesis is initiated from "immediate early" promoters and extended into the "delayed early" and "quasi late" genes.

The question may be raised whether *in vivo* the "delayed early" and "quasi late" genes are also transcribed by the host RNA polymerase. There is convincing evidence that *E. coli* RNA polymerase transcribes large polycistronic units *in vivo* as well.

By size determination of global T4 RNA and by hybridization experiments at various times after infection, the extension of RNA from "immediate early" into "delayed early" sequences was demonstrated quite directly (SAUERBIER et al., 1970; BRODY et al., 1969).

In vivo, part of the transcription of lysozyme mRNA is initiated immediately after infection with bacteriophage T4 (HERRLICH et al., 1971a). When infection is carried out in the presence of rifampicin, an inhibitor of the initiation of transcription, the formation of β-glucosyltransferase and lysozyme is prevented. Addition of rifampicin 45 seconds after infection (at 30°) allows significant synthesis of these enzymes. Therefore, although the active enzymes themselves appear late (5 min for β-glucosyltransferase, 9 min for lysozyme) transcription of their mRNA is initiated in part immediately after infection. It should be noted that no rifampicin-resistant RNA polymerase can be detected after T4 infection (HASELKORN et al., 1969).

When T4 infection is carried out in the presence of chloramphenicol, only "immediate early" species of T4 mRNA can be detected (SALSER et al., 1970; GRASSO and BUCHANAN, 1969; LEMBACH and BUCHANAN, 1970). Therefore one might conclude that the transcription of "delayed early" genes requires a phage-coded protein of the "immediate early" class. However, the polarity phenomenon in which under certain conditions chloramphenicol has been shown to cause either a stop of transcription (IMAMOTO, 1973) or a rapid breakdown of promoter-distal RNA (MORSE, 1970) due to a lack of translation, could explain this. Chloramphenicol pretreatment in suA cells (BECKWITH, 1963) which do not show this breakdown of mRNA (MORSE and PRIMAKOFF, 1970), indeed seem to allow the accumulation of "delayed early" RNA (BLACK and GOLD, 1971). Likewise, when the formation of active proteins is inhibited by the use of amino acid analogues (which allow protein synthesis to continue and therefore do not cause RNA breakdown), "delayed early" RNA can be detected. The fact that methyltryptophan allows some accumulation of "delayed early" RNA (LEMBACH and BUCHANAN, 1970) is in aggreement with this interpretation.

In an *E. coli* mutant defective in K^+ uptake, translation can be inhibited by K^+ depletion. Under these conditions both "immediate early" and "delayed early" RNA could be detected (MORSE and COHEN, 1973).

When the distance of the delayed early gene 43 from its promoter was determined by the UV technique of transcriptional unit mapping (see page 64) it was found that when initiation is limited to the first minute after infection at 30° through the use of rifampicin, it appeared that this gene is distant from its promoter (HERCULES and SAUERBIER, 1973).

Thus, *in vivo*, transcription of "delayed early" and "quasi late" genes can be initiated at "immediate early" promoters as it is *in vitro*.

Beside this mechanism, however, T4 seems to induce an activation mechanism for the transcription of "delayed early" and "quasi late" genes. The activation mechanism may favor specific genes and may not activate the "delayed early" and "quasi late" groups in toto. The reasoning is the following:

α) When rifampicin is added 45 seconds after infection at 30°, the ratios of the rates of synthesis of lysozyme and β-glucosyltransferase were changed as compared to the control (HERRLICH et al., 1971 a). Less lysozyme was synthesized in the presence of rifampicin. We favor the interpretation that there is an additional mechanism of lysozyme gene transcription which does not function in the presence of rifampicin. The additional mechanism would then involve a new rifampicin-sensitive initiation step. The lysozyme gene might be reached from a second promoter later in the phage cycle. The existence of promoters closer to the lysozyme gene was also suggested by the following experiment: When *in vitro* synthesis is carried out at an elevated magnesium concentration (15 mM), lysozyme RNA is initiated from a promoter half as far from the gene (GOLD and SCHWEIGER, 1970).

β) The rIIB region of T4 seems to be transcribed from a promoter to the left end of rIIA and in addition from a promoter between rIIA and rIIB (SCHMIDT et al., 1970). A similar relationship with two promoters has been described for the T4 gene 1 (deoxynucleotide kinase; SAKIYAMA and BUCHANAN, 1973).

γ) The possibility that "delayed early" genes are transcribed by both extension from "immediate early" promoters and a new activation mechanism, is supported by the isolation of a T4 mutant that shows a delay and a decrease in the amounts of "delayed early" proteins synthesized (MATTSON, personal communication).

δ) Further support comes from experiments using the UV technique of transcriptional unit mapping, which show that when T4 infection is allowed to progress without inhibition, certain delayed early genes appear to be adjacent to their promoters at later times after infection (HERCULES and SAUERBIER, 1973).

One should keep in mind that whatever the activation mechanism may be, the host RNA polymerase (at least the β-subunit which is supposed to bind rifampicin; RABUSSAY and ZILLIG, 1969; HEIL and ZILLIG, 1970) is utilized for the transcription (HASELKORN et al., 1969; HERRLICH et al., 1971 a). The data mentioned above favor the induction of a new transcription factor but further clarification is required.

In summary, the sequential induction of T4 prereplicative proteins is caused by the sequential clustering of the genes. In addition, some "delayed early" transcription may be activated or magnified by a phage-induced mechanism. Fig. 4 illustrates our present state of knowledge.

b) The Synthesis of Late Proteins

The prereplicative genes are transcribed mainly from the l strand (L) of T4 DNA (GUHA and SZYBALSKI, 1968) while "late" RNA is mainly comple-

mentary to the r strand (GUHA and SZYBALSKI, 1968; GRASSO and BUCHANAN, 1969). The host RNA polymerase (at least the β subunit) is in some way involved in "late" transcription (HASELKORN et al., 1969). However, the details of "late" transcription are not understood.

It has been known for a long time that the efficient synthesis of "late proteins" as well as of their messengers requires prior DNA synthesis (EPSTEIN et al., 1963; LEVINTHAL et al., 1967). There is disagreement on the requirement of continued DNA replication for the sustained synthesis of late proteins (ZOGRAF et al., 1967; LEMBACH et al., 1969; SAUERBIER and BRÄUTIGAM, 1970; RIVA et al., 1970). In any case, the presence of a maturation protein (gene 55 product) is required and gene 33 product enhances "late" transcription (PULITZER, 1970; BOLLE et al., 1968b). Small T4 specific proteins copurify with the *E. coli* RNA polymerase isolated after T4 infection; the products of genes 55 and 33 may be among these proteins (STEVENS, 1970; GOFF and HORVITZ, cited by LOSICK, 1972). More recently it has been demonstrated that under certain conditions at least small amounts of some late proteins can be synthesized in the absence of DNA replication (SAUERBIER and HERCULES, 1973; CASCINO et al., 1971; BERGER and BRUNER, 1973). One of these conditions is the mutant combination in genes 30 and 46 or 47. It was interpreted to indicate the need of a nuclease action for late transcription (CASCINO et al., 1971). The significance of these observations is unclear. The mechanism of "late protein" induction has not been further characterized. Attempts to synthesize "late proteins" in a DNA dependent *in vitro* system have been unsuccessful (TRAUB et al., 1966).

c) Translational Control

From the discussion of the timing of the various groups of prereplicative phage proteins and the transition from prereplicative to late transcription, it would appear that most of the control of protein synthesis after T4 infection occurs on the transcriptional level. However, translational controls have been implicated in the shut-off of host protein synthesis and in the shut-off of "early" protein synthesis late in the infectious cycle.

Originally, an abrupt termination of host transcription within the first 4 minutes (37°) was reported (ROUVIÈRE et al., 1968; LANDY and SPIEGELMAN, 1968). Using more precise methods, it was shown that approximately 50% of RNA synthesized at 4 minutes after infection was host specific (KENNELL, 1968). Because the capacity to synthesize β-galactosidase after IPTG induction decays more rapidly than does RNA synthesis, translational control was thought to be involved in the cessation of host protein synthesis. Also, superinfection with phage T4 causes the exclusion of RNA phage M12 or f2 (HATTMAN and HOFSCHNEIDER, 1967; YAROSH and LEVINTHAL, 1967). One or more T4 proteins interfere with the synthesis of RNA phage proteins (HATTMAN and HOFSCHNEIDER, 1968). The mechanism by which T4 interferes with RNA phage development is still not well understood. In cell-free systems from cells infected with T4, translation of RNA phage RNA was less efficient than the

overall translation of T4 messenger RNA, while in systems from uninfected cells both templates served equally well in the stimulation of amino acid incorporation (HSU and WEISS, 1969; SALSER and GESTELAND, cited by HATTMAN and HOFSCHNEIDER, 1969). A factor contained in the initiation factor fraction was thought to be responsible for the discrimination between T4 RNA and RNA phage RNA, because the high-salt-wash of ribosomes from uninfected cells could restore f2 RNA translation in crude extracts from T4 infected *E. coli* (SCHEDL et al., 1970). It was found that the binding of the RNA phage RNA to ribosomes and the binding of formylmethionine tRNA to the initiation complex were blocked (DUBE and RUDLAND, 1970; KLEM et al., 1970) and, although translation of the A protein was not affected, the synthesis of the two other proteins (replicase and coat protein) was inhibited (ARGETSINGER-STEITZ et al., 1970). This discrimination could be demonstrated *in vivo* as well: T4 infection blocks the synthesis of f2 phage coat protein (HATTMAN, 1970). The discrimination between RNAs by the translation machinery was extended to different species of T4 mRNA: Ribosomes prepared from cells infected with T4 but harvested late in the infectious cycle, were found to translate "late" T4 mRNA in preference to "early" T4 mRNA and RNA phage RNA. This selection was shown to be due to initiation factor IF3 (POLLACK et al., 1970; LEE-HUANG and OCHOA, 1971). This offered an additional approach to the problem of the early-to-late transition of phage protein synthesis.

Other authors failed to detect *in vitro* discrimination by "T4 ribosomes" against RNA phage RNA (HATTMAN, 1970; GOLDMAN and LODISH, 1972). Instead, a general decrease of protein synthesis was observed irrespective of which mRNA was used, and the ratios of synthesis of the three RNA phage proteins were identical in systems prepared from T4 infected or uninfected cells (GOLDMAN and LODISH, 1972). The reasons for this disagreement are not yet clear. Perhaps slight differences in the conditions of *in vitro* protein synthesis could cause the failure to demonstrate the discrimination.

Discrimination by IF3 was also found in uninfected *E. coli* (LEE-HUANG and OCHOA, 1971; LEE-HUANG and OCHOA, 1972). IF3α is selective for messengers such as MS2, *E. coli* and "early" T4 mRNA, while IF3β promotes specifically the translation of "late" T4 mRNA. An interference factor i from *E. coli* seems to bind to IF3 so that the translation of the coat protein cistron of MS2 RNA is inhibited but the synthesis of T4 proteins continues more or less unchanged (GRONER et al., 1972). More recently, two interference factors —one inhibiting IF3α, the other affecting IF3β—have been described (LEE-HUANG and OCHOA, 1972). The significance of such an interference factor and of changes in IF3 activity to T4 infection is not yet established. An explanation for translation control in T4 infection must account for the fact that "late" proteins of T4 can be synthesized *in vitro* using late T4 mRNA and ribosomes isolated from uninfected *E. coli* (SALSER et al., 1967) and that the time of IF3 inactivation measured so far is too late for an involvement in the host shut-off.

Still another set of T4-induced changes in the translation machinery awaits correlation with the *in vivo* regulation: The pattern of transfer RNA and the properties of valyl tRNA synthetase change after T4 infection (SUEOKA and KANO-SUEOKA, 1964; WATERS and NOVELLI, 1967; KANO-SUEOKA, 1969; WEISS et al., 1968; MCCLAIN, 1970; CHRISPEELS et al., 1968; WILSON, 1973). It is possible that these new tRNAs serve to increase the efficiency of translation of certain T4 genes containing codons which are only recognized by minor species of *E. coli* tRNA.

As in T7 infection, the synthesis of "early" proteins (immediate early and delayed early) is discontinued at later times after T4 infection. Because mRNA homologous to early genes may persist throughout the lytic cycle (BALDI and HASELKORN, 1967; HALL et al., 1964; FRIESEN et al., 1967), translational controls may be involved in the shut-off of "early" T4 protein synthesis. The causative function was hypothesized to be a methylase (SAUERBIER et al., 1971). Because no translatable messenger for deoxynucleotide kinase at late times could be isolated (SAKIYAMA and BUCHANAN, 1971), one could argue that the mRNA may be modified and inactivated, not ruling out that there may be differences in the shut-off of the various early genes. Specific messenger degradation is an alternative explanation.

Like in bacteriophage T7, there are indications that the various messenger species produced by T4 are degraded with different rates (SAUERBIER and HERCULES, 1973). The degradation of select species appears to depend on a T4 function (reg A). The regA mutation prevents the shut-off of synthesis of "early" proteins in DNA negative mutants, but has little effect on the shut-off in the DNA^+ state (WIBERG et al., 1973). This suggests that there may be more than one mechanism for the shut-off in wildtype T4.

Conclusion. The understanding of T4 phage regulation is still far from complete. We have omitted the description of various phenomena which we cannot yet order into a regulatory model, like changes in the host RNA polymerase (ZILLIG et al., 1970) or in the cell membrane (PUCK and LEE, 1955; SILVER, 1965; FURROW and PIZER, 1968; ENNIS and KIEVITT, 1973; HERRLICH et al., 1973). The data reviewed allow the following preliminary scheme: Prereplicative protein synthesis is controlled by sequential transcription and translation of polycistronic units. An additional mechanism for the activation of delayed early genes is suggested. The synthesis of late proteins seems to require a complex set of functions, including DNA replication and the function of gene 55. Translational control in T4 is a fact, but identifying the step which is controlled at the level of translation and identifying the precise mechanism used will be a problem for future investigation.

3. Control of Protein Synthesis in other Bacteriophages

In principle, the *in vitro* synthesis of phage enzymes in an *E. coli* system can be applied to both, *E. coli* and non-*E. coli* phage. Enzyme synthesis was observed using DNAs from various *B. subtilis* phage: $\phi 29$ lysozyme (in col-

laboration with M. SALAS), SPP1 lysozyme (in collaboration with TH. TRAUTNER), SP82 dCMP deaminase and SP5C dCMP deaminase (SCHWEIGER and GOLD, 1970). Only initial studies of phage regulation have been made.

One system among the *E. coli* phages remains to be discussed in this context: Lambda. Lambda DNA is a poor template for *E. coli* RNA polymerase (SCHWEIGER et al., 1969; GESTELAND and KAHN, 1972) which may be due to the need for transcriptional control proteins. These are peculiar to the lambda system and have only recently been investigated by a direct method such as the *in vitro* enzyme synthesis.

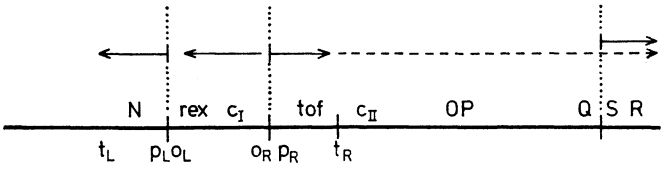

Fig. 5. Scheme of λ transcription. The arrows indicate the direction of transcription. For details of the nomenclature see review by SZYBALSKI et al. (1970)

Three control proteins have been analysed:

α) The lambda repressor: Under repressed conditions, only a small segment of lambda DNA is transcribed by *E. coli* RNA polymerase, the CI-rex region (see review by PTASHNE, 1971; and Fig. 5). The repressor blocks transcription from both promoters, p_L and p_R (Fig. 5; STEINBERG and PTASHNE, 1971; Wu et al., 1971; WU et al., 1972). The repression has also been demonstrated in a coupled transcription-translation system (GESTELAND and KAHN, 1972). It appears that several repressor molecules (as dimers, the monomer having a molecular weight of 28000) can bind to the operator sites and that RNA polymerase is inhibited in three stages: binding, before forming the initiation complex, and after forming the initiation complex (STEINBERG and PTASHNE, 1971; MANIATIS and PTASHNE, 1973).

β) The N gene product: When repression is removed, transcription starts from p_L in leftward and from p_R in rightward direction. In the absence of N gene product *in vivo*, transcription terminates soon, at t_L and t_R (see Fig. 5; and review by SZYBALSKI et al., 1970). The expression of gene R for instance depends on the function of gene N (FRANKLIN, 1971; HERSKOWITZ and SIGNER, 1970; review by THOMAS, 1971). *In vitro*, the synthesis of lambda endolysin (gene R product) showed a long lag period in the DEAE system (unpublished) indicating that its transcription was started at the early promoter (p_R). By using DNA from λN^- phage the requirement for concomitant N product synthesis *in vitro* was demonstrated (GREENBLATT, 1972). The synthesis *in vitro* of endolysin and of anthranilate synthetase directed by λN^- DNA carrying the appropriate genes, became a test system for the N product (GREENBLATT, 1972; DOTTIN and PEARSON, 1973). Because lambda repressor inhibits the N-dependent enzyme synthesis it has been assumed that N gene product

promotes extension of RNA chains rather than new initiation (GREENBLATT, 1972; DOTTIN and PEARSON, 1973).

γ) The Q gene product: Gene Q increases the rate of late RNA synthesis (complementary to genes right of Q) *in vivo* (see review by SZYBALSKI et al., 1970). Q gene product appears to cause new initiation of RNA chains at a promoter site between genes Q and S (HERSKOWITZ and SIGNER, 1970; DAMBLY et al., 1968). Preliminary evidence seems to indicate that gene Q is involved in the appearance of a new RNA polymerizing activity (NAONO and TOKUYAMA, 1970).

The study of lambda by *in vitro* protein synthesis has been restricted to either host enzymes of genes that are fused to lambda and are under lambda control (DOTTIN and PEARSON, 1973), or on lambda endolysin synthesis GREENBLATT, 1972). In addition, several lambda-specific proteins have been synthesized *in vitro* and analysed by gel electrophoresis, one of them possibly being the tof gene product (GESTELAND and KAHN, 1972). Although only these few proteins can be assayed, the large collection of lambda mutants and of genetic techniques should be able to be combined with cell-free protein synthesis experiments in order to elucidate the mechanisms of the lambda control functions.

B. E. Coli Protein Synthesis

1. The Regulation of the Lac Operon[3]

The lactose operon of *Escherichia coli* is the group of jointly controlled genes which has been characterized genetically to the largest extent (see reviews by BECKWITH, 1970; and by ZUBAY and CHAMBERS, 1971). Although the *lac* operon model was proposed some years ago (PARDEE et al., 1959; JACOB and MONOD, 1961), only recently has the final proof of the model been made; that was by cell-free enzyme synthesis (ZUBAY et al., 1970a). In addition, the DNA dependent *in vitro* system defined new essential features of the *lac* control system (ZUBAY, 1969).

a) Negative control

The lactose operon is subject to coordinate negative and positive control. The negative control is exerted by the *lac* repressor whose existence had been postulated in the original hypothesis (JACOB and MONOD, 1961). The repressor is a protein of 150000 molecular weight with four identical subunits (GILBERT and MÜLLER-HILL, 1966; RIGGS and BOURGEOIS, 1968). As suggested from genetic data, the repressor does indeed bind to DNA containing the operator region (GILBERT and MÜLLER-HILL, 1967; RIGGS et al., 1968). Allolactose, the inducer of the *lac* operon (JOBE and BOURGEOIS, 1972), lactose which is converted to allolactose and does not act directly (JOBE and BOURGEOIS, 1973),

3 Again, the discussion of the *lac* operon will have to be limited to advances made in conjunction with cell-free enzyme synthesis.

or IPTG, an inducer which is not metabolized, all apparently cause an increase in the dissociation constant of the repressor-DNA complex and bring about release of the DNA. These experiments suggested that negative control in the *lac* operon is exerted at the level of transcription. To prove this, cell-free enzyme synthesis was used (ZUBAY et al., 1967; ZUBAY and LEDERMAN, 1969). Repressor-containing extract inhibited the synthesis of the α-fragment of β-galactosidase by 50% and the repression was reversed by the addition of IPTG. IPTG had no effect on systems devoid of repressor. The repressor was also shown to act in systems synthesizing the complete enzyme. The inhibition was raised to 95% by the addition of cAMP (CHAMBERLIN and ZUBAY, 1969); cAMP apparently increases the correct initiation of transcription from the promoter (see page 85; and OHSHIMA et al., 1970). In the absence of cAMP the influence of the repressor seems to be limited by the high portion of incorrect initiation. The kinetics of enzyme synthesis as a function of the time of addition of IPTG suggested strongly that repressor inhibited the initiation of transcription (ZUBAY et al., 1970a) and that inducer acted only before RNA chain initiation. When RNA synthesis was started in the presence of excess repressor but without inducer and further initiation of RNA synthesis was then blocked by rifampicin, the subsequent addition of IPTG did not stimulate enzyme synthesis (ZUBAY et al., 1970a). Thus, the repressor blocks transcription prior to the formation of a stable rifampicin-resistant initiation complex. This result is at variance with data from another laboratory showing that IPTG stimulates lac transcription starting from a preinitiation complex formed in the presence of repressor (CHEN et al., 1971). This would mean that the dissociation of the repressor-DNA complex occurs at a rate faster than the inactivation of the preinitiation complex by rifampicin. Repressor and RNA polymerase have, according to these results, non-overlapping binding sites on the DNA. The sites, however, may be overlapping in a promoter mutant (*lac* p^s) (RIGGS et al., 1970) where IPTG stimulation is lower in a similar experiment with this mutant DNA (CHEN et al., 1971).

What, in fact, is the order of controlling sites in the *lac* operon? The region of the *lac* DNA which is genetically defined as the operator region, maps between the promoter and the *lac* z gene (IPPEN et al., 1968). More recent evidence from λ and T phage suggests that the operator region is located within a non-transcribed area between the RNA polymerase binding site (true promoter or entry site) and a chain initiation site (BLATTNER et al., 1972; SCHÄFER, 1972; SCHÄFER et al., 1973). In *lac*, this seems to be slightly different. Messenger RNA synthesis begins in the repressor-protected region of the DNA (GILBERT, personal communication). The location may explain the mechanism of repressor action: RNA polymerase can possible bind at the correct entry site and still be blocked during the "drifting" to the site of chain initiation by the repressor. Thus, even if the operon were joined to another such that it still had its own operator but were dependent on the other promoter, repressor could stop transcription of that operon. Such a case has been described for a fusion of the *lac* and *trp* operons (REZNIKOFF et al., 1969).

The *lac* repressor has its counterpart in the lambda repressor (see page 81) and information on the lambda repressor suggests a similar mechanism of action (STEINBERG and PTASHNE, 1971; WU et al., 1971; MANIATIS and PTASHNE, 1973).

The binding of *lac* repressor to DNA was quantitated .Under the conditions of cell-free enzyme synthesis, the dissociation constant (K_m) for the binding of repressor to DNA was determinated to 1.6×10^{-9} moles^{-1} liters (ZUBAY et al., 1970a). The i^q repressor used here binds about 1/10 of normal (ZUBAY, personal communication). The K_m values found in DNA repressor (wildtype repressor) binding experiments by the glycerol gradient technique are 10^{-12} moles^{-1} (GILBERT and MÜLLER-HILL, 1967) and by the filter technique are 2×10^{-13} moles^{-1} liters (RIGGS et al., 1970). However, the K_m value depends strongly on the ionic conditions and these values are likely to be spurious, while the high ionic strength of the cell-free system probably reflects more closely the conditions present *in vivo* (ZUBAY et al., 1970a).

Similar methods led to estimates of the stoichiometry of the repressor reaction. Each repressor molecule reacts with one operator molecule and with two inducer molecules (ZUBAY and LEDERMAN, 1969). Because repressor monomers bind to inducer (RIGGS and BOURGEOIS, 1968), but not to operator, it was suggested that each tetramer repressor molecule carries four inducer and two operator binding sites (ZUBAY and LEDERMAN, 1969) and a dimer would then form the functional unit.

b) Positive Control: Catabolite Repression

Glucose, added to the medium of a growing culture of *E. coli*, inhibits the formation of β-galactosidase (MONOD, 1947) and of other inducible enzymes (MAGASANIK, 1961). DEPENDING on the state of the lac operon in the cell and the duration of the effect, different types of glucose inhibition can be distinguished (MAGASANIK, 1970): the strongly inhibiting effect of glucose added to a culture growing on another carbon source, has been called transient repression (20–30 minutes) and the long-lasting effect of glucose has been called catabolite repression. The glucose effect is mimicked by other metabolizable carbon sources such as glycerol, glucose-6-phosphate, fructose, galactose (MCFALL and MANDELSTAM, 1963) and the most severe repression is found with the combined addition of glucose and gluconate (BUETTNER et al., 1973).

It has long been clear that catabolite repression was different from the negative control system described above. Repressor and operator mutations isolated at that time did not alter the sensitivity of their operons to catabolite repression (MAGASANIK, 1961) although evidence from several laboratories supported the idea that catabolite repression affects gene expression at the transcriptional level (NAKADA and MAGASANIK, 1964; TYLER and MAGASANIK, 1969; JACQUET and KEPES, 1969). However, the mechanism remained obscure through 20 years of intensive study. The detection of cAMP (see literature in MAKMAN and SUTHERLAND, 1965) and cell-free enzyme synthesis have produced the key to an understanding of the mechanism of catabolite repression.

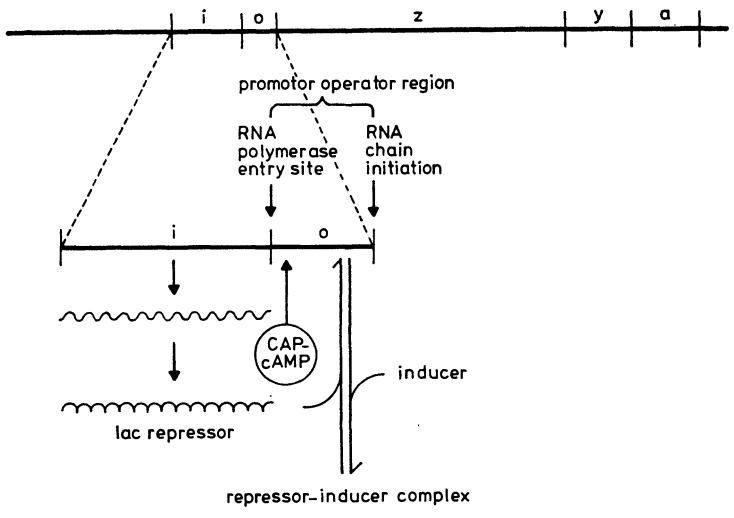

Fig. 6. Model of *lac* operon control

The line of experiments leading to our present understanding of catabolite repression began with the observation that glucose causes the loss of cAMP from bacterial cells into the medium (MAKMAN and SUTHERLAND, 1965). The idea that cAMP might be involved in repression of enzyme synthesis proved to be correct: high concentrations of cAMP outside the cells reversed the transient and permanent effects of glucose on gene expression (PERLMAN and PASTAN, 1968a; ULLMANN and MONOD, 1968; PERLMAN and PASTAN, 1968b; review by PASTAN and PERLMAN, 1970). Even escape enzyme synthesis in lysogenic cells after phage induction is catabolite-repressible and cAMP reverses repression (RAHMSDORF et al., 1973). Still, an indirect involvement of cAMP in catabolite repression was possible.

A break came when the direct stimulation of β-galactosidase synthesis *in vitro* by cAMP was demonstrated (CHAMBERS and ZUBAY, 1969). The extent of stimulation was particularly high (1300% of control) in a system derepressed with IPTG. As discussed above, cAMP improves faithful initiation and thereby enhances repressor action. This is suggestive of a transcriptional action mechanism of cAMP. Indeed, the lag period needed for the action of cAMP, the failure to see an effect of cAMP after the addition of rifampicin and the similarity of results with enzyme synthesis as a function of time of addition of either rifampicin or IPTG or cAMP, all indicate that cAMP acts on the transcriptional level, more precisely at the initiation of transcription (ZUBAY and CHAMBERS, 1969). cAMP stimulated also lac mRNA synthesis in the preincubated S30 system (CROMBRUGGHE et al., 1970). A new class of promoter mutants which rendered the *lac* operon insensitive to glucose effects, confirmed that catabolite repression is a matter of initiation of RNA (SILVERSTONE et al., 1969; PERLMAN et al., 1960).

Does cAMP act directly on RNA polymerase or is there a yet unidentified link? It was originally thought that cAMP would either inactivate a "repres-

sor"-type molecule which is common to all catabolite sensitive operons or that it would somehow help the RNA polymerase to initiate (SCHWARTZ and BECKWITH, 1970). These authors selected *E. coli* mutants for inability to grow on various carbon sources; the existence of these mutants is predicted by both models (SCHWARTZ and BECKWITH, cited by ZUBAY, 1969; SCHWARTZ and BECKWITH, 1970). These mutations resulted in a shut-off of all genes subjected to the glucose effect. Those with intact cAMP metabolism could either have a "super-repressor" exerting, "super catabolite repression" or could lack some factor which combines with cAMP. The latter was shown to be true by cell-free enzyme synthesis (ZUBAY, 1969; ZUBAY et al., 1970b; EMMER et al., 1970). Such mutants with intact cAMP production were lacking a protein factor required for "turning on" the *lac* operon since a protein synthesizing system from a factor-defective mutant did not support the synthesis of β-galactosidase. The addition of factor-containing extracts restored the capacity to synthesize β-galactosidase only in the presence of cAMP (ZUBAY, 1969; ZUBAY et al., 1970b; EMMER et al., 1970). The protein variously called CAP for catabolite gene activator protein, or CGA for catabolite gene activator, or CRP for cAMP receptor protein was partially purified using as assay the ability to restore β-galactosidase synthesis in CAP-deficient cell extracts (ZUBAY et al., 1970b) or the binding to cAMP (EMMER et al., 1970). CAP protein has a molecular weight of 40000–45000 and consists of two subunits of 22000 daltons (RIGGS et al., 1971). Cyclic GMP inhibits the binding of cAMP to CAP protein and also inhibits β-galactosidase synthesis to the same extent suggesting that cAMP binding to CAP protein is an essential step in activating catabolite-repressed genes.

The CAP protein-cAMP system is involved in the transcription of the *lac* operon (ERON et al., 1971; CROMBRUGGHE et al., 1971a; CROMBRUGGHE et al., 1971b), a result expected from the earlier experiments. CAP protein binds to DNA (RIGGS et al., 1971; NISSLEY et al., 1972) and the binding is greatly strengthened by cAMP and inhibited by cGMP again suggesting that binding to DNA is involved in the relief of catabolite repression. The *lac* promoter region contains independent binding sites for CAP protein and for RNA polymerase (ERON and BLOCK, 1971; BECKWITH et al., 1972). DNA from a promoter mutant which is partially resistant to catabolite repression did direct the synthesis of some β-galactosidase without the CAP protein-cAMP system (CROMBRUGGHE et al., 1971a). Promoter mutants not dependent on CAP-cAMP were interpreted as "superpromoters" (ERON and BLOCK, 1971). The mechanism of CAP-cAMP action seems to be to help RNA polymerase holoenzyme to initiate at the normal promoter as soon as repressor has been removed.

The essential elements for controlled transcription in a purified system are *lac* DNA, RNA polymerase (holoenzyme), cyclic AMP binding protein, cyclic AMP, *lac* repressor and inducer (CROMBRUGGHE et al., 1971b; ERON et al., 1971). However, other experiments give some doubt as to the completeness of the control system: ppGpp has been reported to stimulate the transcription of the *lac* operon in the presence of ribosomal wash (CROMBRUGGHE et al.,

1971a; ZUBAY et al., 1971) and the synthesis of ribulokinase from the *ara* operon (ZUBAY et al., 1971), but there was no effect on a purified transcription system and on galactokinase synthesis in a complete transcription-translation system (CROMBRUGGHE et al., 1971b). Determination of cAMP concentrations *in vivo* did show, in most cases, an inverse relationship between cAMP level and the intensity of catabolite repression (BUETTNER et al., 1973). However, in transient repression and in the very severe catabolite repression by glucose plus gluconate, the authors failed to see such a correlation. The isolation of a mutant which requires exogenous cAMP for growth even on a rich medium, may suggest an additional regulatory role for cAMP (OHNISHI et al., 1972). Another function, alt, was found by isolating mutants on arabinose from a strain defective in CAP-cAMP (either cAMP or CAP defective) (SILVERSTONE et al., 1972). This class of mutations compensates for the loss of the CAP-cAMP system. In summary, the essential factors for *lac* transcription are known, but additional control functions involved in *lac* transcription cannot be ruled out.

We have discussed the molecular mechanism of catabolite repression but have neglected the question of how cAMP and CAP levels are regulated in *E. coli*. This very basic problem has not yet been solved.

2. Other Operons of E. coli

a) The Regulation of the Arabinose Operon, First Example of a Positive Control

The arabinose operon in *E. coli* consists of three structural genes: *ara*A, *ara*B, and *araD*, and a control gene *ara*C (SHEPPARD and ENGLESBERG, 1966; review by ENGLESBERG, 1971). Several lines of evidence, mainly through work with stable merodiploids, deletions and complementation analysis, suggested that the *ara*C locus codes for a regulatory protein which is needed for transcription of the operon. This regulatory protein was postulated to exist in two functional states, P1 and P2, both being in equilibrium with each other. P2 reacts with a specific site on the DNA (*ara*I) thereby activating the operon. Full expression, however, occurs only when P1 is not blocking the *ara* operator site. Thus, the same protein appears to exert activating and repressing properties (ENGLESBERG et al., 1969). L-arabinose (the inducer) binds to the *ara*C product apparently favoring the active state. The order of genes in the operon is *ara*D *ara*A *ara*B *ara*I *ara*O *ara*C. Superimposed upon the regulation by the *ara*C product is control by catabolite repression (ZUBAY et al., 1971a).

In vitro synthesis of ribulokinase (*ara*B) directed by DNA from phage ϕ80 carrying the *ara* operon (ϕ80d*ara*) in a system prepared from a ribulokinase deletion mutant, demonstrates the requirement of the inducer L-arabinose and cyclic 3′,5′-AMP (ZUBAY et al., 1971a; YANG and ZUBAY, 1973). The synthesis depends on the presence of the *ara*C protein (GREENBLATT and SCHLEIF, 1971;

YANG and ZUBAY, 1973). *ara*C product is also synthesized in the λd*ara* dependent *in vitro* system causing a delay of ribulokinase appearance (YANG and ZUBAY, 1973). This lag period is reversed by the addition of preformed *ara*C product whose stability can be increased by the addition of L-arabinose and of the serine protease inhibitor p-toluenesulfonylfluoride (YANG and ZUBAY, 1973). The antagonistic effect of D-fucose lends some support to the idea that *in vivo ara*C protein is indeed a repressor as well as an activator. With DNA from a fucose resistant C^c mutant, ribulokinase synthesis is not inhibited by D-fucose (GREENBLATT and SCHLEIF, 1971).The *in vivo* dominance of C^+ over C^c which led to the model of P1 being a repressor (SHEPPARD and ENGLESBERG, 1967) could be imitated *in vitro:* Increasing amounts of C^+ containing extract produced an increasing sensitivity to D-fucose which was interpreted as the manifestation of repressor activity of C protein (GREENBLATT and SCHLEIF, 1971).

b) In vitro Studies on the Tryptophan Operon

The structural genes of the tryptophan biosynthetic enzymes are clustered in an order which corresponds to the biosynthetic sequence (IMAMOTO et al., 1966). The genes have been designated, in the order of transcription and translation: E, D, C, B, A. A repressor gene (*trp*R) has been identified which is not in the same genetic region as the structural genes (MORSE and YANOFSKY, 1969) and which is dispensible for cell growth. Repression is also governed by an operator locus (*trp*O) which is at the left side adjacent to E (HIRAGA, 1969). The genetic data suggest a repressor mechanism similar to the one in the *lac* operon, but no direct evidence has been available.

The *in vitro* synthesis of *trp* enzymes is another good example of the faithfulness of cell-free enzyme synthesis. The appearance of tryptophan synthetase (*trp*B and *trp*A) and of anthranilate synthetase (*trp*E and *trp*D) *in vitro* parallels the kinetics *in vivo* and both enzymes are synthesized in equimolar amounts, as they are *in vivo* (POUWELS and VAN ROTTERDAM, 1972).

In vitro enzyme synthesis served as a test for the activity of *trp* repressor (ZUBAY et al., 1972). φ80 DNA was used which carried the operator-promoter region of the *trp* operon fused to the *lac* genes z, y, a (REZNIKOFF et al,. 1969). In this fused operon, the *lac* genes are repressed *in vivo* by addition of tryptophan (in *trp*R^+ cells) and *in vitro* the synthesis of β-galactosidase is repressed when extracts containing *trp* repressor are used. β-galactosidase synthesis became a test for a partial repressor purification (ZUBAY et al., 1972). DNA amount dependence experiments suggest that the repressor acts at the level of transcription. *trp* operon regulation *in vitro* is independent of cyclic AMP (ZUBAY et al., 1972; POUWELS and VAN ROTTERDAM, 1972) and in a purified transcription system, tryptophan has been proven to be the corepressor (ROSE et al., 1973).

c) On the Repression of the Arginine Pathway

The nine structural genes are controlled by the *arg*R gene product (GORINI et al., 1961; MAAS, 1961). The *arg*R gene product interacts with seven operator sites. Among them is the operator site responsible for the *arg*E gene which is one of the independent units (GLANSDORFF and SAND, 1965; CUNIN et al., 1969; JACOBY, 1972). *Arg*E codes for the enzyme N-α-Acetyl-L-Ornithinase. *In vitro* synthesis of this enzyme is repressed by extracts from *arg*R$^+$ cells while β-galactosidase synthesis is not affected (URM et al., 1973). However, extracts from an *arg*R$^-$ strain also showed some repressing activity which was interpreted as inactive repressor becoming partially active when present in excess. Each cell contains about 200 molecules of *arg* repressor, 20 times more than is present for the tryptophan or lactose operon (URM et al., 1973).There is no conclusive evidence yet as to whether arginine or arginyl-tRNA or some other compound is the actual corepressor in the *arg* pathway regulation (CELIS and MAAS, 1971).

d) Polarity in vitro

A nonsense mutation in a promoter-proximal gene of a polycistronic transcription unit can cause a reduced rate of expression of promoter-distal genes. This phenomenon is called *polarity* (FRANKLIN and LURIA, 1961; JACOB and MONOD, 1961 b). In bacteria, this effect has been found to be associated with reduced amounts of messenger RNA specified by DNA regions distal to a polar mutation. On the basis of this observation two possible models of polarity have been formulated: the termination of protein synthesis by the nonsense codon could either cause a premature termination of transcription (IMAMOTO and KANO, 1972; IMAMOTO, 1973) or, alternatively, could be followed by the rapid degradation of the non-translated mRNA distal to the nonsense codon (MORSE et al., 1969; HIRAGA and YANOFSKY, 1972). More experimental results seem to favor the possibility of rapid mRNA degradation. Mutations in a gene designated suA (BECKWITH, 1963; SCAIFE and BECKWITH, 1966) have been found to relief polarity *in vivo* (MORSE and PRIMAKOFF, 1970). Extracts from cells carrying this mutation seem to exhibit less endonuclease activity tested with T4 mRNA than did extracts from the parent strain (KUWANO et al., 1971). This nuclease is thought to be required for the degradation of the non-protected part of the RNA which would thus not be available for translation. The degradation would, according to the model, be prevented by the reinitiation by ribosomes at the next cistron border (NEWTON et al., 1965). Detailed mapping of mutations in the β-galactosidase gene and in the i gene and correlation of the map position to the degree of polarity suggests that, at certain points on the messenger RNA, probably at internal AUG codons, reinitiation can take place (ZIPSER, 1970; PLATT et al., 1972). If the polarity-causing nonsense codon is close to the next reinitiation AUG, polarity is less distinct. When the distance between stop and restart is long, polarity is pronounced, Thus, the degree of polarity appears to depend on the distance between termination and reinitiation of protein synthesis (ZIPSER, 1970).

Polar effects were not observed when DNA carrying polar mutations was used to direct enzyme synthesis *in vitro* (see T7 DNA-directed enzyme synthesis, page 72, and WETEKAM et al., 1972). Although no polarity has been seen after T7 infection *in vivo*, one would expect that nonsense mutations in the left part of the early transcriptional unit of T7 DNA would induce polar effects upon the translation of distal genes *in vitro*. The cell-free system from uninfected cells should contain the polarity system, but no polarity was found (unpublished). Also DNA carrying mutations in the *gal* operon which show polarity *in vivo*, lost the "polar" property *in vitro* (WETEKAM et al., 1972). This difference between the *in vivo* and *in vitro* expression is particularly striking with mutations of the insertion type which exhibit extremely strong polar effects *in vivo* independent of their map position within the gene (see review by STARLINGER et al., 1973).

The reasons for the *in vitro* relief of polarity have recently been elucidated. A protein factor present in extracts from wildtype *E. coli* but not in extracts from *E. coli* suA$^-$ restores polarity with both DNAs carrying insertions or amber codons (WETEKAM and EHRING, 1973). The "polarity" factor is apparently lost in the preparation of the cell-free protein synthesis system. By means of RNA-directed *in vitro* synthesis of the galactose enzymes, the polarity-restoring activity has been shown to act also on preformed mRNA (SCHUMACHER and EHRING, 1973). These results clearly support the model of RNA degradation and are not compatible with a polarity model involving termination of transcription.

Other results using a pure transcription system with DNA from an insertion mutant in the galactose operon, were discussed in favor of the termination model of polarity (CROMBRUGGHE et al., 1973). Although the two inserted sequences IS 1 and IS 2 (HIRSCH et al., 1972; FIANDT et al., 1972) are equally polar *in vivo*, termination of transcription was found only with DNA carrying IS 2, not with DNA carrying either IS 1 or a strongly polar nonsense mutation. Possibly termination of transcription is a property peculiar to insertion IS 2.

A special aspect of polarity is displayed by insertions located in the control region of the *gal* operon (WETEKAM et al., 1972; WETEKAM and EHRING, 1973). *In vivo*, these mutations result in low levels of synthesis of all three galactose enzymes, not affected by the addition of inducer (D-fucose). *In vitro*, however, the operon is fully expressed and the synthesis of gal enzymes is regulated by inducer and cAMP. Since the repressor and the CAP protein bind to the promoter region, it appears that the promoter and operator elements are not inactivated by the insertion but that the insertion was rather integrated into a DNA sequence between the promoter and the first structural gene of the gal operon. The inserted sequence, however, is transcribed (WETEKAM and EHRING, 1973).

e) Synthesis of tRNA in vitro

Although not directly related to the topic of this review, it should be mentioned briefly that a DNA-directed cell-free system has been used for the

Table 1. Lag periods of messenger and enzyme synthesis *in vitro* (in minutes at 37°)

	mRNA	enzzyme	Reference
T4 β-glucosyltransferase	2	5	SCHWEIGER and GOLD, 1969b
T4 lysozyme	5	10	SCHWEIGER and GOLD, 1969b
T7 DNA ligase	3.3	7	SCHERZINGER et al., 1972a
T7 lysozyme	4.5	8	SCHWEIGER et al., 1971
β-galactosidase	2[a]	5[a]	ZUBAY and CHAMBERS, 1969
Anthranilate synthetase	—	3.5	POUWELS and VAN ROTTERDAM, 1972
Tryptophane synthetase	—	6.5	POUWELS and VAN ROTTERDAM, 1972

[a] The time needed for initiation of RNA synthesis was subtracted.

synthesis of su^+_{III} tyrosyl-tRNA under the direction of $\phi 80psu^+_{III}$ DNA (ZUBAY et al., 1971b). The tRNA was isolated after synthesis and its biological activity confirmed in a system that synthesizes β-galactosidase only in the presence of sufficient suppressor tRNA.

II. Cell-Free Preparations for the DNA-Directed Synthesis of Enzymes and Characteristics of *in vitro* Synthesis

A. Description of Cell-Free Systems

DNA-directed protein synthesis was observed originally by measuring the overall amino acid incorporation (TRAUB and ZILLIG, 1966; LEDERMAN and ZUBAY, 1967). DNA stimulated the incorporation above a certain background level largely due to the presence of nucleic acids endogenous to the system. Techniques were, therefore, developed to remove the endogenous template nucleic acids. RNA and DNA were removed by *preincubation* (originally described by MATTHAEI and NIRENBERG, 1961) of the total cell-free preparation, by *precipitation* with protaminesulfate (WOOD and BERG, 1962) or by *chromatography* of the protein on *DEAE-cellulose* in combination with preincubation of the ribosomes (TRAUB and ZILLIG, 1966).

For the *in vitro* synthesis and detection of specific enzymes by their activities the presence of endogenous nucleic acids is no severe problem. The endogenous template should, however, not contain the information for the enzyme to be detected. Added template RNA and DNA compete well with the endogenous nucleic acids.

For any DNA-directed enzyme synthesis *in vitro*, the conditions in the living cell should be more or less exactly copied. This is best approached in an extract prepared simply by disruption of the cells. Indeed such a crude extract supports the synthesis of enzymes (SCHWEIGER et al., 1972). Unlike in intact cells, nucleases and proteases are free to attack nucleic acids and the products

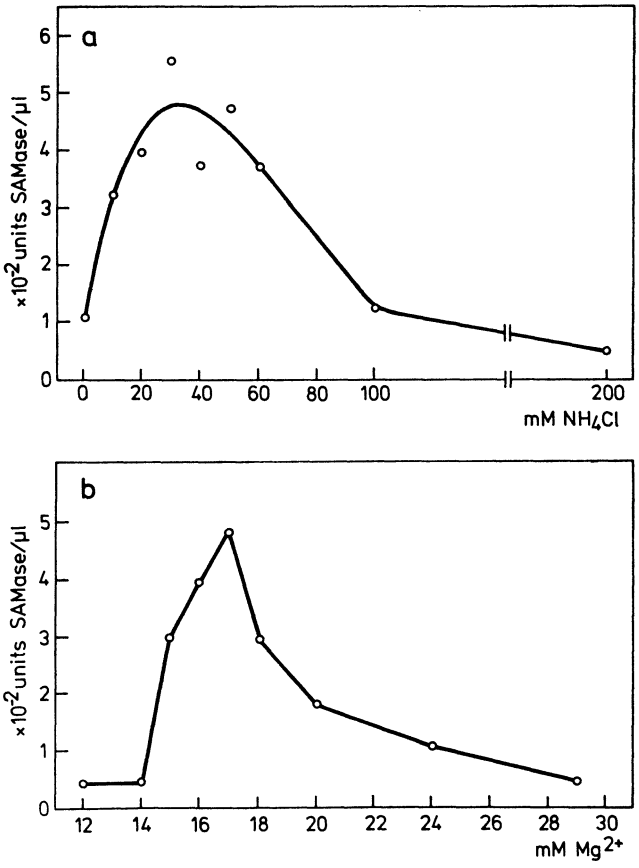

Fig. 7a and b. Magnesium and salt dependence of enzyme synthesis in a crude protein synthesizing system. (Experiment together with H. J. RAHMSDORF.) Crude extracts were obtained by lysing the cells with the Brij-lysozyme treatment (GODSON and SINSHEIMER, 1967). The pellet from 50 ml of exponentially growing *E. coli* 514 of $O.D._{600} = 0.4$ (or any other K-strain) was suspended in 0.1 ml 25% sucrose in 0.01 M tris HCl pH 8.1 (final volume approximately 0.2 ml). 20 µl 6.4 mg/ml lysozyme in 0.25 M tris HCl pH 8.1, plus 20 µl 20 mg/ml EDTA were added. After 2 minutes in ice, a mixture of 30 µl 5% Brij 58 in 0.01 M tris HCl pH 7.2 and 30 µl 0.1 M $MgCl_2$ was added. The cells lysed by stirring several times on a whirlmix. 20 µl of crude extract was diluted to 50 µl at the following concentrations of components (neglecting the material from the cell sap): sucrose 3.3%, lysozyme 0.17 mg/ml, EDTA 1.43 mM; Brij 58 0.2%; tris HCl, pH 8.0, 59.1 mM; K-acetate 50 mM; amino acids 0.2 mM each; ATP 2 mM; UTP, CTP, GTP 0.5 mM each; PEP 20 mM; dithiothreitol 2.5 mM; tRNA 500 µg/ml; T3 DNA 50 µg/ml; and (a) 17 mM $MgCl_2$ plus varying concentrations of NH_4Cl, (b) 30 mM NH_4Cl plus varying concentrations of magnesium (note the presence of EDTA). After 40 minutes at 37°, aliquots were tested for SAMase activity

in such an extract. In order to avoid this and for a better control of the reaction conditions (salts, dependence on various constituents of the protein synthesizing machinery), further purifications are advisable. We will briefly describe the available systems, and, then, compare in more detail the two systems most widely used for DNA-directed enzyme synthesis.

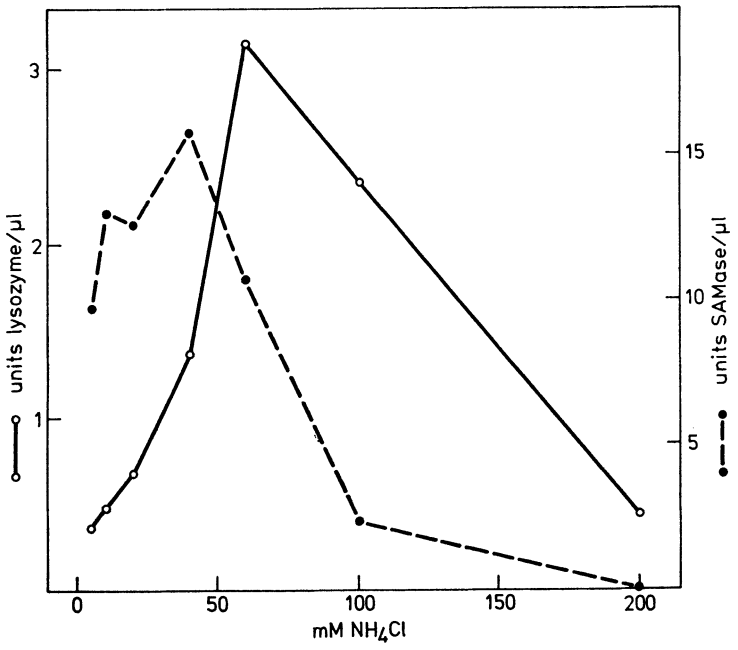

Fig. 8. Salt dependence of enzyme synthesis in an S30 system. (Experiment together with H. J. RAHMSDORF.) 50 ml of an exponentially growing culture of strain XA 7007 (suA) in rich medium were harvested at $O.D._{600} = 0.4$. After washing once with TMA buffer, the cells were suspended in 0.4 ml TMA buffer and lysed by ultrasonication. The debris were removed. The S30 incubation mixture contained, in addition to 20 µl of the S30 extract, the following components in a final volume of 0.05 ml: tris HCl pH 8.0 51 mM; K-acetate 50 mM; amino acids 0.2 mM each; ATP 2 mM; UTP, CTP, GTP 0.5 mM each; PEP 20 mM; dithiothreitol 2.5 mM; tRNA 500 µg/ml; T3 DNA 50 µg/ml; $MgCl_2$ 11 mM (the S30 extract adds to 15 mM $MgCl_2$ in toto); varying concentrations of NH_4Cl. After 40 minutes at 37°, aliquots were tested for lysozyme (o——o) and SAMase (•——•) activity respectively

1. Crude Systems

Cells disrupted by the freeze-thawing method, by ultrasonication and by the Brij-lysozyme treatment (GODSON and SINSHEIMER, 1967), all yield extracts which successfully support enzyme synthesis *in vitro* (SCHWEIGER et al., 1972; and unpublished). Because of the dilution as compared to the cell, salts and substrates must be added (Fig. 7).

2. S30 Extract without further Processing

An S30 extract (a cell extract after removal of the cell debris) supports enzyme synthesis under the direction of added DNA. Similar to the crude system, the S30 contains the salt and substrates from the cell sap and, because of the dilution during cell disruption, additional salts and substrates are needed (Fig. 8).

Table 2. Preparation of cell-free systems and constituents of protein synthesizing mixtures

Preincubated S30 system (ZUBAY et al., 1970a)		DEAE system (GOLD and SCHWEIGER, 1971; HERRLICH and SCHWEIGER, 1974)	
a) Bacteria			
K12 strains		all K12 strains	
Grown in rich medium		grown in rich medium	
At 28°, to mid-log phase		at 28°, to early-log phase	
Cells washed twice in tris HCl 0.01 M, Mg-acetate 0.014 M KCl 0.06 M, mercaptoethanol 0.006 M pH 8.2		cells washed twice in TMA buffer (tris HCl 0.01 M, $MgCl_2$ 0.01 M, NH_4Cl 0.022 M, dithiothreitol 0.001 M, 5% (v/v) glycerol, pH 7.5)	
b) Preparation of system			
50 g in 65 ml above buffer (but with 0.006 M dithiothreitol instead of mercaptoethanol)		6 g cells plus 6 ml TMA buffer	
Aminco pressure cell lysis		lysis with glass beads in Sorvall Omnimix	
2 × 30 minutes 30000 g		20 minutes 80000 g, the supernatant is subjected to 200000 g for 80 minutes	
Supernatant plus 8 ml containing 6 mmols tris HCl, pH 8.2, 0.06 mmols dithiothreitol, 0.17 mmols $Mg(ac)_2$, 0.6 µmols 20 amino acids, 0.048 mmols ATP; 0.54 mmols Na_3PEP, 0.16 mg PEPkinase, preincubated at 37° for 80 minutes in darkness		supernatant adsorbed to DEAE cellulose (0.9 mval/g; 2 g dry weight) and eluted by approx. 10 ml 0.25 M NH_4Cl in TMA buffer	
Dialysed at 4° for 18 hrs against the above buffer (but with K-acetate)		pellet suspended at 1500 O.D.$_{260}$/ml in TMA buffer and used directly, or preincubation, followed by resedimentation	
c) Incubation mixtures			
Tris-acetate pH 8.2	0.044 M	tris-HCl pH 8.0	0.056 M
Dithiothreitol	0.0014 M	dithiothreitol	0.0025 M
K-acetate	0.055 M	K-acetate	0.05 M
20 amino acids	0.00022 M	20 amino acids	0.0002 M
ATP	0.0022 M	ATP	0.002 M
CTP, UTP, GTP	0.00055 M	CTP, UTP, GTP	0.0005 M
Phosphoenolpyruvate	0.021 M	phosphoenolpyruvate	0.02 M
NH_4-acetate	0.027 M	NH_4Cl	0.111 M
Mg-acetate	0.0147 M	$MgCl_2$	0.011 M (varying)
$CaCl_2$	0.0074 M	—	
tRNA	100 µg/ml	tRNA (stripped)	500 µg/ml
Pyridoxine HCl	27 µg/ml	—	
Triphosphopyridine nucleotide	27 µg/ml	—	
Flavine adenine dinucleotide	27 µg/ml	—	

Table 2 (continued)

Preincubated S30 system (ZUBAY et al., 1970a)		DEAE system (GOLD and SCHWEIGER, 1971; HERRLICH and Schweiger, 1974)	
Folinic acid	27 μg/ml	Ca-leucovorin (150 μg/ml in some cases)	
p-aminobenzoic acid	11 μg/ml	—	
Ø80dlac DNA	50 μg/ml	phage DNA	50 μg/ml
Cyclic 3′,5′-AMP	0.0005 M	—	
S30	6.5 mg protein/ml	supernatant protein ribosomes	4 mg/ml 150 $O.D._{260}$/ml
(Last component added after prewarming all other components)		(all components mixed at 0°)	
d) Some properties of the systems			
Total monovalent ions	0.147 M	total monovalent ions	0.237 M
Total divalent ions	0.022 M	total divalent ions	0.011 M
Ionic strength μ =	0.213	ionic strength μ =	0.270
Leucine incorporation (per total incubation period)	5 nmols/ml	leucine incorporation (per total incubation period)	20–100 nmols/ml (with preincubated ribosomes)

3. Preincubated S30 System

ZUBAY and coworkers (ZUBAY and CHAMBERS, 1969) have established a modification of the S30 system of MATTHAEI and NIRENBERG (1961), a preincubated S30 system. This system found wide application for the study of synthesis of *E. coli* enzymes. The S30 is mixed with a small volume of a solution containing all the components needed for translation (amino acids, ATP, ATP-regenerating system) and preincubated at 37° for 80 minutes. The S30 extract is then dialyzed against a desired buffer at 0°. Table 2 summarized the preparation of the S30 system and lists the constituents of the incubation mixture.

4. S30 System from Thermophilic Bacteria

In an S30 extract from thermophilic bacteria, the endogenous DNA can be efficiently digested by incubation with pancreatic DNase at low temperature (G. BAUER, W. SIEGERT and P. H. HOFSCHNEIDER, personal communication). The DNase is then destroyed by preincubation at the growth temperature of the bacteria. The system has been used for the synthesis of coat proteins under the direction of $\phi\mu$-4 phage DNA (RABUSSAY et al., 1969), the products being detected by gel electrophoresis and immunoprecipitation (BAUER et al., personal communication).

5. S100 System

An S30 is separated into S100 protein (supernatant from a 100000 g — 2 hours centrifugation) and ribosomes (pelleted in this centrifugation). The

incubation conditions are identical to those of the S30 system. The S100 protein may be dialysed to remove the low molecular weight components of the cell.

6. DEAE-System

This is the most purified system available for DNA-directed enzyme synthesis (Table 2). The preparation is essentially as follows: S100 protein is adsorbed to DEAE-cellulose and eluted in one step by 0.25 M NH_4Cl (in TMA buffer)—a procedure which removes basic proteins and nucleic acids. The resulting protein fraction is stored in liquid nitrogen and used without removing the salt. The ribosomes are either preincubated or used directly. This so-called "DEAE-system" contains little protease, RNase and DNase. It is, therefore, not necessary to use *E. coli* strains lacking RNase I. The protein fraction and the preincubated ribosomes contain only small amounts of nucleic acid. Salt and substrate conditions depend only on the additions from the outside. If ribosomes are not preincubated, they are more active in enzyme synthesis, but stimulate leucine incorporation to a higher background level because of the endogenous nucleic acid (TRAUB and ZILLIG, 1966; and unpublished).

7. A Comparison between the "DEAE-System" and the Preincubated S30 System

These two cell-free preparations are the ones which have found the widest application for DNA-directed enzyme synthesis *in vitro*. Because they were developed for quite different purposes (phage enzyme synthesis—*E. coli* enzyme synthesis), a detailed comparison will be of interest.

Growth of cells, preparation of the system and the composition of the *in vitro* protein synthesizing mixture are summarized in Table 2. The reader's attention should be drawn to the following differences between the two systems: The time of harvest of cells is different. Both systems are free of low molecular weight constituents of the cell and are low in endogenous nucleic acids—both in contrast to the crude systems. The preincubated S30 system, however, contains more proteins than the "DEAE-system". These may have positive (proteins which the "DEAE-system" may have lost) or negative (RNase etc.) effects on enzyme synthesis. The preincubated S30 system contains calcium and several coenzymes which are not required in the DEAE-system. The final ionic strength and concentration of magnesium is different. The total polypeptide synthesis as determined by the incorporation of leucine is lower in the preincubated S30 system. The "DEAE-system" synthesizes phage enzymes with higher yield, but the synthesis of *E. coli* enzymes under the direction of ϕ80dlac DNA or λdgal DNA is much less than in the preincubated S30 system. The reasons for this are not yet clear.

Table 3. Dependence on initiation factors of T3 DNA directed enzyme synthesis in vitro (experiment together with H. J. RAHMSDORF)

SAMase synthesis was performed in a DEAE system as described in Table 2. The ribosome fraction was, however, obtained by prolonged centrifugation at 200000 g (5 hours). The ribosomes were resuspended in TMA buffer and 1:10 diluted into ribosome washing buffer (0.1 M tris HCl pH 7.8, 50 mM KCl, 10 mM Mg-acetate, 5% glycerol, 1.1 M NH_4Cl, 1 mM dithiothreitol). After stirring at 0° for 3 hours, the washed ribosomes were sedimented at 200000 g for 8 hours. After suspension in TMA, part of the ribosomes were washed a second time in the same manner, but stirred at 0° for 8 hours. The sediment was resuspended in TMA at 1500 $O.D._{260}$/ml. The supernatants were combined, diluted 1:3.9 and passed through DEAE cellulose. The filtrate was treated with 16.7 g $(NH_4)_2SO_4$ per 100 ml, the initiation factors were precipitated by 26 g additional $(NH_4)_2SO_4$ per ml, dissolved in TMA and dialysed against TMA buffer. The incubation mixtures for protein synthesis contained either crude ribosomes or adequate amounts of initiation factors and/or washed ribosomes.

Complete system minus ribosomes	SAMase activity/μl
Plus crude ribosomes	360
Plus washed (1×) ribosomes	40
Plus washed (2×) ribosomes	2
Plus initiation factors	4
Plus washed (1×) ribosomes + initiation factors	267
Plus washed (2×) ribosomes + initiation factors	254
Plus crude ribosomes + initiation factors	510

B. Variations in the Composition of the System

1. Changes in the Preparation of Cellular Components

Crude ribosomes or preincubated ribosomes have been subjected to methods of further purification. Chromatography of ribosomes on DEAE-cellulose has not yet been tested for enzyme synthesis (TRAUB and ZILLIG, 1966). By washing ribosomes with 1 M NH_4Cl in a suitable buffer, a protein fraction is dissociated from the ribosomes which contains most of the initiation factors. Enzyme synthesis becomes dependent on the addition of this fraction (Table 3). The initiation factors can also be separated on DEAE-cellulose and added back individually to the enzyme synthesis mixture (unpublished). The ribosomal subunits can be further disintegrated and reconstituted (TRAUB and NOMURA, 1968; NOMURA and ERDMANN, 1970). *In vitro* reconstituted 30S ribosomes synthesize polyphenylalanine under the direction of poly U and they are also active in enzyme synthesis (EGBERTS et al., 1972). RNase III is removed by a similar ribosome washing procedure and the system becomes dependent on the addition of RNase III (HERCULES et al., 1974; and unpublished).

2. Additional Components

Various components have been added to either an S30 system or the "DEAE-system", e.g. RNA polymerase, CAP factor, repressors, regulatory proteins. In each case one must observe whether the conditions optimal for enzyme synthesis are also optimal for the action of the added component. For

proteins, such as CAP protein, *lac*, *trp*, or *arg* repressors or *ara*C product this seems to be true (ZUBAY et al., 1970b; ZUBAY and LEDERMAN, 1969; ZUBAY et al., 1972; URM et al., 1973; GREENBLATT and SCHLEIF, 1971). However, to see repressor action, one may have to lower the amount of DNA in order to have a more favorable repressor to DNA ratio.

Other proteins may not function under the conditions mentioned. For instance, T7 phage RNA polymerase works only at low ionic strength. In order to measure enzyme synthesis which is mediated by T7 RNA polymerase, an alternative composition of the protein synthesis mixture has been described (SCHERZINGER et al., 1972b).

3. Separation of Transcription and Translation

a) Uncoupled Transcription and Translation in One Tube (SCHWEIGER et al., 1971; HERRLICH and SCHWEIGER, 1974)

This method allows one to influence transcription or translation separately, to leave out one or another component or to study the effect of parts of the translatory machinery on transcription without concomitant translation. Also ionic strength or magnesium concentrations can be changed from one step to the other.

In principle, RNA is synthesized with RNA polymerase and template DNA present. RNA synthesis is interrupted by addition of actinomycin D or of RNase-free DNase and then incubation is continued in the presence of the translational apparatus (Table 2).

b) Complete Separation

RNA is synthesized *in vivo* or *in vitro* and the RNA is extracted by phenol, precipitated and added as messenger RNA to either one of the enzyme synthesizing systems (SALSER et al., 1967; MILLETTE et al., 1970).

The magnesium concentrations must be optimized for both methods a) and b).

C. Requirements

Most of the components listed in Table 2 are required for enzyme synthesis *in vitro* (see also Table 4).

1. Ribosomes

Enzyme synthesis of course needs the ribosomal particles plus several factors that are usually associated with the ribosomes. A concentration dependence can be measured in systems where ribosomes are added as a separate fraction. Increasing amounts of ribosomes yield increasing enzyme synthesis up to a ribosome concentration of 600 μg/ml (Fig. 9). When preincubated ribosomes are used, the linear part of the dependence curve is longer, probably because some copies of an essential protein (until now not determined

Table 4. Requirements of enzyme synthesis

The effect of various omissions on SAMase synthesis *in vitro* is shown. The system was prepared as described in table 2. The ribosomes were not preincubated. Therefore, no requirement for tRNA can be demonstrated and the ribosomal fraction contains some supernatant protein.

	SAMase units/μl
Complete system	155
Minus DNA	0.4
Minus nucleotide triphosphates	0.2
Minus tRNA	152
Minus protein fraction	16
Minus ribosomes	0.2
Complete system plus Ca-leucovorin	176

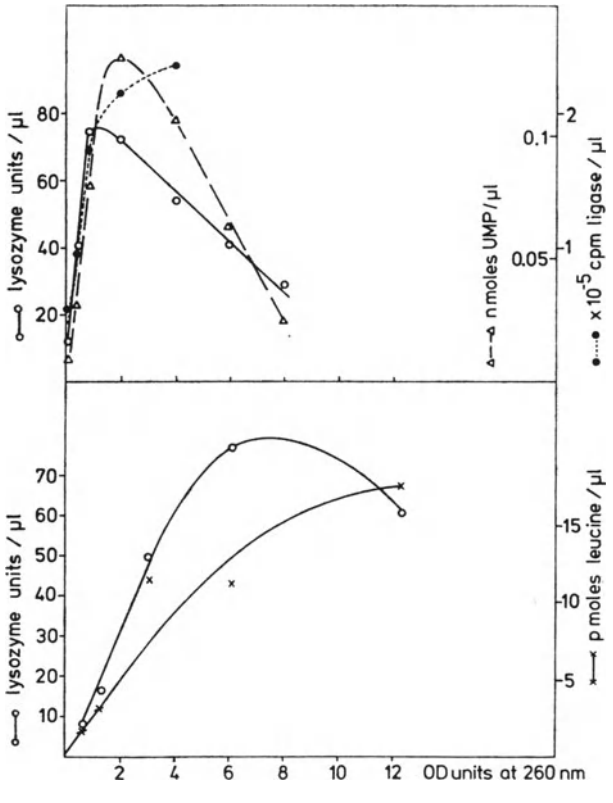

Fig. 9. Dependence of enzyme synthesis *in vitro* on ribosomes. Protein synthesis was performed in the "DEAE system" as described in Table 2. The amount of ribosomes was varied ($O.D._{260}$ units per 50 μl incubation mixture). Upper graph: crude ribosomes were used. Lower graph: preincubated ribosomes were added. For a description of the enzyme assays see HERRLICH and SCHWEIGER (1974)

which) have been inactivated during preincubation. Preincubation removes at the same time most of the endogenous template RNA. Preincubated ribosomes still contain initiation factors. Careful washing with 1 M ammonium chloride removes the initiation factors to a large extent without destroying the ribosomes. Salt-washed ribosomes require the addition of the initiation factor fraction which also contains RNase III or of purified initiation factors and RNase III in order to be competent for enzyme synthesis (Table 3; HERRLICH et al., 1974; and unpublished). As mentioned above, reconstituted S30 ribosomal particles from split proteins and RNA, regain part of their function both in polyU-dependent polyphenylalanine synthesis and in enzyme synthesis (EGBERTS et al., 1972).

From the concentration dependence curve described above, an approximate measure of the activity of the ribosomes can be obtained. The number of rounds of translation by ribosomes in T7 DNA-directed enzyme synthesis was also estimated: Crude ribosomes translate—on the average—10 times, preincubated ribosomes translate the messenger twice. The data must be taken with great reservation, because they depend on a large number of assumptions.

2. tRNA

In a crude extract or S30 system, no additional tRNA is required. The S30 or crude extract contains saturating amounts of tRNA. The DEAE-system is partially dependent on the addition of tRNA: the protein fraction is almost free of tRNA, but tRNA is attached to the ribosomes. In fact, preincubated ribosomes still contain sufficient tRNA to allow 30–50% of the leucine incorporation which can be achieved upon addition of tRNA. However, some tRNA species are apparently limiting since meaningful protein synthesis is reduced to 5% (GOLD and SCHWEIGER, 1969a). Response to tRNA addition is improved by further purification of the ribosomes.

Since an S30 and even a DEAE-system contains some tRNA, nonsense codons in the mRNA are suppressed when the system is prepared from suppressor tRNA carrying strains, and to avoid suppression, the cell-free system must be prepared from suppressor minus bacteria. Using such a system, DNAs containing amber mutations do not direct synthesis of the corresponding enzymes when tRNA from suppressor-negative bacteria is added to the reaction mixture (HERRLICH and SCHWEIGER, 1971), but active enzymes are formed when tRNA from a suppressing strain is added. Similarly mRNAs prepared from cells infected with T7 or T4 phage amber mutants direct the synthesis of enzymes of the mutated genes only when suppressor tRNA is added (unpublished; GESTELAND et al., 1967). These messenger RNAs are present in intact form in T phage infected cells, although bacterial messengers are degraded in uninfected cells where the RNA distal to an UAG codon is almost not detectable (MORSE and PRIMAKOFF, 1970; MORSE and GUERTIN, 1971; IMAMOTO and KANO, 1971). Infection with T7 or T4 phage changes the physiology of the cell and as a result RNA breakdown or synthesis is no longer

influenced by a nonsense codon. In this connection, it should be mentioned that the average half life of messenger RNA is prolonged in T4 or T7 infected cells (see pages 72 and 80; SAUERBIER, unpublished; SUMMERS, 1970). The system T4 amber RNA-suppressor minus tRNA serves an additional purpose: it has been used for the identification of unknown suppressor tRNA (BRUENN, 1972).

Enzyme synthesis *in vitro* depends on the presence of N-formyl-methionine $tRNA_{fmet}$. However, in systems prepared from normal cells there is no requirement for a formyl donor, since the cells contain an excess of the formyl donor N^{10}-formyltetrahydrofolic acid, stuck primarily to the ribosomes after disruption. Similar to the case with other tRNA species, the requirement for formylmethionyl $tRNA_{fmet}$ can only be detected after special treatment of the cell-free system. Treatment of the cells with the antibiotic trimethoprim before the preparation of the cell-free system causes starvation for the formyl donor needed for the synthesis of N-formylmethionyl $tRNA_{fmet}$. Trimethoprim inhibits *E. coli* dihydrofolate reductase (BURCHAL and HITCHINGS, 1965), so that any tetrahydrofolate that is oxidized—for instance in thymidilate synthesis—is not regenerated by reductase. A cell-free system perpared from such starved cells shows a dependence on formyl donor addition (GOLD and SCHWEIGER, 1969b). Addition of N^5-formyltetrahydrofolic acid (folinic acid) stimulates enzyme synthesis up to 25 times, depending on the magnesium ion concentration used and on the cell-free system. The requirement for a formyl donor of the cell-free protein synthesis system can be overcome by N-formylmethionine $tRNA_{fmet}$. By labeling experiments it could be shown that the methionine is incorporated into the N-terminal position of newly synthesized protein. Unformylated methionine $tRNA_{fmet}$ stimulated protein synthesis much less (approximately 25% as much). The level of residual formyldonor was not determined in the cell-free system and the stimulation of enzyme synthesis by methionyl $tRNA_{fmet}$ might be due to transformylation or it could be caused by the ability of methionine $tRNA_{fmet}$ to function as initiator tRNA (GOLD and SCHWEIGER, 1969b). Formyl donor is essential for both early as well as late T4 enzyme synthesis.

3. Protein Fraction

In the "DEAE system" the protein fraction is obtained by chromatography of the supernatant S100 on DEAE-cellulose. There are differences in the saturating amounts of the protein fraction for synthesis of various enzymes. Thus, although maximal T7 lysozyme synthesis is reached with 60 μg protein per standard reaction (0.050 ml), for maximal ligase formation slightly more is necessary (90 μg) (Fig. 10) and maximal synthesis of T7 RNA polymerase needs more than 140 μg protein fraction. (For maximal leucine incorporation as much as 200 μg protein fraction is needed.) The reasons for these differences are unknown.

The protein fraction contains the enzymes and factors needed for translation. In particular, the content of tRNA charging enzymes and of the

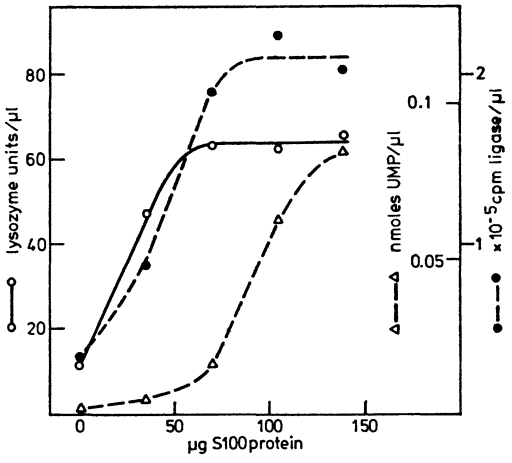

Fig. 10. Dependence on S100 protein of enzyme synthesis. Experiment as in Fig. 9 except that the amount of DEAE protein fraction was varied

elongation factors Tu, Ts and G, have been tested (unpublished, together with J. GORDON). The protein fraction also contains RNA polymerase holoenzyme. It is not yet known whether other transcriptional factors like repressors or CAP protein are present in sufficient amounts. RNA polymerase itself may become a limiting factor, especially when the S100 has been obtained by prolonged centrifugation: then RNA polymerase must be added. Also, in the uncoupled system, purified RNA polymerase is used for transcription and not the protein fraction, which is later added together with ribosomes for the second part of the incubation (the translation) (SCHWEIGER et al., 1971; HERRLICH and SCHWEIGER, 1974).

4. Template

In a preincubated system, either S30 or the DEAE system, nucleic acids as templates are required when measuring amino acid incorporation into polypeptides or enzyme synthesis. The saturating amount to give maximal synthesis, for a given system, must theoretically be a function of the number of promoters available and would therefore vary from one DNA to another. In the preincubated S30 system, ϕ80dlac DNA saturation for β-galactosidase synthesis is reached at above 50 µg/ml reaction mixture (ZUBAY et al., 1970a). In the DEAE system, the values range from 20 µg/ml for T4 (GOLD and SCHWEIGER, 1969a; SCHWEIGER and GOLD, 1969a) to 60 µg/ml for T7 DNA (Fig. 11). When RNA is used as template, the saturation amounts depend on the type of RNA. Total RNA isolated from T7 infected cells does not saturate the cell-free system at 800 µg/ml. In contrast, T7 RNA produced in vitro by purified *E. coli* RNA polymerase, leads to maximal enzyme synthesis at 16 µg/ml (Fig. 12). This difference is probably due to the high proportion of ribosomal and transfer RNA in the nucleic acid preparations isolated from cells.

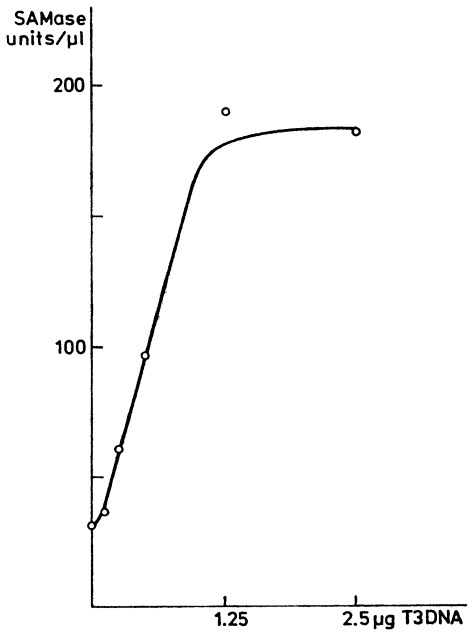

Fig. 11. Dependence on template of DNA directed enzyme synthesis. SAMase synthesis was performed in the presence of varying amounts of T3 DNA per incubation mixture. Incubation mixture as in Table 2. Enzyme assay as described by HERRLICH and SCHWEIGER (1974)

Not only DNAs and RNAs from coli phages direct the synthesis of enzymes, but also templates from unrelated organisms. The efficient production of enzymes under the direction of DNAs from B. subtilis phages demonstrates that "foreign" DNAs can be transcribed and translated with a high degree of fidelity (Fig. 13 and 14; SCHWEIGER and GOLD, 1970).

However, not all templates can be successfully introduced into the *E. coli* system. On the basis of amino acid incorporation, our laboratory was able to define three classes of template DNAs (SCHWEIGER et al., 1969). 1. DNAs which were good templates *in vitro*. These originated from various bacteria and bacteriophages. 2. Several template DNAs from bacteria and bacteriophages had a low template activity, among them for instance Lambda and *E. coli* DNA. This low template activity is due to inefficient transcription since the RNA made is translated. 3. In contrast to these two groups, DNAs from all eukaryotic organisms tested did not direct the synthesis of protein although transcription by *E. coli* RNA polymerase was normal as far as the absolute amount of UMP incorporated is concerned. Instead, the translation of the newly synthesized RNA was deficient. This translational block was also detected when RNA was isolated from various cells. Again RNAs from eukaryotic organisms did not stimulate amino acid incorporation into polypeptides in the *E. coli* system while bacterial RNA did (HERRLICH et al., 1967). The reverse was observed in a cell-free preparation from rat liver. The

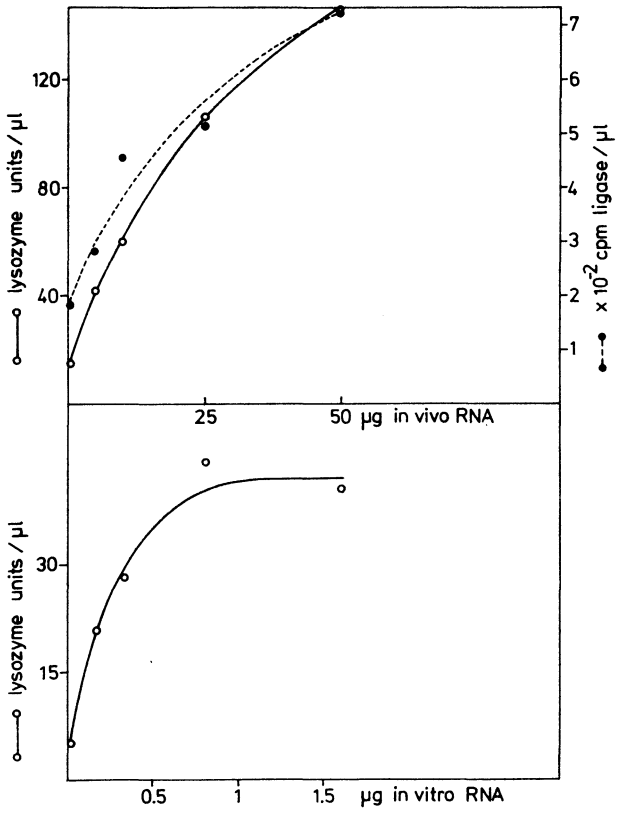

Fig. 12. Dependence on template of RNA directed enzyme synthesis *in vitro*. A DEAE system of the composition described in Table 2 was started with varying amounts of RNA per incubation mixture. The isolation of "*in vivo*" RNA (in this case 10 minutes past T7+ infection) has been described (HERRLICH and SCHWEIGER, 1974), for the preparation of "*in vitro*" RNA (by *E. coli* RNA polymerase on T7 DNA) see SCHWEIGER et al. (1971)

experiments indicated the existence of two classes of protein synthesis, a bacterial and a non-bacterial class (SCHWEIGER et al., 1969). Class selection appears to occur at the recognition of RNA by ribosomes. This class hypothesis is supported by the structural differences between the two ribosome systems, and by their different sensitivities to various antibiotics. RNA from animal viruses may even inhibit *E. coli* protein synthesis (OLSON et al., 1968). More recent results prove, for a specific protein, that hemoglobin messenger RNA is not translated correctly in the *E. coli* system, and add to the body of evidence that ribosomes recognize only their cognate messenger (NOLL et al., 1972). Also peptides synthesized *in vitro* with SV40 and polyoma DNA did not band identically with proteins isolated from infected cells (CRAWFORD et al., 1971).

Several authors, however, have reported the synthesis of proteins under the direction of templates from animal and plant viruses in an *E. coli* system

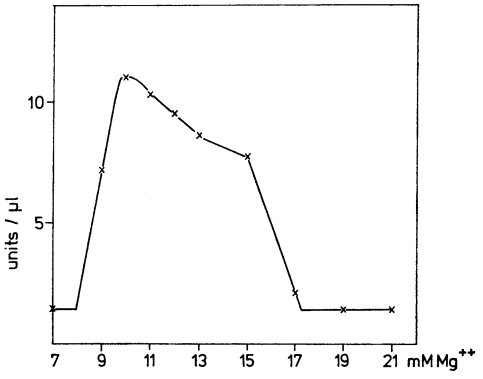

Fig. 13. Dependence of $\phi 29$ DNA directed synthesis of lysozyme *in vitro* on Mg^{2+} concentration. (Experiment together with Drs. M. SALAS and E. VIÑUELA.) The reaction mixtures for enzyme synthesis were incubated at various concentrations of $MgCl_2$. The template DNA was prepared from *B. subtilis* bacteriophage $\phi 29$. All other reaction conditions were as in Table 2

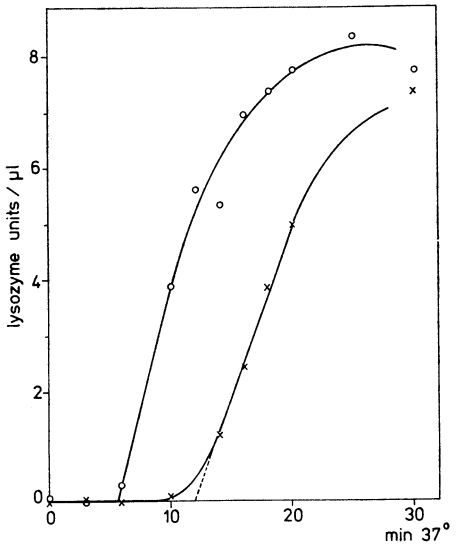

Fig. 14. Kinetics of synthesis of $\phi 29$ lysozyme *in vitro*. The reaction mixtures for $\phi 29$ DNA directed protein synthesis were incubated in the presence of either 11 or 16 mM Mg^{2+}. Aliquots were removed at various times (as indicated) and kept on ice with 50 µg/ml chloramphenicol until the enzyme assays were performed. o——o Lysozyme synthesis at 16 mM Mg^{2+}. ×——× Lysozyme synthesis at 11 mM Mg^{2+}

(TSUGITA et al., 1962; CLARK et al., 1965; SCHWARTZ, 1967; VAN RAVENSWAAY CLAASEN et al., 1967; LAYCOCK and HUNT, 1969; REKOSH et al., 1970; SIEGERT et al., 1972; RICE and FRAENKEL-CONRAT, 1973; CRAWFORD and GESTELAND, 1973). $Q\beta$ RNA was faithfully translated by cell-free extracts from eukaryotic cells (MORRISON and LODISH, 1973). Possibly some viral RNAs form an additional (intermediate) class of RNA-ribosome recognition.

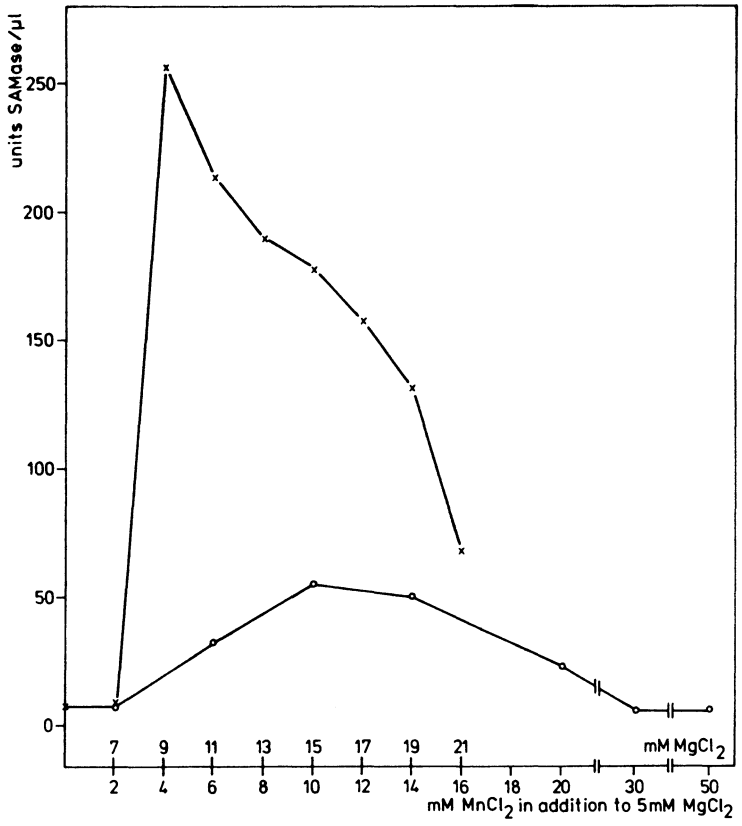

Fig. 15. Dependence of T3 DNA directed SAMase synthesis on Mg^{2+} and Mn^{2+} concentration. SAMase synthesis was performed in the "DEAE system" as described in Table 2. Synthesis was allowed to proceed at various concentrations of $MgCl_2$ or $MnCl_2$. In the cases of manganese addition a basal concentration of 5 mM $MgCl_2$ was present in all samples because the system itself had been prepared with TMA buffer

Exceptional cases, in this respect, are the systems from mitochondria and chloroplasts. DNAs from these organelles (although they originate from eukaryotic organisms) stimulate amino acid incorporation in a cell-free system from *E. coli* (RABUSSAY et al., 1970). And the reverse is true as well: A cell-free system from mitochondria accepts template DNA of the bacteriophages T7 and T3 and synthesizes T7 and T3 specific enzymes (RICHTER et al., 1972). The protein synthesizing machinery in these organelles including the RNA polymerase must be bacteria-like and the ability to recognize bacterial signals on DNA and RNA seems to be conserved throughout phylogeny. Also the mitochondrial DNA seems to carry signals that are similar to those in bacterial DNA.

5. Divalent Ions and Polyamines

The concentration of divalent ions is particularly critical as is the composition of ionic species. Magnesium cannot be replaced by manganese except at

the cost of a considerable decrease in enzyme synthesis (Fig. 15). Polyamines, however, substitute well for part of the magnesium, as discussed below (HERRLICH et al., 1971b).

The concentration of divalent ions needed for optimal enzyme synthesis depends on the concentration of other components in the system like ribonucleic acids, phosphoenolpyruvate, nucleoside triphosphates and DNA and possibly even on the glass ware used. Therefore, synthesis of the same enzyme in different systems may have different magnesium optima. Within the same system, different magnesium optima are observed for different enzymes (SCHWEIGER and GOLD, 1969b) and they also depend on whether DNA or RNA is used as template (SCHWEIGER et al., 1971). This will be described in more detail: Using the preincubated S30 system, optimal DNA-directed enzyme synthesis occurs at 14–15 mM magnesium [tested for β-galactosidase (ZUBAY et al., 1970a), uridyltransferase and galactokinase (WETEKAM et al., 1972)]. In the DEAE system optimal magnesium concentrations range from 10–15 mM. Some enzymes are synthesized best at 10–11 mM Mg^{2+} (under the direction of DNA) whereas others, for instance T4 lysozyme, are formed best at 15 mM (SCHWEIGER and GOLD, 1969). Optimal magnesium concentration of enzyme synthesis *in vitro* and the time of synthesis of these enzymes *in vivo* during normal phage development, seem to be correlated. The typical "early" enzymes have *in vitro* Mg^{2+} optima of 10–11 mM, while enzymes which are synthesized in the late phase of phage development, have *in vitro* synthesis optima of 15 mM Mg^{2+}. A third group of enzymes proved to be synthesized *in vitro* under both magnesium concentrations, at 11 and 15 mM (T7, T3 lysozymes [SCHWEIGER et al., 1971]; SP82 dCMP deaminase [SCHWEIGER and GOLD, 1970]; ϕ29 lysozyme [Fig. 13]). For these enzymes it could be demonstrated that they are induced *in vivo* via both, an early and a late mechanism (HERRLICH et al., 1971a). In kinetic experiments, the synthesis of these enzymes occurs with a longer lag period in the presence of 11 mM Mg^{2+} than in the presence of 15 mM Mg^{2+} (see also Fig. 14). The delay in appearance at 11 mM is due to a transcriptional process and it has been shown that RNA polymerase recognizes different promotors at 11 versus 15 mM Mg^{2+} (under these specific conditions in the DEAE system) (GOLD and SCHWEIGER, 1970; SCHWEIGER et al., 1971; SCHERZINGER et al., 1972a). RNA dependent enzyme synthesis occurs at about 11 mM Mg^{2+} in the DEAE system and at 13 mM Mg^{2+} in the uncoupled system (SCHWEIGER et al., 1971; and unpublished). Again note: specific molarities get their meaning only in connection with a specific method.

From all these data it should be summarized that the observation of the magnesium concentration is of critical importance in all attempts to synthesize an enzyme in vitro (SCHWEIGER and GOLD, 1969b; ZUBAY et al., 1970a).

Magnesium can, to some extent, be substituted by spermidine or putrescine (HERRLICH et al., 1971b). These polyamines have a differential influence on DNA directed enzyme synthesis *in vitro*. Spermidine for instance stimulates

the T3 DNA dependent formation of SAMase and the T3 or T7 DNA dependent synthesis of phage RNA polymerase, as compared to the synthesis in the presence of magnesium alone, but inhibits the synthesis of T3 and T7 lysozyme (HERRLICH et al., 1971 b). Similar observations with RNA dependent enzyme synthesis suggest that the polyamine effect concerns a translational process. The differential effect of spermidine on translation suggests the possibility of translational control of these enzymes.

In addition to magnesium, the concentration of calcium salts appears to be critical in the preincubated S30 system (Table 2; ZUBAY, personal communication).

6. Monovalent Ions

The composition of monovalent ions is not very critical in bacterial systems. Ammonium and potassium salts both chlorides and acetates are in use. However, variations in the optimal concentration of monovalent ions have been observed (Fig. 8).

D. Characteristics of Enzyme Synthesis *in vitro*

1. Product Analysis

a) Characterization of RNA

The most stringent test for messenger RNA is of course its translation into specific proteins. Synthesis of enzymes is the best indication that the transcription unit had been read properly and that the RNA contained the correct translational signals. T7 RNA which was synthesized *in vitro* by E. coli RNA polymerase on T7 DNA, under salt and substrate conditions similar to the DEAE system, was isolated and purified. The RNA contained mainly one species of molecular weight 2.2×10^6 daltons (MILLETTE et al., 1970). 85% of the RNA ended with U at the 3′ terminus, indicating that transcription was terminated specifically. In the cell-free system, the RNA coded for several proteins (MILLETTE et al., 1970; SCHWEIGER et al., 1971; SCHERZINGER et al., 1972a) (see also page 65). The RNA product is therefore a correct copy of this part of the codogenic DNA strand and carries the proper signals for initiation and termination of translation. In addition, these experiments demonstrate that *E. coli* RNA polymerase does not need any other proteins for correct transcription. T7 RNA needs, however, posttranscriptional processing by RNase III (DUNN and STUDIER, 1973; HERCULES et al., 1974).

b) Characterization of the in vitro Synthesized Protein

The appearance of enzyme activity is itself a good indication that a complete protein has been properly synthesized.

T3 and T7 Lysozyme. T3 and T7 lysozyme synthesis under the direction of the corresponding DNAs was performed in a DEAE system. The product consists of free enzyme and most probably ribosome-bound enzyme based

upon its behavior on Sephadex G 100 (unpublished). The ribosome-bound enzyme is probably bound non-specifically due to the fact that lysozyme is a basic protein. The enzymes synthesized *in vitro* were also compared with their *in vivo* counterparts and with egg white lysozyme as far as enzymatic activity is concerned. Lysozyme converts murein (peptidoglycan) into muropeptides (PRIMOSIGH et al., 1961). Analysis of these muropeptides allows the unequivocal determination that an enzyme exerts lysozyme activity and also helps to differentiate between lysozymes of different origin. Presumably the ratio of muropeptides produced by digestion with different types of lysozyme (especially the ratio C3/C4) reflects their individual kinetics of action. The ratio C3/C4 differs between egg white lysozyme and T3 lysozyme (Table 5), while egg white lysozyme and T2 lysozyme produce the same C3/C4 ratio (W. LEUTGEB, unpublished). This type of evidence showed that the lysozymes synthesized *in vitro* under the direction of T4, T7 or T3 DNA were identical to the corresponding enzymes *in vivo*. In this experiment, cell walls from *E. coli* C labeled with ^{3}H-2,6-diaminopimelic acid, were used. The solubilized radioactive material was chromatographed by the procedure for analysis of muropeptides described by LEUTGEB and SCHWARZ (1967). Separate fractions of the solubilized material were also hydrolyzed and then chromatographed in order to determine the amount of ^{3}H-diaminopimelic acid and ^{3}H-lysine. ^{3}H-diaminopimelic acid is, to some extent, decarboxylated *in vivo* to ^{3}H-lysine. When the cell wall preparation is incubated with trypsin, mainly ^{3}H-lysine-containing material is released (Table 5), while the lysozymes synthesized *in vitro* liberate almost exclusively ^{3}H-diaminopimelic acid-containing muropeptides (Table 5). The remaining 2% lysine-containing material is due to the low background of protease in the DEAE system. These results clearly demonstrate that specific lysozymes are synthesized in the *in vitro* protein synthesizing mixtures (LEUTGEB, HERRLICH and SCHWEIGER, unpublished; SCHWEIGER and GOLD, 1969a).

β-Glucosyltransferase. Native β-glucosyltransferase from T4-infected cells transfers a glucose residue from UDPG to the hydroxymethyl side chain of deoxyhydroxymethylcytidine in intact unglucosylated T4 DNA. The *in vitro* synthesized enzyme did not hydrolyze UDP-glucose in the absence of DNA. The reaction product (when unglucosylated T4 DNA was added) was DNase-sensitive and all the radioactivity was associated with hydroxymethyl-dCMP upon enzymatic digestion and chromatography (GOLD and SCHWEIGER, 1969a). The Km-values for UDP-glucose binding and DNA binding were similar to the native enzyme.

dCMP-Deaminase. This is, next to the β-galactosidase monomer, the largest enzyme synthesized *in vitro*. The native enzyme of bacteriophage T4 is subject to allosteric regulation by a stimulating effector, dCTP, and by an inhibitor, dTTP. The enzyme synthesized *in vitro* behaves similarly (SCHWEIGER and GOLD, 1970; TRIMBLE et al., 1972). dCMP deaminase from *B. subtilis* infected with phage SP82 does not respond to these effectors; neither does the enzyme produced *in vitro* under the direction of SP82 DNA (SCHWEIGER and GOLD, 1970). Beside enzymatic activity, the detection of allosteric regula-

Table 5. Muropeptide patterns after "lysozyme" digestion of cell walls. (% found in muropeptide resp. DAP and lysine per 100% total radioactivity)

	Egg white lysozyme	In vitro synthesized T7 "lysozyme"	In vitro synthesized T3 "lysozyme"	Trypsin
C3	24	50	52	various spots
C4	21	5	3	that do not
C5	18	28	31	correspond to
C6	37	17	14	muropeptides
DAP	98	98	98	17
Lysine	2	2	2	83

E. coli cell walls were labeled with ^{3}H-2,6-diaminopimelic acid and adsorbed to filter paper disks (GOLD and SCHWEIGER, 1970). Egg white lysozyme, "in vitro" enzyme and trypsin respectively were incubated in 0.5 ml 0.1 M NH_4 acetate with the cell wall preparation. Incubation was continued until most of the radioactivity was excorporated. The supernatant was removed from the disk and lyophilized. Part of the material was chromatographed together with non-radioactive carrier muropeptides on Macherey-Nagel 2214 (descendent, perpendicular to the direction of the paper fibers). The eluant was n-butanol-acetic acid-water = 4:1:5 (v/v) and chromatography was allowed to proceed for 36 hours.

Ninhydrin-positive spots and all the rest of the chromatogram were counted in a scintillation spectrometer. Percent of total radioactivity was calculated for each muropeptide. The ratio C5/C6 depends on the amount of carboxypeptidase which is present in the *E. coli* system. The carboxypeptidase converts C6 to C5 (see LEUTGEB and SCHWARZ, 1967). The ratio C3/C4 is specific for the enzyme. The other part of the lyophilized original material was hydrolyzed by 6 N HCl at 100° for 16 hours. The hydrolysate was chromatographed on paper with Rhuland's mixture. The amounts of diaminopimelic acid (DAP) and lysine were determined. *In vivo* DAP is partly decarboxylated to lysine. The label appears therefore partly in protein. Trypsin removes, from total cell wall preparations, mainly lysine-containing fragments while lysozyme can only produce the muropeptides. The low amount of lysine-material solubilized by lysozyme may be either due to contaminating proteases in the *E. coli* system or to unspecific removal of undigested cell wall from the filter paper.

The muropeptides found after T7 and T3 lysozyme treatment cochromatograph with those described by LEUTGEB and SCHWARZ, 1967. It has not been proven, however, that the cleavage products arc in fact identical. The parentheses ("lysozyme") should stress that the type of lysozyme-like cleavage has not been examined further. But because the pattern is so different from the obtained with egg white or T4 lysozyme, it serves as a good characteristicum for comparing the *in vivo* and *in vitro* enzymes.

tion is the best proof available for the correctness of the *in vitro* synthesized proteins.

α-Fragment of β-Galactosidase. The α-fragment can be measured by *in vitro* complementation with a mutant (deletion) enzyme deficient in the α-fragment. This assay was adapted to *in vitro* protein synthesis by including the deficient enzyme in the preincubated S30 system used for α synthesis. The yield of α-fragment was higher when complementation was allowed to proceed during protein synthesis than when the deficient enzyme was added afterwards. 98% of the resulting β-galactosidase activity sedimented with 10.5 S which corresponds to β-galactosidase dimers. The α-fragment had apparently been

released from the ribosomes and had complemented the z gene fragment which itself sediments at only 9.7 S (as dimer) (DE VRIES and ZUBAY, 1969).

β-Galactosidase. β-galactosidase itself has been made *in vitro* and subjected to sedimentation analysis. Depending on how much enzyme was synthesized *in vitro*, the activity sedimented as a monomer or dimer (7.4 S or 10.2 S respectively). Upon further concentration, the *in vitro* product formed tetramers (16.6 S) similar to the native enzyme (ZUBAY and CHAMBERS, 1969). The nature of the enzymatic activity of the *in vitro* enzyme is unequivocally defined by the specificity of the substrate. Further purification of β-galactosidase synthesized *in vitro* was achieved and a structural and kinetic analysis of both the *in vitro* enzyme and the natural enzyme showed identity (CHAMBERS and MANLEY, 1973).

In Summary. In vitro synthesized protein is released from the ribosomes as completed polypeptide chains. It folds to the normal three-dimensional structure so that it becomes enzymatically active and is subject to allosteric regulation. The "*in vitro* enzyme" carries the specific properties encoded in the DNA added as template.

2. Kinetics of Synthesis

a) Speed of Transcription and Translation

Transcription proceeds *in vitro* at 37° with a speed of 15–28 nucleotides/sec (RICHARDSON, 1970; MILLETTE and TROTTER, 1970; ROSE et al., 1970). The speed of translation was determined to be 1.6 aa/sec at 25° and 3.0 aa/sec at 31° (WILHELM and HASELKORN, 1970) which would correspond to 6 aa/sec at 37°, and 5 aa/sec at 37°, respectively (SCHERZINGER et al., 1972a). Translation thus seems to trail slightly behind transcription. Beside direct methods of determination, the speeds of transcription can also be calculated from the lag periods of appearance of mRNA or enzyme (Table 1 and Fig. 2) (SCHERZINGER et al., 1972a; ZUBAY and CHAMBERS, 1969; POUWELS and VAN ROTTERDAM, 1972).

b) Lag Periods of Synthesis

The initiation of specific phage messenger RNA synthesis does not show a distinct lag period, whether tested *in vitro* (GOLD and SCHWEIGER, 1970; SCHWEIGER et al., 1971; SCHERZINGER et al., 1972a), or *in vivo* (HERRLICH et al., 1971a). The initiation of β-galactosidase specific mRNA synthesis *in vitro* takes off with a lag period of 2–3 min; this lag period is not understood (ZUBAY and CHAMBERS, 1969). The first appearance of finished messengers also has a lag period (Table 1) which reflects the distance on the DNA between the promotor and the promotor-distal end of the gene under observation. From the length of the lag period and the rate of RNA polymerization, this distance can be determined. In case the position of the promoter on a polycistronic message is known, as for instance in T7, where the only promoter

for host RNA polymerase is close to one end of the genome (DAVIS and HYMAN, 1970), this method may be used to map gene positions (SCHERZINGER et al., 1972; and Fig. 2).

In DNA dependent *in vitro* synthesis of protein, it takes even longer for the enzyme to appear than for the messenger (Table 1 and Fig. 2) (SCHERZINGER et al., 1972a; ZUBAY and CHAMBERS, 1969). This was also observed when enzyme folding was allowed to proceed after protein synthesis (stopped by chloramphenicol treatment) (POUWELS and VAN ROTTERDAM, 1972). In T4 DNA directed lysozyme synthesis, the lysozyme messenger is completed at 5 minutes (37°) and the enzyme appears at 10 minutes (GOLD and SCHWEIGER, 1970); T7 lysozyme messenger appears *in vitro* at 4 minutes and the enzyme at 7.5 minutes (SCHWEIGER et al., 1971); and it takes 5 minutes for the first completed β-galactosidase messenger to appear and 8–9 minutes for the enzyme activity (ZUBAY and CHAMBERS, 1969). Assuming in each case, that the folding of the peptide chains had been completed at the time the enzyme assay was started, we have to conclude that the amino acid polymerization takes longer than RNA synthesis in these systems. This may be in contrast to *in vivo* conditions where RNA synthesis and protein synthesis are supposed to follow each other closely.

T7 RNA-dependent enzyme synthesis shows a shorter lag period than DNA-dependent synthesis (MILLETTE et al., 1970). This may be explained by the fact that in the coupled system, ribosomes appear to start at the 5′-end of a polycistronic messenger RNA (MILLETTE et al., 1969); similar results are obtained from *in vivo* experiments (MORSE et al., 1969) and from translation of RNA bacteriophage RNA (LODISH, 1968). The finding *in vivo* of intercistronic restarts subsequent to the introduction of a nonsense codon (PLATT et al., 1972) suggests that these internal AUG codons are normally masked. Since in the DEAE system, transcription is twice as fast as translation and translation start points would theoretically be exposed, these must be available only to ribosomes which started at the original 5′-end of the total messenger. Another explanation is offered by the finding that the T7 RNA is processed prior to translation by RNase III which is contained in the ribosomes (DUNN and STUDIER, 1973; HERCULES et al., 1974). This step may be limiting *in vitro* and thereby cause lag periods when T7 DNA (or polycistronic messenger) is used as template.

A recent report could not correlate the sequence of appearance of T4 proteins *in vivo* with the sequence *in vitro* (TRIMBLE et al., 1972a). One possible explanation for this inconsistence is that enzymes synthesized *in vitro* need various times for "maturation", as discussed above (POUWELS and VAN ROTTERDAM, 1972; HERRLICH and SCHWEIGER, unpublished). Kinetic measurements *in vitro* must therefore include a "safety incubation" in the presence of chloramphenicol to allow "maturation" to be completed. Other authors find good correlation between *in vitro* and *in vivo* synthesis of proteins (by UV-measurements, messenger characterization, genetic mapping, SCHERZINGER et al., 1972a; BLACK and GOLD, 1971).

c) Duration of Linear Synthesis

RNA dependent enzyme synthesis in the DEAE system is linear for 7–8 minutes. The half life of lysozyme messenger RNA appears to be approximately 7 minutes. A similar stability was measured for total RNA (unpublished data). This half life is longer than the one *in vivo* (ADESNIK and LEVINTHAL, 1970; SCHWEIGER et al., 1972). The in vitro system may lack nucleases (DEAE cellulose chromatography of the S100 protein) or part of the natural mRNA degrading enzymes (WETEKAM and EHRING, 1973).

DNA-dependent enzyme synthesis is linear for 40–60 minutes (at 37°) in both the DEAE- and the preincubated S30-system (GOLD and SCHWEIGER, 1969a; SCHWEIGER and GOLD, 1969a; ZUBAY et al., 1970a). Linearity, in this case, is a function of repeated reinitiations by RNA polymerases. Synthesis finally levels off as the substrates become limiting since by the addition of new substrates and fresh ribosomes, the synthesis can be restarted (unpublished).

3. Controls (see also Table 2)

Effective controls are omissions of ribosomes, template, nucleoside triphosphates, amino acids and magnesium. Omissions of protein fraction, tRNA and of monovalent ions also inhibit to some extent. Enzyme synthesis depends of course on incubation at biological temperatures. The final yields of enzymes were equal within the temperature range of 28° to 37°, but were progressively less at temperatures below 28°. The speed of synthesis, of course, varies with temperature. Other controls are: addition of templates which do not carry the information for the protein to be tested, and the use of various antibiotics.

4. Yields of Enzyme Synthesis

An interesting question for the validity of results obtained with cell-free protein synthesis, is whether the enzyme synthesized is only a random product out of a large mass of nonsense peptides—in other words: whether RNA polymerase and the translational apparatus efficiently recognize true start and stop signals. The faithfulness of transcription and translation *in vitro* can be estimated. Various data must be obtained. Thus, from the specific enzymatic activities of purified enzymes, the absolute amounts of enzyme protein synthesized *in vitro* are calculated. From leucine incorporation data the total amount of protein synthesized, including the enzyme under observation plus all other proteins, complete or incomplete, can be estimated. We know also the gene size of the enzyme we observe, and in both T7 and T4, the total stretch of DNA that is transcribed by *E. coli* RNA polymerase. In Table 6 two ratios are formed: yield of enzyme to total protein, and gene size to total template. A comparison gives information on how much senseful protein is synthesized. In T4 DNA dependent enzyme synthesis, a large number of proteins must be synthesized from a total molecular weight of 50×10^6 daltons codogenic DNA (early). Correspondingly, each individual enzyme makes up only little of the total amount of protein. Even so the percentage of senseful protein is high at

Table 6. Yields of enzyme synthesis *in vitro*

		μg/ml	% of total protein	% of senseful protein
T7 RNA polymerase	exp. I	4.54	11.35	26.7
	exp. II	17.8	44.5	104.0
T7 lysozyme	exp. I	1.47	3.68	55.3
	exp. II	2.0	5.0	75.3
	exp. III	3.44	8.14	122.3
T4 β-glucosyltransferase			0.1–0.2	17–35
T4 lysozyme			0.05–0.1	8.2–17

The yields of enzyme synthesized *in vitro* per ml of cell-free system were calculated from the specific activities of the purified enzymes (ZILLIG et al., 1966; CHAMBERLIN et al., 1970). It was assumed that T7 lysozyme has the same specific activity as T4 lysozyme. The data on T4 enzyme yields had been described elsewhere (SCHWEIGER and GOLD, 1970). In the same cell-free mixtures, the amount of total protein synthesized was determined by the incorporation of ^{14}C-leucine into TCA precipitable material. To the amount of total protein the yields of various individual enzymes were related (column 2). In order to estimate the percentage of senseful protein synthesized, we calculated the portion of a gene of the total stretch of DNA transcribed (28–30% in T7; 40% in T4). The data in column 2 were divided by this factor, assuming that all proteins were synthesized with equal efficiency.

10–30%. In T7 enzyme synthesis where only 25–30% of the DNA is transcribed into one polycistronic messenger, each protein makes up a large proportion of total protein synthesized. The yield of senseful T7 protein is amazingly high: 25–100%. Although these calculations must be interpreted with caution, they definitely indicate that cell-free protein synthesis is an efficient process and compares well with the *in vivo* conditions.

How does the rate of *in vitro* enzyme synthesis compare to *in vivo* synthesis? Under optimal conditions, 0.2 μg β-galactosidase per ml is synthesized within 60 minutes of incubation (CHAMBERS and MANLEY, 1973). Compared to maximal *in vivo* synthesis, this corresponds to an efficiency of 0.06%. In φ80d*arg* directed synthesis of N-α-acetyl-L-ornithinase, the *in vitro* rate was approximately 0.09% of the *in vivo* rate (URM et al., 1973). T7 infected cells produce about 7×10^4 units of lysozyme per minute and per mg of ribosomes assuming that 3×10^{10} cells contain 1 mg (1500 O.D.$_{260}$) of ribosomes. The *in vitro* rate is 4×10^2 units per minute and per mg of ribosomes or 0.57% of the *in vivo* rate. All these data are of course approximations. Rates of enzyme synthesis in both the preincubated S30 system and the DEAE system are of the same order of magnitude.

5. On the Mechanism of Protein Synthesis

a) Initiation of Transcription

Correct transcription of phage DNA by *E. coli* RNA polymerase needs holoenzyme (BURGESS et al., 1969; HINKLE and CHAMBERLIN, 1970). In

addition, beside the complete enzyme, correct initiation of RNA synthesis by *E. coli* polymerase needs an ionic strength of 0.2–0.3, a defined magnesium concentration and a DNA with the correct promoter regions. Transcription of *E. coli* DNA by *E. coli* RNA polymerase requires, in addition, cyclic AMP and cyclic AMP binding protein. In the absence of cyclic AMP, initiation at the correct promoter site is less specific (ZUBAY et al., 1970). Lambda repressor and *lac* repressor, both seem to inhibit the initiation of transcription (ZUBAY et al., 1970; WU et al., 1971; GESTELAND and KAHN, 1972).

b) Initiation of Translation

Cell-free enzyme synthesis confirmed that the synthesis of senseful proteins is started with formylmethionyl-$tRNA_{fmet}$ (GOLD and SCHWEIGER, 1969b). From cell-free protein synthesis several lines of evidence support the view that the specific recognition of mRNA by ribosomes and their cofactors occurs in front of the initiation codon. A first suggestion came from experiments demonstrating group specificity of protein synthesis (SCHWEIGER et al., 1969). Subsequently, a more elaborate hypothesis was introduced, involving initiation factor IF_3 and interfering factor i. It is supposed that i binds to IF_3 and thereby introduces cistron selectivity (GRONER et al., 1972). Initiation factors are needed for specific enzyme synthesis *in vitro* (Table 3). In addition, it seems that the initiation of translation is influenced by spermidine (HERRLICH et al., 1971b).

c) Elongation of Transcription and Translation

The exact mechanism of RNA chain elongation is as yet unclear. Two possible models have been suggested: 1. The strands are separated by the RNA polymerase (melting model; FUCHS et al., 1967) or 2. the incoming nucleoside triphosphates are aligned in the correct sequence by interaction with both strands (triple strand model; STENT, 1958; ZUBAY, 1962; RILEY, 1970). Experiments using heteroduplex DNA as templates for enzyme synthesis *in vitro*, demonstrate that the non-codogenic strand has none or almost no influence on the transcription product, favoring strongly the synthesis via local DNA melting (HERRLICH et al., 1972; WETEKAM, 1972). By the same type of experiments, the "sense" strand of a DNA can be determined.

Also the elongation of translation in its detailed relationship to transcription awaits further investigations (see also remarks on this subject by IMAMOTO, 1973b).

d) Termination of Transcription

Transcription of natural templates like T4 and T7 DNA by *E. coli* RNA polymerase yields defined RNA products at both high and low ionic strength and without the help of additional factors (MILLETTE et al., 1970). At the ionic strength of the *in vitro* protein synthesizing systems (preincubated S30 or

DEAE system), the RNA is released and RNA polymerase starts a new cycle. The RNA released is of constant size distribution and the main species carries at the 3'-end uridine in the case of T7, and 66% uridine, 21% adenosine in the case of T4 (MILLETTE et al., 1970). We have to conclude that *E. coli* RNA polymerase holoenzyme is itself able to recognize this specific termination signal on DNA. Beside this termination mechanism, a factor-dependent mechanism has been suggested (ROBERTS, 1969; SCHÄFER and ZILLIG, 1973a and b). These factors produce shorter RNA chains. However, since the transcriptional units in T4 and T7 *in vivo* are as long as those found in the factor-independent termination *in vitro* (HERRLICH et al., 1971a; HAUSMANN and HÄRLE, 1971), it has been proposed that a post-transcriptional cleavage produces the *in vivo* T7 mRNA pattern (see page 66; MILLETTE et al., 1970). Recently, such post-transcriptional cleavage has in fact been found (DUNN and STUDIER, 1973; HERRLICH et al., 1974).

e) Phage Polymerase, Mitochondrial Polymerase

T7 phage RNA polymerase is a single polypeptide chain of the molecular weight 100000 (CHAMBERLIN et al., 1970), T3 phage RNA polymerase is one polypeptide of about 90000 molecular weight (unpublished). Both enzymes initiate, synthesize senseful RNA and terminate without the help of any additional factors (SCHERZINGER et al., 1972b). The optimal ionic strength is low. Mitochondrial RNA polymerase is another enzyme consisting of one polypeptide chain. By preparation of a mitochondrial protein synthesizing system, phage DNA dependent enzyme synthesis proved that mitochondrial RNA polymerase recognizes the same initiation and termination signals as does *E. coli* RNA polymerase (RICHTER et al., 1972).

6. Additions of Non-Physiologic Compounds

The following compounds have been tested and found to inhibit enzyme synthesis by less than 10%, in the concentration noted:

5% glycerol (shifts magnesium optimum to higher values)
5% sucrose
1% DMSO
0.5% Brij 58
50 mg/ml bovine serum albumin
0.5 $O.D._{260}$ T7 phage particles
1 mM formaldehyde

7. *In vitro* Synthesized Enzymes at the Date of this Review

Table 7 lists all enzymes that have been synthesized *in vitro* to date.

Table 7. DNA directed enzyme synthesis in vitro

Enzyme	Template	Authors	Remarks
(Early antigen)	T4	TRAUB et al., 1966	immuno-logical assay
(Lysozyme)	T4	SALSER et al., 1967	T4 RNA directed
S-adenosylmethionine-hydrolase	T3	SCHWEIGER, 1967; HERRLICH and SCHWEIGER, 1970	
(α-fragment of β-galactosidase)	Ø80dlac	DEVRIES and ZUBAY, 1967	fragment
β-galactosidase	Ø80dlac	LEDERMAN and ZUBAY, 1968	
α- and β-glucosyl-transferase	T4, T2, T6	GOLD and SCHWEIGER, 1969a; and unpublished; YOUNG and TISSIÈRE, 1969	
(Alk. phosphatase)		DOHAN et al., 1969	not detected as enzyme, RNA dep.
Lysozyme	T4, T2, T6 T3, T7 Ø80, Ø29, SPP-1 Lambda	SCHWEIGER and GOLD, 1969a; HERRLICH and SCHWEIGER, 1970 unpublished unpublished and GREENBLATT, 1972	
dCMP deaminase	SP82, SP5C T4	SCHWEIGER and GOLD, 1970 SCHWEIGER et al., cited by SALSER et al., 1967; SCHWEIGER and GOLD, 1970; TRIMBLE et al., 1972	
Transacetylase of the lac operon	Ø80dlac	ZUBAY et al., 1970a	
RNA polymerase	T7, T3	GELFAND and HAYASHI, 1970; HERRLICH and SCHWEIGER, 1971	
Galactokinase, uridyltransferase, epimerase of the gal operon	λpgal	PARKS et al., 1971; WETEKAM et al., 1971; WETEKAM et al., 1972	
1-Ribulokinase	Ø80dara	ZUBAY et al., 1971; GREENBLATT and SCHLEIF, 1971	
DNA ligase	T3, T7	SCHERZINGER et al., 1972a	
Tryptophan synthetase anthranilate synthetase	Ø80ptrp	POUWELS and VAN ROTTERDAM, 1972	
Anthranilate synthetase	Ø80 imm$^{\lambda}$ptrp	DOTTIN and PEARSON, 1973	
dCMP hydroxy-methylase, thymidylate synthetase, dihydrofolate reductase	T4	TRIMBLE et al., 1972	
Deoxynucleotide kinase	T4	TRIMBLE et al., 1972; NATALE and BUCHANAN, 1972	

Brackets indicate examples of RNA dependent enzyme synthesis or of DNA dependent synthesis of enzyme fragments and antigenic determinants.

Post Scriptum

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The literature for this review was collected until May 1973. We regret oversights.

Abbreviations used

TMA-buffer	0.01 M tris HCl pH 7.5, 0.01 M $MgCl_2$, 0.022 M NH_4Cl, 0.001 M dithiothreitol, 5% (v/v) glycerol
PEP	phosphoenolpyruvate
EDTA	ethylenediaminotetraacetic acid
SAMase	S-adenosylmethioninehydrolase
TCA	trichloroacetic acid
DEAE cellulose	diethylaminoethyl cellulose
IF	initiation factor
S30	supernatant after centrifugation at "30000 g", ribosome-containing, but free of debris
S100	ribosome-free supernatant (after centrifugation at "100000 g")
ATP, GTP, UTP, CTP	nucleotide triphosphates
UDPG	uridine-diphosphoglucose
IPTG	isopropyl-β-D-thiogalactopyranoside
lac operon	lactose operon
trp operon	tryptophan operon
ara operon	arabinose operon
gal operon	galactose operon
arg operon	arginine operon
cAMP	cyclic 3′,5′-AMP
cGMP	cyclic 3′,5′-GMP
CAP	catabolite gene activator protein
DAP	2,6-diaminopimelic acid

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