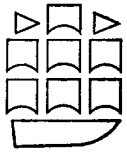
STRATEGY OF THE VIRAL GENOME

A Ciba Foundation Symposium

Edited by
G. E. W. WOLSTENHOLME
and
MAEVE O'CONNOR



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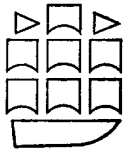

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Symposium on Strategy of the Viral Genome held 26–28 January, 1971

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The Foundation's house at 41 Portland Place, London, has become well known to workers in many fields of science. Every year the Foundation organizes six to ten three-day symposia and three to four shorter study groups, all of which are published in book form. Many other scientific meetings are held, organized either by the Foundation or by other groups in need of a meeting place. Accommodation is also provided for scientists visiting London, whether or not they are attending a meeting in the house.

The Foundation's many activities are controlled by a small group of distinguished trustees. Within the general framework of biological science, interpreted in its broadest sense, these activities are well summed up by the motto of the Ciba Foundation: *Consocient Gentes*—let the peoples come together.

CHAIRMAN'S INTRODUCTION

J. H. SUBAK-SHARPE: STRATEGY OF THE VIRAL GENOME

Department of Virology, University of Glasgow

A virus is essentially a molecule of nucleic acid—the genetic material—enclosed within a protein-rich protective coat which allows the viral genome to be transported from one host to another. In size viruses range from simple bacteriophages like R17 with a diameter of 20 nm and a particle weight of $3 \cdot 6 \times 10^6$ daltons to the complex poxviruses like vaccinia with a diameter of 250 nm and a particle weight of 4×10^9 daltons. Uniquely, all viruses pass through a stage in which the genetic substance is the only material link connecting one generation with the next. They are only able to replicate in the complex environment of the living cell and no virus possesses the independent capacity to synthesize proteins. All the simple precursors and energy-rich molecules needed for the synthesis of viral macromolecules are usually synthesized by host cell enzymes. To produce progeny virus the infecting viral genome diverts the anabolism of the host cell from the making of normal host constituents towards the synthesis and assembly of viral components. These materials which are foreign to the uninfected cell are synthesized partly by the pre-existing metabolic apparatus of the cell and partly by new virus-specified macromolecules which are not destined to become part of the progeny virus particles.

This poses the fundamental question: what is the source of every bit of genetic information needed to specify the many specialized proteins and nucleic acids in the infected cell which catalyse and control metabolism, energy provision, macromolecular synthesis, and finally the production of completed progeny virus? Our discussions during the next three days will, I trust, provide some of the answers.

It is probably unnecessary to do so but I must first remind you that this symposium will be interested first of all in hard facts. If, because nothing better is available, soft information has to be given, it should be pointed out by the contributor that he regards this or that as soft information. Second, I hope that you will all do your best to avoid the use of misleading terminology. Evaluation and assessment can be led astray far too easily by such terminology. Third, let us not allow ourselves to be misled by over-

optimistic reasoning, attractive though such reasoning may be, for it only rarely leads to true insight and usually retards scientific progress.

The symposium title is *Strategy of the Viral Genome*, although it may be more appropriate to think in terms of several strategies. By definition, strategy is employed to win wars, tactics to win battles. Usually our research concerns problems at the tactical level, but here we are trying to obtain insight into the organization of the viral genome in the widest sense. To achieve our objective I regard it as our tasks now:

(1) To recognize the different types of information coded by virus. At one stage or another, of course, we may want to define how we decide whether or not a protein or nucleic acid is coded by a virus. But these definitions have already been made and agreed and are probably familiar to all of us.
(2) To comprehend (and this is difficult, and involves the danger of over-optimistic reasoning) why some types of information may be needed by some but not all groups of viruses.
(3) To discern an overall viral genome strategy, or a limited number of strategies for rational groupings of viruses. Here it is valueless to adopt a 'splitter' mentality and consider every virus to have a uniquely different strategy. It should prove possible to choose the approach of a 'lumper' and agree that distinctive major types of strategy are probably employed, and then to describe and enumerate these.

For example, the small lytic RNA viruses could be considered to employ one type of general strategy, the RNA tumour viruses another and the DNA tumour viruses a third. Viruses whose genome exists as several pieces, like reovirus, could represent a fourth strategy, the large DNA lytic viruses a fifth one, and the small single-stranded DNA viruses another, and so on. But is the problem that simple? Some of us may feel that this particular scheme deals inadequately with the small RNA viruses. Clearly viruses whose genome is the plus (the translatable) strand must employ a different strategy to those where the virion nucleic acid is the minus strand. These are matters I hope we shall be discussing.

Before we can evaluate strategies we must decide what is most relevant and in particular distinguish between essential and non-essential genetic information. Caution will be needed, for in the laboratory we may come to regard as non-essential some viral functions and viral genetic material which under natural conditions may be essential. For example, when we grow virus in a particular host we have no guarantee that this is the normal host

outside the laboratory. Moreover in our experiments we often infect cells under very artificial conditions—usually when they are, or have just been, growing in log phase. But in nature the initially infected cells are probably in the resting phase, when only very low amounts of many enzymes found in log phase are present. For this reason one may in many instances discover apparently unnecessary duplication between virus-coded and cell-coded macromolecules, but strategically this situation may be essential to ensure survival for the virus. In addition, what at first seems to be duplication may prove to be pseudo, as is the case with herpes virus-specified thymidine kinase which appears really to be a deoxypyrimidine kinase rather than a thymidine kinase and consequently is different from the mammalian enzyme.

It may assist our discussions if I attempt a preliminary summary of the different types of molecules for which information may be carried by a virus.

(1) The structural components of the virion. This group includes the coat proteins and nucleic acid which are part of the virion, and also the internal components and virion enzymes that ensure the particle's ability to initiate infection. (Virion enzymes never function in the same infectious cycle as the genome which coded for them, but in the succeeding one.) Some virion constituents seem to function both as structural components and as control elements, for example the fibre and the penton base of adenovirus.

(2) The non-virion proteins which function in the same infectious cycle as the genome which programmes them. This group includes many different enzymes, numerous immunologically detectable antigens whose functions may or may not be known, and also control elements.

(3) Then there are proteins of dual origin, some of whose polypeptide chains are host-specified while others are virus-specified. In the case of modifying factors the association with the host-specified protein may be quite loose.

(4) There are of course the virus-specified messenger RNA species but, more pertinent to our discussion, we now know that transfer RNA can sometimes be virus-specified.

(5) There remains a further heterogeneous group consisting of 'virus-specified molecules with unknown function' and 'potentialities of the viral genome which are not fully understood'. What for example is the function of 'VA' RNA in adenovirus-infected cells? Why does one end of turnip yellow mosaic virus RNA have valyl amino acid acceptor potentiality? Is it coincidence that the genome of small

RNA bacteriophage has between two genes the apparent potential to code for a hexapeptide? What information is at the start and at the end of the viral genome? Why is the double-stranded DNA interrupted in one strand in some viral genomes, but not in others?

Even if we do not take up many of these points I hope we will keep them in mind. Now let us start to discuss in some depth the 'Strategy of the Viral Genome'.

SOME ENZYMES SPECIFIED BY DNA PHAGES

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IN 1950 it was generally accepted that viruses had genes that determined proteins, but we thought that viral genes only controlled virus structures. These structures controlled functions such as the adsorption of a virus to a host, and some other genetically determined phenotypic expressions, such as the size and appearance of a viral plaque, were obscure enough not to need other hypotheses. Until 1952 the biochemical data had not revealed any obviously qualitative aberrations of metabolic behaviour; virus-infected cells made proteins and nucleic acids just as normal cells did, and made them in approximately similar amounts (Cohen 1947). We thought that although viral genes confiscated the then unknown intermediates of protein synthesis for the synthesis of viral structural proteins, the existing metabolic activities of the infected host were adequate for virus multiplication (Cohen 1952).

The detection in 1952 of a new pyrimidine, 5-hydroxymethylcytosine, in the DNA of the T-even bacteriophages (Wyatt and Cohen 1952) and our inability to find a comparable compound in the uninfected host, *Escherichia coli* (Wyatt and Cohen 1953), provided cause for thought. Nevertheless, any clarification of the alternatives required a knowledge of the mechanism of biosynthesis of the pyrimidines and of the origin of thymidine. This led to work with the thymineless mutant (Cohen 1953), which mysteriously lost the power to multiply when held in the absence of thymine and could not be kept alive with hydroxymethylpyrimidines (Barner and Cohen 1954). When we infected this organism with T2 or T5 in the absence of thymine, the infected cell could then make thymine (Barner and Cohen 1954, 1959). Even after this experience we merely listed numerous possible explanations of this acquisition of function, including the now well-known concept that viruses may possess genes which may direct the synthesis of totally new, metabolically significant proteins.

This idea became almost inescapable when it was shown in 1957 that the

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enzyme for the synthesis of 5-hydroxymethyldeoxycytidylate (dHMP), that is the dCMP hydroxymethylase found in T-even phage-infected cells, could not be detected in uninfected *E. coli* (Flaks and Cohen 1957; Cohen 1961, 1968). It was also shown that thymidylate synthetase (Flaks and Cohen 1957, 1959) increased sharply in infected cells. These reactions are presented in Fig. 1.

As is well known, biochemists went on to detect many virus-induced enzymes in phage-infected cells. Among the many new questions stemming from these observations were the *de novo* origin of these enzymes,

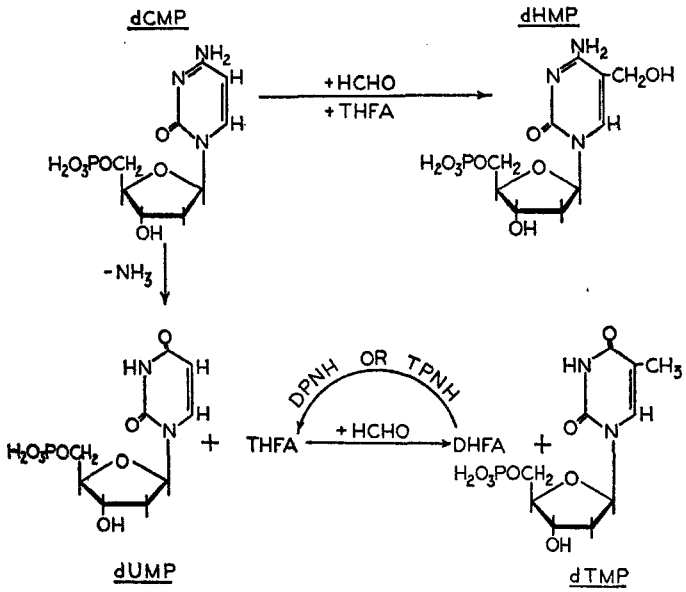

FIG. 1. Three reactions for which enzymes are induced in T-even virus infection of *Escherichia coli* (Cohen 1968).

their genetic determination by virus or host or both, their roles and essentiality in virus multiplication and the control of their elaboration. Another was whether such phenomena occurred in other systems of virus infection and it can be stated that virus-induced enzymes are to be found in most, if not all, viral systems in which RNA is the genetic determinant, and in many DNA virus systems. In some of the latter, such as φX174 and SV40, where virus-induced enzymes have not yet been detected, the number of viral genes exceeds the numbers of proteins present in the viruses and some of these genes are known to relate to essential, but as yet incompletely defined, metabolic functions. Obviously we are far from understanding many aspects of metabolism, particularly DNA replication

and membrane activities, and it is precisely these areas which are crucial to the multiplication of DNA viruses, if not of all viruses.

This change in our views on the contributions of the virus to its own multiplication suggested that viral disease might be more readily controlled by human intervention. If viruses used a pre-existing metabolic machinery exclusively it would be difficult to block viral multiplication without damaging the host cells generally. However, if viruses caused the appearance of new enzymic apparatus in infected cells, a carefully tailored

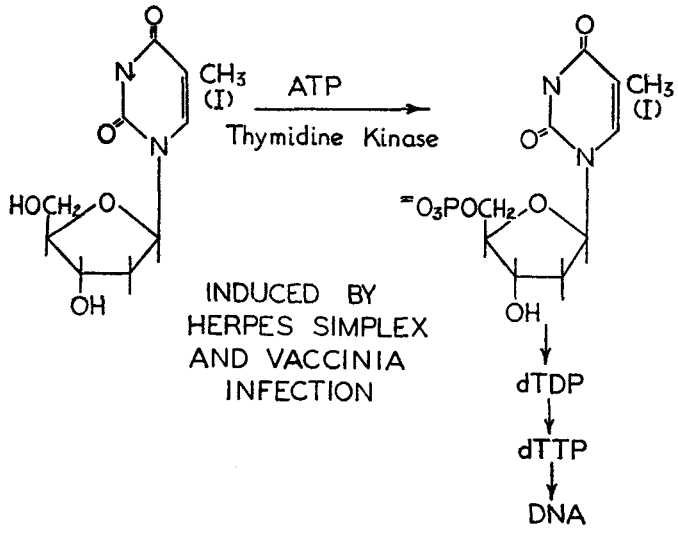

FIG. 2. The phosphorylation of 5-iododeoxyuridine by thymidine kinase in animal cells infected by herpes simplex virus or vaccinia virus.

chemical might jam such new enzymes selectively without damaging uninfected cells.

Although this possibility has not been pursued explicitly, we can now indicate two practical consequences of this advance in virology. The first is the clinical use of 5-iododeoxyuridine in herpetic keratitis, which takes advantage of the increase in thymidine kinase found in herpes virus infections of corneal cells. As shown in Fig. 2, this enzyme, which is believed by Buchan and co-workers (1970) to be coded by the DNA of herpes virus, phosphorylates the nucleoside to a nucleotide which enters virus DNA and affects its ability to sustain virus multiplication. Uninfected corneal cells have a very low rate of incorporation of iododeoxyuridine into DNA. The problems that arise in proving rigorously that an enzyme that increases during infection is virus-determined will be considered briefly below.

The second consequence is the discovery of an RNA-dependent DNA polymerase in RNA tumour viruses, which is being used to seek the presence of a virus as the aetiological agent in human leukaemia. That such an enzyme is present and may have differential sensitivity to some rifampicin derivatives has been recently reported by Gallo, Yang and Ting (1970). Thus, these results too suggest that the phenomenon of virus-induced enzymes, whose study started with the discovery of a new and still quite rare compound in a quite unusual phage, must be considered as a possible approach to the control of virus disease generally, as well as providing insights into the nature of viruses and genes, and their multiplication, control and expression.

That the biological phenomenon will be useful in clarifying the metabolic systems themselves is already evident and I shall discuss some areas, such as the role of membranes, in which this clarification must take place before virology itself can advance.

VIRUS-INDUCED ACQUISITION OF ENZYME FUNCTION AND ITS *DE NOVO* SYNTHESIS

After the discovery of dCMP hydroxymethylase and clarification of the reaction it catalyses, we showed that the enzyme appears early in infection in conditions requiring protein synthesis (Flaks, Lichtenstein and Cohen 1959). We investigated whether this meant that the enzyme was synthesized entirely *de novo* after infection, that is, whether the entire polypeptide chain (or chains) was assembled without a contribution from polypeptides of the uninfected host cell. This task of purifying the enzyme from prelabelled infected cells took about four years (Pizer and Cohen 1962; Mathews, Brown and Cohen 1964) and we were able to show that all the amino acids of the virus-induced enzyme were assembled after infection.

While this work was in progress, leading to a partial purification of the enzyme and the determination of some kinetic parameters and its molecular weight (Pizer and Cohen 1962), Wiberg and co-workers (1962) found mutants of the virus deficient in production of the hydroxymethylase under restrictive conditions. This indicated that a viral gene controlled the production of the enzyme in some measure. Nevertheless, at least two other hypotheses are possible, even if a viral gene controls the appearance of an enzyme.

(1) The enzyme is associated with a non-dissociable moiety that inactivates the catalytic function. Protein synthesis after infection might produce a totally different enzyme which removes the inhibitory fragment, as in the conversion of pepsinogen to pepsin.

(2) The enzyme is incomplete before infection. It consists of an inactive polypeptide precursor to which is added, by protein synthesis, a completing and activating fragment.

Both alternatives, although presented on the assumption that there is one polypeptide per enzyme molecule, can readily be modified to indicate that our new enzyme is made up of essential subunits. Although no evidence has been obtained yet for the first hypothesis, three examples of the second possibility have been demonstrated recently, that is, the appearance of virus-induced activities has been shown to involve the addition after infection of newly synthesized protein to pre-existing host proteins. In each example the rigorous demonstration of the existence of an enzyme with several subunits has necessitated extensive and careful enzyme purification, with all the tedium and manual labour such work implies.

In the first example, infection of *E. coli* with T4 resulted in the appearance of a new valyl-tRNA synthetase. The new activity is associated with a molecule which, compared to the host enzyme, is more heat- and urea-resistant and has a larger volume and higher sedimentation rate. Appearance of the new activity necessitates protein synthesis and phage mutants exist that cannot produce this new activity, which is not essential for normal phage production (Neidhardt *et al.* 1969). When the enzyme from deuterium-labelled cells was sedimented in caesium chloride at least 90 per cent of the phage-induced enzyme was formed from polypeptide chains present in the cell before infection (Chrispeels *et al.* 1968). It has now been demonstrated that the new highly purified valyl-tRNA synthetase consists of the host protein (mol. wt. 100 000) and a new virus-induced protein (mol. wt. 10 000) (Marchin and Neidhardt 1970).

In recent, somewhat comparable discoveries (Kondo, Gallerani and Weissmann 1970; Kamen 1970), it was shown that the replicase induced by the RNA phage, Qβ, consists of one phage-specific and three host-specific polypeptide chains. Although at least one of the latter is essential for enzymic activity, none are known subunits of DNA-dependent RNA polymerase of *E. coli*. Nevertheless the recognition that these host subunits are present in the virus-induced enzyme has recently led to some insights into the transcription of normal ribosomal genes in *E. coli* DNA.

According to Stevens (1970), the core enzyme of DNA-dependent RNA polymerase of *E. coli*, consisting of α, β, and ω after removal of σ, is altered after T4 infection. Isotopic amino acids appear uniquely in a new 'ω' subunit, which has a molecular weight of 8–10 000, and concomitantly the normal ω subunit is lost from the core enzyme. The functional significance of this replacement is unclear.

SYNTHESIS OF VIRUS-INDUCED ENZYMES *IN VITRO*

As Fig. 3 shows, in addition to the synthesis in T-even phage systems of one or more groups of early enzymes which catalyse reactions essential for DNA synthesis, there is a later synthesis of other proteins, many of which are involved in phage structure and assembly (Cohen 1968). Some of these events have recently been produced in cell-free systems. At the same time our knowledge of the mechanisms of protein synthesis has advanced—knowledge which has been both derived from phage systems and used to explore these systems. For example, the study of transcription on T4 DNA

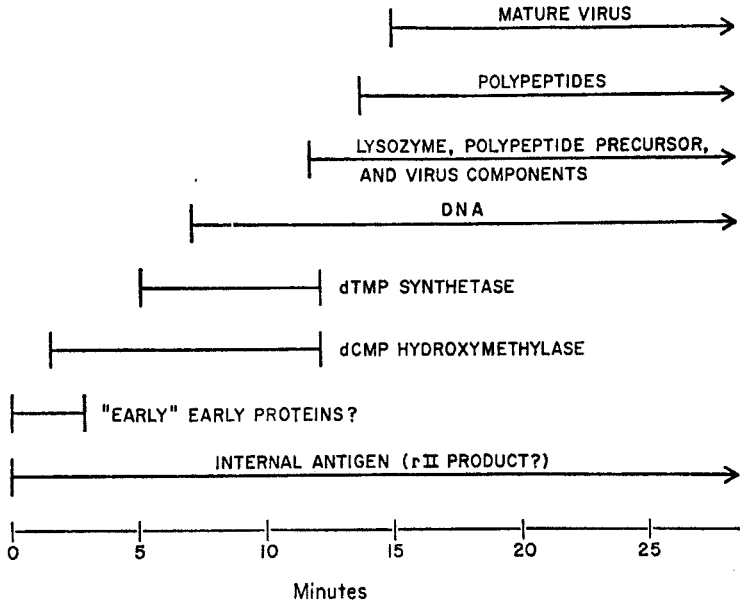

FIG. 3. The timing and sequence of early proteins, DNA and late proteins in T-even r^+ phage infection of *E. coli* strain B in a glucose-mineral medium at 37°C (Cohen 1968).

led to the detection of sigma factors in normal and phage-infected cells (Walter, Seifert and Zillig 1968; Bautz, Bautz and Dunn 1969; Travers and Burgess 1969) and has in turn led to questions about the role of such factors in the control of the different times of appearance of the virus-induced proteins. Study of the structure of tRNA led Sueoka and Kano-Sueoka (1964) and Hsu, Foft and Weiss (1967) to detect new species of tRNA in T4 and T5 infection. The role of these new tRNA species in the control of virus multiplication is far from clear. A sharp change in the composition of ribosomes after infection was shown in an isolated system. This change may help to explain the arrest of synthesis of host proteins

after T-even phage infection. The exclusion of MS2 by infection with T-even phage is also explained by inhibition of the formation of initiation complexes with *E. coli* RNA and MS2 RNA (Klem, Hsu and Weiss 1970).

Since almost every part of the transcription and translation systems is altered in some respect by infection, the fact that it is possible to make both early and late T4-induced proteins *in vitro* takes on a heightened significance. Presumably an adroit exploitation of these systems should tell us something of the roles of the new proteins and tRNA's in transcription and translation. The complexity of these interactions between new and old metabolic machinery during infection is underlined by the continued use of host mechanisms in infection. These include unchanged α and β subunits of RNA polymerase (Stevens 1970), the gross conservation of ribosomes (Cohen 1947; Brenner, Jacob and Meselson 1961), the continuous requirement for a given ribosomal protein during infection (Cafferata and Haselkorn 1970), and the requirement for formylmethionine in protein initiation (Miovic and Pizer 1968).

Salser, Gesteland and Bolle (1967) have demonstrated the synthesis of T4 lysozyme and head protein peptides in a cell-free system derived from uninfected cells programmed by RNA isolated from infected cells. Despite the elegance of this and subsequent studies, a role for new phage-induced tRNA contained in the RNA used in these syntheses has not been clearly excluded in these experiments or in other *in vitro* studies of protein synthesis (Brawerman *et al.* 1969; Coolsma and Haselkorn 1969; Schweiger and Gold 1969; Gold and Schweiger 1970).

The potential value of the *in vitro* approach is suggested by the combined use of T4 DNA and RNA- and protein-synthesizing systems from uninfected cells to study transcription and translation, leading to the production of both early enzymes (α- and β-glycosyl transferases) and late proteins (lysozyme). At 11 mM-Mg^{++} both groups of proteins were produced in an order similar to that observed in an *in vivo* system. However it could be shown that the delay in DNA-dependent lysozyme synthesis was removed by increasing the magnesium ion concentration from 11 mM-Mg^{++} to 15 mM-Mg^{++} (Gold and Schweiger 1970). Furthermore, the increase in Mg^{++} concentration markedly reduced the production of glucosyl transferase.

But has such a result any significance for the control of lysozyme production *in vivo*? That is, can lysozyme production *in vivo* in fact be switched on by the development of an appropriate intracellular concentration of certain cations? The *e* gene for lysozyme is contiguous with early genes and appears to be transcribed early because of inadequate

termination (Schmidt *et al.* 1970), but will this result also apply to truly late genes, namely those whose RNA products are initiated late, after the activity of gene 55 is expressed (Pulitzer and Geiduschek 1970)? It is obviously easier to explore a catalytic protein than a structural protein but why have *in vitro* studies concentrated almost exclusively on lysozyme, the mRNA of which has long been known to be transcribed early (Bautz *et al.* 1966)? Surely a number of other catalytic proteins can be described as truly late functions. These may be found both in the structural proteins themselves, for example the dATPase of sheath subunits, and in enzymes essential to virus assembly.

DETECTION OF CATALYTIC AND STOICHIOMETRIC FUNCTIONS

An ingenious study of the dominance of wild-type genes over amber alleles in T4 (Snustad 1968) has revealed that some otherwise undefined genes involved in T4 morphogenesis control the synthesis of catalytic

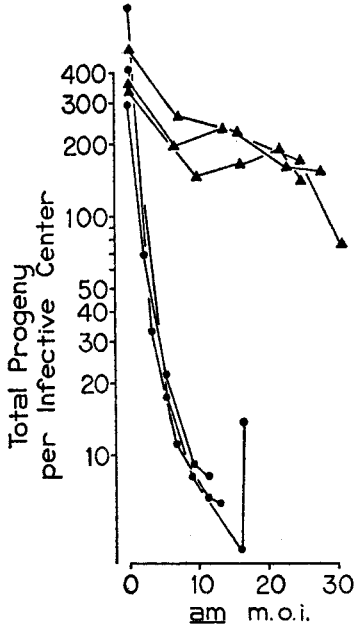

FIG. 4. The effect of varying the *am*/wild-type input ratio on burst size (Snustad 1968). Since the wild-type multiplicity of infection (m.o.i) is constant (0·5), the *am*/wild-type input ratio in the potential yielders (wild-type infected cells) is essentially equal to the *am* m.o.i. ▲ = *am* B24 (gene 1); ● = *am* B270 (gene 22).

functions. Snustad has postulated that the catalytic product of a single wild-type gene in the presence of many amber alleles will still be sufficient to maintain a large burst size in a mixed infection of wild-type and amber mutants, whereas a product itself used in virus structure, that is, a stoichiometric function derived from a single wild-type gene, would not permit the

development of many progeny. By this test, we clearly have many genes coding for unknown products which are presumed to have a catalytic function (Table I). Are there any exceptions to this? In Fig. 4 the slow

TABLE I

ANALYSES OF AMBER GENES (SNUSTAD 1968)

Gene	*Response*	*Known gene-product or mutant phenotype description*
	(A) *Genes coding for known products*	
1	Catalytic	Deoxyribonucleoside monophosphate kinase
30	Catalytic	Polynucleotide ligase
42	Catalytic	dCMP hydroxy-methylase
43	Catalytic	DNA polymerase
56	Catalytic	dCTPase-dUTP
24	Stoichiometric	Minor head component
22		
23	Stoichiometric	Major head component
18	Stoichiometric	Major tail-sheath component
34	Stoichiometric	Tail-fibre components
36		
37		
	(B) *Genes coding for unknown products*	
33	Catalytic	Maturation defective
55		
44	Catalytic	DO
45		
41	Catalytic	DS
62	Catalytic	
32	Stoichiometric	
46	Catalytic	DA
47	Catalytic	
59	Catalytic	
39	Catalytic	DD
52		
58, 61		
60		
63+?	Catalytic	DD+'labile factor'
31	Catalytic	Head morphogenesis
20	Stoichiometric	
21	Stoichiometric	
	(B) *Genes coding for unknown products (contd.)*	
38	Catalytic	Tail-fibre morphogenesis
35	Stoichiometric	
57	Stoichiometric	
26	Catalytic	Tail morphogenesis
28	Catalytic?	
51	Catalytic	
10	Ambiguous	
7	Intermediate	
5	Stoichiometric	
6	Stoichiometric	
8	Stoichiometric	
25	Stoichiometric	
27	Stoichiometric	
29	Stoichiometric	
48	Stoichiometric	
53	Stoichiometric	
54	Stoichiometric	
4	Catalytic	Head completion, tail completion, joining heads to tails, and particle stabilization
50	Catalytic	
65	Catalytic	
2	Stoichiometric	
9	Stoichiometric	
11	Stoichiometric	
12	Stoichiometric	
13	Stoichiometric	
14	Stoichiometric	
15	Stoichiometric	
16	Stoichiometric	
17	Stoichiometric	
49	Stoichiometric	
64	Stoichiometric	

DO=no DNA synthesis; DS=some DNA synthesis; DA=arrested DNA synthesis; DD=delayed DNA synthesis.

decrease of burst size with decreasing input of gene 1, controlling the catalytic deoxyribonucleoside monophosphate kinase, can be contrasted with the rapid decrease with decreasing input of gene 22, controlling a stoichiometrically required minor head protein.

After a review of the literature on T-even phage polypeptides and structural proteins (Cohen 1968), I had suggested that the major head protein, the gene 23 product, as it appears in phage might be different in size from that synthesized initially. Furthermore, many facts suggested that a proteinase was at work, possibly the product of gene 31 which has been implicated both in the production of phage polypeptides and the solubilization of head proteins. Several recent papers have in fact demonstrated the reduction in molecular weight of the gene 23 product before it is packaged into phage (Hosoda and Cone 1970; Laemmli *et al.* 1970; Dickson, Barnes and Eiserling 1970). As a result of this method of analysis Snustad has designated (see Table I) gene 31 as controlling a catalytic product. At least 17 such genes designate catalytic products; their precise activities are still unknown. Many of these ill-defined activities are very much concerned with functions of phage assembly but others appear to relate to relatively early essential metabolic functions.

Do any of these relate to genes controlling σ, ω, tRNA, etc., or are these products not essential for phage multiplication? If the latter, these genes could not be analysed by Snustad's method because they do not give rise to amber mutants. Of course, many genes giving rise to apparently inessential catalytic functions have been detected. Such genes designate enzymes such as thymidylate synthetase, valyl-tRNA synthetase and dihydrofolate reductase (Mathews and Cohen 1963). The dihydrofolate reductase is particularly interesting and puzzling since it is a structural component of the T4 phage tail plate (Kozloff *et al.* 1970), as well as playing a role in the maintenance of thymine synthesis and such functions as protein initiation. Of course the dihydrofolate reductase-deficient mutants described as *wh* may produce and contain an inactive enzyme still capable of serving as structural protein in the tail plate. In this case there may well be amber mutants, deficient in tail assembly and defined as controlling stoichiometric functions, which should nevertheless be examined for deficiency in the ability to induce dihydrofolate reductase. We should then examine assembly-deficient amber mutants whose genes have been located near the *td* locus. (Mutants in gene 63, which is located close to this site and controls an early catalytic protein implicated in tail attachment, should be examined for precisely this deficiency.)

MEMBRANE FUNCTIONS

As work has developed in phage multiplication, the chemical problems have become ever more varied and sophisticated. From the elementary exploration of nucleic acids and proteins with a technology limited to some

simple colour reactions, a Kjehldahl estimation and the mere incorporation of one or another radioactive isotope, every area of intermediary metabolism has now been reached. The ultimate complexity has now been attained and lipids and membranes must be explored, an activity which may even require the redistillation of solvents.

PM2, a bacteriophage which infects a marine pseudomonad, not only contains a double-stranded circular DNA, but is also coated by lipid. It appears that phosphatidylethanolamine is the only phosphatide present in the phage and that all this lipid was made in the bacterium before infection (Espejo and Canelo 1968). Since bacterial phospholipids are concentrated in membranes, it has been inferred that multiplication and assembly are very much associated with the bacterial membrane. Indeed studies on the physiology of multiplication of this virus (Datta and Franklin 1969) have indicated that marked changes in membrane-bound enzymes and proteins during infection lead to decreases in respiration, oxidative phosphorylation, etc.—systems which are preserved in T-even phage infections. Datta and Franklin (1969) suggested that ". . . virus-specific proteins are inserted into the cellular membrane; this may or may not be accompanied by the displacement of some normal cellular membrane proteins. The altered cellular membranes, containing virus-specific proteins, may then form the outer shell of the virion." In this more manageable system we may have a tool useful in understanding some steps in infections by some animal viruses, such as myxoviruses and some RNA tumour viruses.

Although the T-even phages are not coated with lipid, much evidence has now accumulated to suggest that multiplication of these and other viruses is also associated with the bacterial membrane. According to Simon (1969), intracellular phages are attached to the bacterial membranes by thin fibres (short-tail fibres) extending from their base plate and it appears that the terminal assembly of completed phage occurs at this step. The gene 12 product involved in associating phage and membrane is bound in its entirety to the membrane. That other stages of synthesis and assembly involve the membrane is suggested by the following findings:

(*a*) Parental virus DNA (T4, λ and φX174) becomes associated with the bacterial membrane after infection (Earhart *et al.* 1968; Levine, Levine and Nisman 1969; Knippers and Sinsheimer 1968; Levine and Sinsheimer 1969).

(*b*) A membrane-bound DNA polymerase and some newly synthesized proteins in the membrane have been detected after infection by T4

(Frankel *et al.* 1968). Some of these proteins will perhaps prove identical to those found by other techniques to be associated with replicating viral DNA (Miller and Kozinski 1970; Miller and Buckley 1970; Alberts 1970).

(*c*) In infection by gene 31 mutants of T4, the protein designated by gene 23 is bound to the cell envelope (Kellenberger, Eiserling and Boy de la Tour 1968).

(*d*) Canavanine-treated cells accumulate altered proteins at the membrane and become incapable of synthesis of T-even phage mRNA (Schachtele, Anderson and Rogers 1968).

In the latter instance the authors propose "... that there are a limited number of specialized membrane sites in the bacterium at which DNA replication is organized and that detachment from or jamming of the genome at these sites stops transcription." The existence of such an essential bacterial site has also been postulated in the multiplication of φX174 as the site of association of the parentally-derived replicative form of the virus DNA (Stone 1967; Yarus and Sinsheimer 1967; Lindquist and Sinsheimer 1968). That the bacterial membrane is considered to fulfil comparable functions for bacterial DNA replication and transcription is no longer an odd notion.

It may also be useful to indicate here some of the relations of the membrane to phenomena of penetration, permeability and lysis. Of great interest for several decades has been the early arrest of synthesis of host polymers during T-even infection. As indicated above (p. 11), host ribosomes are modified by virus-induced proteins to prevent formation of initiation complexes with host mRNA (Klem, Hsu and Weiss 1970). Nevertheless the fact that phage ghosts devoid of DNA also arrest host synthesis has suggested an effect comparable in many respects to the effects of colicin E1, which alters the membrane and affects energy metabolism by some unknown mechanism (Luria 1970). Although a phospholipase activity has been reported in some colicins including E1 (Cavard *et al.* 1968), no one has ever assayed for such activity in phage ghosts or for changes in phospholipids after attachment of ghosts to bacteria.

The activities of phage ghosts have recently been summarized in some detail by Duckworth (1970). As described in that review, the apparently reversible metabolic arrest may be explained by effects in which the bacterial chromosome is separated from the membrane, as a result of rapid osmotic changes (Cohen 1968) or by allosteric changes in the membrane (Changeux and Thiéry 1967). The irreversible changes instituted by

whole phage appear to be provoked by the proteins induced after insertion of viral DNA, and perhaps to some extent by enzymes or other substances associated with phage DNA, as in recent unpublished reports of lysozyme as an internal protein and the presence of an endonuclease in the T4 capsids. It is not yet clear whether the lytic activity other than lysozyme detected by Emrich and Streisinger (1968) and by Yamazaki (1969) is present in ghosts, i.e. in the outer virus structure, or is injected with the DNA. It may be mentioned that although exogenous spermidine inhibits lysis by ghosts (Buller and Astrachan 1968) it does not prevent inhibition of induced enzyme synthesis by such particles (Duckworth 1970).

Since *e* gene mutant (lysozyme-deficient) phages are infectious, lysozyme is not essential for penetration. Lysis to release internal virus may be effected by exogenous lysozyme. Whether the new enzyme detected by Yamazaki (1969) on virus particles fulfils a role in penetration is not known.

Transient leaks in cells and their repair after infection have been known for some time and it has been inferred that a membrane-repair mechanism exists. Thus the premature lysis of K12 (λ) by *rII* mutants in minimal media is believed to relate to unrepaired damage to the membrane; this can be forestalled or possibly repaired in the presence of suitable concentrations of Mg^{++} or spermidine.

Other T-even phage genes, *pr* and *q*, control resistance to acridine dyes such as proflavine and quinacrine, respectively, and affect the penetrability of the membrane to these compounds. The normal *pr* gene product appears to increase membrane penetrability, whereas infection with *pr* phage mutants reduces sensitivity to proflavine, perhaps permitting the completion of normal repair. It is possible that repair is effected by host enzymes.

It has been shown that lysis is provoked by an arrest of respiration, which is a membrane function. This effect on respiration precedes a marked terminal increase in membrane permeability which permits the emergence of lysozyme to destroy the encircling corset of bacterial cell wall (Mukai, Streisinger and Miller 1967; Séchaud, Kellenberger and Streisinger 1967). Nevertheless, lysis can occur even in the absence of lysozyme when infection is effected by a phage mutant in the *s* gene which appears to control repair of the bacterial cell wall (Emrich 1968).

The degradation of the bacterial membrane to permit release of lysozyme to act upon the wall is under the control of a phage gene, *t*, mutation of which results in lysis-defective mutants (Josslin 1970). The nature of this effect is not yet clear, but perhaps some insight into all these problems is provided by recent studies on phospholipid metabolism in infection.

PHOSPHOLIPID METABOLISM IN INFECTION

Infection by T4 phage permits the continuing synthesis of phospholipids similar to those of the uninfected host, albeit at a somewhat reduced rate (Furrow and Pizer 1968). However, the relative rates of incorporation of ^{32}P into phosphatidyl glycerol (PG) and phosphatidylethanolamine (PE) are changed. The ratio of PG to PE shifts fairly early in infection from 0·35 in the uninfected cells to 0·90 in infected cells. This change, which may be related to resealing of the membrane, is prevented by chloramphenicol.

Although r^+ and r phage show slight differences in their overall rates of phospholipid synthesis, no effects have been detected in the relative rates of synthesis of these major phospholipids. Nevertheless it has been reported that *rII* mutants cause an increased relative rate of ^{32}P incorporation into cardiolipin (see Buller and Astrachan 1968). A similar effect on cardiolipin synthesis leading to severe membrane damage, arrest of macromolecular synthesis and lysis is provoked specifically by oleate starvation in *E. coli* K12 strains requiring this fatty acid for growth (Henning *et al.* 1969). Similar changes in phospholipid synthesis are also provoked by cyanide or partial anaerobiosis (Peterson and Buller 1969).

In extending these results with T2 and T6, Rampini (1969) has observed an increase in lysophosphatidylethanolamine and cardiolipin about 15 minutes after infection. Phospholipase A, an enzyme which converts PE to its lyso-derivative and liberates free fatty acid, has been detected in *E. coli* (Okuyama and Nojima 1969). Indeed a marked increase in free fatty acid derived from cellular phospholipid begins to appear 10–13 minutes after infection by T4 and continues until lysis is complete (Cronan and Wulff 1969). At this point, 10–15 per cent of the cell lipid has been converted to free fatty acid, with a 50-fold increase over the amount of free fatty acid in uninfected cells. Lysophosphatide is also degraded and appears to lose its remaining fatty acid. This effect is reported to need protein synthesis and is inhibited transiently under conditions in which lysis is inhibited. Thus at least two toxic substances, free fatty acid and a lysophosphatide (the latter in small amounts), are generated immediately before lysis. The interrelations of these substances, the synthesis of cardiolipin, anaerobiosis and membrane damage obviously present difficulties in assigning primary causal roles in the provocation of lysis. Nevertheless it appears that the membrane and wall are both participating actively in dissolution and repair throughout infection. These reactions are maintained in precarious balance until metabolic arrest and lysis, a balance which may be effected by products controlled by virus genes *rII*, *e*, *s*, *t*, *pr* and *q*, as well as by cell enzymes which include the relatively unexplored *E. coli*