demonstrated a shielding effect of the ribosome on nascent proteins; ribosome-bound nascent chains 30–35 residues long were found to be protected from external proteolytic attack. Protamine, 31–32 residues long, may therefore be protected from attack by a putative methionine-removing enzyme until the chain is completed and released into the cytoplasm. Other contributory circumstances may be the difficulty of cleavage of the Met–Pro peptide bond; in general, X–Pro bonds are resistant to digestion, but this enzyme is specific only for dipeptides. Also, as shown by Ling and Dixon25, at the stage of differentiation at which protamine synthesis is maximal, there has been a considerable reduction of the spermatid cytoplasm and ribosome content. The levels of cytoplasmic enzymes, including the methionine-removing enzyme, may therefore become limiting at this stage.

The involvement of methionine in the initiation of protein synthesis in eukaryotic cells is not yet firmly established although the presence of a formylatable species of Met-tRNA with the properties of an initiator tRNA in mouse ascites cells11,12 is certainly consistent with such a role. Our observations of methionine involvement in the synthesis of the very unusual, sperm-specific polypeptide, protamine, suggest a mechanism where removal of the N-terminal methionyl residue may become limiting in the transient incorporation of this amino-acid is readily observed.

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Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4

by

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BACTERIOPHAGES of the T-even type are complex structures containing many different proteins and specified by many genes. Using an improved technique of gel electrophoresis, many hitherto unknown proteins have been found in bacteriophage T4 and some of these have been identified with specific gene products. Four major components of the head are cleaved during the process of assembly, apparently after the precursor proteins have assembled into some large intermediate structure.

Using an improved method of gel electrophoresis, many hitherto unknown proteins have been found in bacteriophage T4 and some of these have been identified with specific gene products. Four major components of the head are cleaved during the process of assembly, apparently after the precursor proteins have assembled into some large intermediate structure.

Bacteriophage T4 is therefore no longer a self-assembly system in the narrow sense, because the bonding properties of the various components become altered during the assembly process.

The product of gene 22, the proteins of genes 20, 21, 22, 24, 31, 40 and 66 are required to determine the size and shape of the head-shell (refs. 4 and 5 and unpublished work of P. A. Eislering, E. P. Geiduschek, R. H. Epstein and E. J. Metter). To several of these genes shape-specifying functions have been tentatively assigned24. Gene 22 is associated with the formation of the hemispherical capsheath. Gene 31 somehow modifies or activates the major capsid protein. Gene 66 with the elongation of the particle and gene 40 with the formation of the hemispherical capsheath.

Structural Components of the Phage

Many phage proteins can be separated with our improved method of disk-electrophoresis in sodium dodecyl sulphate (SDS). This system, to be described in detail elsewhere (U. K. L. and J. V. Maizel), combines the high resolution power of disk-electrophoreses25 with the capability of SDS to break down proteins into their individual polypeptide chains26. The proteins are also separated according to their molecular weight; the 48 was first reported for a continuous system27. All the proteins the genetic
grated into the particle. The pattern of a total lysate of wild type infected proteins with molecular weights less than about 50,000 also shows a head to tail junction. "Early labelled" phages were prepared identically, but the label was added 1 min and chased 6 min after infection by the addition of 3 ml of 3 per cent 'casamino acids'.

"Ghosted" phages and head particles. Ten ml. cultures were grown and infected as described above ("Labelled lysates") but infected with 15 ml. of a 6/18 infected suspension containing 500,000 particles per ml. The infected cultures were concentrated by low speed centrifugation 35 min after infection and the pellets were resuspended in 1 ml. neutral phosphate buffer containing 10-3 M MgSO4, 20 µg deoxyribonuclease and a drop of chloroform. They were resuspended by repeated pipetting, and incubated for 15-30 min at room temperature before layering on a CsCl gradient. Ghosts were then prepared by the Spinco 'SW25.2 rotor' with 0.8 ml. layers, and the following solution (in neutral phosphate buffer and 10-3 M MgSO4) was layered on the last CsCl step to prevent precipitation of soluble proteins at the CsCl interfaces:

\[ \text{CsCl} \times \text{water} + 0.1 \% \text{SDS} \]

The samples (0.2-0.3 ml.) contained the final concentrations ("final sample buffer"):

\[ \begin{align*}
0.0625 \text{ M Tris-Cl} \\
0.192 \text{ M glycine} \\
0.1 \% \text{SDS}
\end{align*} \]

The proteins were fixed in the gel with 50 per cent trichloroacetic acid (TCA) overnight, stained for 1 h at 37° C with a 0.1 per cent Coomassie brilliant blue solution made up freshly in 50 per cent TCA. The gels were destained by repeated washing in 7 per cent acetic acid. Autordiagrams of gels were prepared by a modified version (U. K. L. and J. V. Maizel, unpublished) of Fairbanks et al.'s (autodiagrams are shown in Figs. 1-7).
separation of the released proteins and the ghosted particles by centrifugation was not complete. Some of these proteins, however, are extracted into the supernatant quantitatively by the freezing and thawing procedure.

Only the protein IP*, and perhaps some low molecular weight proteins which migrate with the marker dye, are structural phage components synthesized at early times (early proteins). This can be seen in the gel pattern of purified phage labelled at early times (Fig. 1). IP* is also labelled at late times. All the other structural phage components are synthesized only late in infection. Some of the principal late proteins show up on the autoradiogram, probably because of residual incorporation of radioactive amino-acids following the chase with unlabelled amino-acids.

Identification of Gene Products

The products of genes 18, 19, 20, 22, 23 and 24 were identified by comparing the gel pattern of extracts of cells infected with wild type phage with those infected with amber mutants in various genes. The identification of the tail and tail fibre proteins will be described later (J. King and U. K. L.). Amber mutations produce only fragments of the protein chain of the mutant genes on infection of restrictive bacteria1. These fragments migrate differently in the gel from the wild type protein. The molecular weights of the proteins identified are listed in Table 1. Fig. 2 is the autoradiogram from dried and sliced gels for various mutants. The product of gene 20 is identified by its absence in the gel pattern of a 20-defective lysate (Figs. 2 and 3a), and the product of gene 22 by its absence in the gel pattern of a 22-defective lysate (Figs. 2 and 3b). Note the amber fragment of mutant B270 in gene 22, This fragment was also identified by Hosoda and Levitt. The product of gene 23 is easily identified by its absence in the gel pattern of a 23-defective lysate (Fig. 2). It can also be seen that P23 overlaps with two minor tail components. If the 23-defective lysate is analysed on gels of lower acrylamide concentration another important observation is made. A band, P23*, which is detectable in variable amounts in the outer head defective lysates is completely missing in the 23-defective lysate (Fig. 3a). This band, P23*, overlaps with P24 in Fig. 2, but is better separated from P24 in less concentrated gels (Fig. 3b). As with the product P23, the product of P24 was identified by its absence in the gel pattern of a 24-defective lysate (Figs. 2f and 3e).

So far, no missing bands have been found in the gel patterns of 31 or 21-defective lysates (Fig. 2e and f), but analysis of the 21-defective lysate on gels of lower acrylamide concentration (Fig. 3b), which resolves higher molecular weight proteins better, clearly shows that a band is missing. This protein, however, is the product of gene 10, a baseplate gene. The mutant N90 in gene 10 in fact carries a second mutation in gene 10 (mutant B265).

In comparing the gel pattern of the head-defective lysates (Fig. 2a–f) with that of wild type (Fig. 2g), further important differences are observed, which shed light on the precursor-product relationship of the head components.

The principal fraction of the gene 23 product has a molecular weight of 56,000 in all the head defective lysates, but in wild type or tail-defective lysates it is almost twice the size of P23 (Fig. 3a). The position of P23*, with a molecular weight of 48,000, Small but significant amounts of P23* are also observed in head-defective lysates (Figs. 3a–f). In lysates prepared identically about 20 per cent of the total P23 is converted to P23* in the 20-defective lysate, 10 per cent in 21 and 2-3 per cent in 22, 24 and 31-defective lysates, as determined from densitometer tracings of the autoradiographs.

The bands P22 and IP are both absent or considerably less intense in the wild type gel pattern (each overplus with two other proteins). A new band, IP*, is seen at the bottom of the gel, which is completely missing in the head defective lysates (Fig. 2a–f).

The band P24, which is found in all head-defective lysates is missing from the wild type pattern, but a new band, P24*, which migrates slightly faster is observed. This is difficult to visualize in Fig. 2, but will become evident in Fig. 6.

Also included in Fig. 2 is the gel pattern of two tail defective lysates. The product of gene 18 (mol. wt 69,000), the principal protein of the tail sheath15, is identified by its absence in the 18-defective lysate (Fig. 2a) and the product of gene 19 (mol. wt 18,000) by its absence in a 19-defective lysate (Fig. 2c). P19 is thought to be the chief component of the tail tube14. This demonstrates that the differences in the head proteins are not related to tail attachment, for the gel patterns of the tail-defective lysates are identical with that of wild type.

Evidence will be presented that the proteins P23, P24, P23 and IP are cleared from infected cells and are precursors to proteins P23*, P24* and IP* found in the final head structure. (The cleavage product of P24 was not detected.) This precursor-product conversion is strongly inhibited by mutations in genes 20, 21, 22, 23, 24 and 31. Two important conclusions can be drawn:

(a) the aberrant head-related structures—single and free

* Henceforth, lysates of cells infected at restrictive conditions with amber mutants in various genes will be referred to as, for instance, a "21-defective lysate", where the amber mutant used was in gene 21.
multi-layered polyheads and \( \tau \)-particles and lumps known to be produced in these mutant infected cells—clearly consist of the precursor protein P23; \( \beta \) because P22 is not cleaved in these mutant infected cells, but is required for polyhead and \( \tau \)-particle formation, as has been established by genetic means; it is strongly suggested that P22 is incorporated into these structures as such.

Kinetics of the Cleavage Reactions

The following experiments were designed to study the precursor relationship of the proteins P23, P24 and IP with P23*, P24* and IP*, respectively. In this experiment the infected cells were pulse-labelled with radioactive amino-acids for a short period (1 min) and the modification of the various proteins was then followed by analysis of the samples taken at intervals in SDS gels. The results of autoradiography of the dried gels are presented in Fig. 4.

Cleavage of P23. It is readily seen in Fig. 4 that most of the P23 protein is at the position of P23 immediately following the pulse of the radioactive amino-acids. It then rapidly disappears, and a new band, P23*, appears simultaneously. That P23 is cleaved and gives rise to P23* is suggested by the fact that both are principal components and this is reinforced by the absence of P23 and P23* from the gel patterns of a 23 defective lysate (Fig. 5d). The kinetics of the cleavage reaction P23→P23* are plotted in Fig. 5a. Cleavage is very rapid: about 50 per cent of the precursor is cleaved within the first 2 min following chase of the label. P23* appears at about the same rate, in a satisfactorily correlated way. The total labelled protein in P23 and P23* is also plotted in Fig. 5a. The total label increases during the first minute, which reflects the completion time of the chase of the labelled amino-acid, but finally the total falls off by 50–30 per cent. This final decrease of the total labelled protein can be nicely explained, for a cleavage from about 60,000 to 46,500 corresponds to a loss of about 30 per cent by weight of protein.

Cleavage of P22 and IP. In the pulse-chase experiment of Fig. 4, two other protein bands, P22 and IP, disappear with time. (The disappearance of P22 in wild type infected cells has also been observed by M. Showe, personal communication.) A new band, IP*, appears at the bottom of the gel pattern. The kinetics of these cleavage reactions are plotted in Fig. 5b. P22 disappears with approximately the same initial rate as P23; about 50 per cent is cleaved 2–3 min following chase of the labelled amino-acids. I have not found a band in the gel pattern which may be derived from P22. Hosoda and Levintal reported indirect evidence that P22 is a structural phage component, but they considered the possibility that P22 might become altered during head formation. Evidence for the precursor-product conversion IP→IP* is provided by the observation that the disappearance of IP and the appearance of IP* is coordinated in time (Fig. 5b). Furthermore, the total label lost at the IP position is recovered in the IP* band. The reaction is slower than that of P23 and P22. Only about 50 per cent of the total label at the IP position disappears. This could be explained either by another protein band overlapping with the IP band or synthesis in excess. IP* cannot arise from P22. IP* must be derived from a precursor which is synthesized early, for I have shown (Fig. 1) that IP* is strongly labelled in the "early labelled" phage preparation. P22, however, is reported to be synthesized at late times only, which I have confirmed. The precursor conversion of IP→IP* has also been observed in a pulse-chase experiment in which the label was added between 4 and 5 min following infection, thus labelling only early proteins (results not shown). The band IP was easily recognized in these gels and the disappearance of IP and the appearance of IP* were again correlated in time. This quantitative agreement and the absence of other unaccounted changing bands in the gel pattern support the argument for the IP–IP* relationship. Of course, a final proof awaits chemical analysis.

It was also observed that, although the pulse was performed between 4 and 5 min after infection, cleavage of IP starts only
because the total label in IP* is two or three times larger than the 1'24-+1'24* reaction is not likely to be detected by the bands are about equal, but the small loss of protein weight by to 1'24*. The integrated absorbance values over these two densitometric measurements. 1'24 could not give rise to 11'*, this time and it is therefore thought that the cleavage of 1'24 is easily distinguished from the small amount of 1'23* which contain the amber fragments, but these are absent in its polypeptide, they observed peptides in 23-defective lysates which is cleaved off in the protein 1'23*: These observations may be derived from the N-terminal end of the amber fragment which is cleaved off in the protein P23*. In establishing the co-linearity of gene 23 and its polyepitope, they observed peptides in 23-defective lysates which contain the amber fragments, but these are absent in wild type lysates or in purified phage particles. These peptides may be derived from the N-terminal end of the amber fragment, which is cleaved off in the protein P23*.

at late times (after 17 min). Phage assembly starts at about this time and it is therefore thought that the cleavage of IP is linked to phage assembly. Cleavage of P24. P24* (mol. wt 43,500) appears coordinately with P23* and P22* (Fig. 4) (the kinetics are not plotted). The precursor product relationship P24—P24* is more difficult to demonstrate, because P24 migrates only slightly faster than P23*, but the following experiment proves that P24 is missing in a pulse-chase wild type lysate. P24 separates somewhat better from P23* in a gel of lower concentration. The samples of the pulse-chase experiment were analysed on 8 per cent acrylamide gels and four time points are presented in Fig. 6. P23 is easily distinguished from the small amount of P23* existing immediately after the chase of the radioactive label (Fig. 6, 15 min). P24 disappears at the same time as P24* appears while P23* increases. One might argue that P24 is obscured by the heavy P23* band. This possibility was excluded by adding a lysate (23-defective lysate) containing P24 to the final samples of the pulse-chase experiment. P24 was then detected and it can be concluded that measurements of P24 are reliable, thus showing that P24 most likely gives rise to P24*. The integrated absorbance values over these two bands are about equal, but the small loss of protein weight by the P24—P24* reaction is not likely to be detected by the densitometric measurements. P24 could not give rise to IP*, because the total label in IP* is two or three times larger than that of P24 and P24 is synthesized late. Moreover, the precursor relationship of P24—P24* is considerably strengthened by recent observations on head maturation genes (my unpublished results). P24 does not seem to be cleaved at all in 50-defective cells and, indeed, no P24* is found. Cleavage of P22, P23 and P24 occurs in 50-defective cells, although at a reduced rate.

**Face of the Small Fragments**

Where are the small fragments of these cleavage reactions? The expected molecular weights for the small fragments stemming from P23, IP and P24 would be about 10,000, 2,500 and 1,500 respectively. Peptides of this size are not sieved on 10 per cent acrylamide gels and migrate with the marker dye (unpublished results of U. K. L. and J. V. Maizel). Attempts to find at least the 10,000 molecular weight fragment from P23 on gels of higher acrylamide concentration have failed. Possibly the fragments are further broken down to undetectable sizes. Fragments of P22 also have not been detected. Acid-soluble components which are derived from an acid-insoluble precursor are known to exist in T4 infected cells? Two are associated with the phage particle and they are released with the DNA from the head upon osmotic shock? The genetic determinants of one of these internal peptides has been mapped recently and lies in the neighbourhood of genes 20 and 21 (ref. 18). My results definitely rule out gene 20, which is incorporated unmodified into normal phage. Unfortunately, I have not discovered the product of gene 21 in the gel pattern. These results do not rule out the possibility that one of the internal proteins is derived from the small cleavage fragment of P22, P23 or P24. The appearance of the internal peptides seems indeed to be coordinated with the cleavage reactions of P23, P22 and P24. Genes 20, 21, 22, 23, 24 and 31, which affect the cleavage of P23, P22 and IP, are known also to affect the appearance of the internal peptides? It has been pointed out to me by S. Brenner and A. Stretton that the cleavage point must occur at the N-terminal end of the P23 protein. In establishing the co-linearity of gene 23 and its polypeptide, they observed peptides in 23-defective lysates which contain the amber fragments, but these are absent in wild type lysates or in purified phage particles. These peptides may be derived from the N-terminal end of the amber fragment, which is cleaved off in the protein P23*.

**Fig. 5. Kinetics of cleavage of P23, P22 and IP.** The kinetics of cleavage were measured using a microdensitometer (double beam recording microdensitometer, Joyce-Loebl) to record the autoradiogram. The exposure of the autoradiogram was chosen so that the absorbance of the band to be measured did not exceed 1 unit. The abscissa represents the integrated absorbance over the relevant peaks. a: A — A, integrated absorbance over the P23 peak; O — O, integrated absorbance over the P22 peak; O — O, total absorbance in P23 and P22. b: A — A, integrated absorbance over peak P22; O — O, integrated absorbance over peak IP; O — O, integrated absorbance over peak IP*.

**Fig. 6. Cleavage of the product of gene 24 (8 per cent acrylamide gels).** Some samples (15, 16, 19 and 26 min) of the pulse chase experiment of Fig. 4 were analysed on 8 per cent acrylamide gels. Only the relevant part of the gel pattern is shown.
Cleavage occurs in a Large Structure

The observation that all the proteins P20, P21, P22, P23, P24 and P25 are required for efficient cleavage of P23, P22, P24 and P25 suggests that these precursor proteins aggregate first to form an oligomeric structure, and are cleaved subsequently, rather than being cleaved first, and then assembled. The following experiments support this view. The experiment is based on the observation that most of the precursor proteins are soluble and non-precipitable when treated with SDS at room temperature, but that phage particles are not totally disrupted, as is also true in urea (1). The gel pattern of purified phage treated with SDS at room temperature is compared with completely degradated phage, boiled for 1 min in SDS (Fig. 7a and b). Only a small fraction of P23* is extracted from phage with SDS at room temperature. Most of the proteins stay at the top of the gel or enter the gel as high molecular weight aggregates. Some proteins are not extracted at all. Note, however, that a few proteins are almost quantitatively extracted from the phage particles. The samples from the pulse-chase experiment treated in SDS at room temperature only are shown in Fig. 7. P23 disappears with time but P25* appears, suggesting that P23 enters an SDS-resistant structure before being cleaved. Of course, these experiments cannot rule out the possibility that P23* is converted to an SDS-resistant structure so rapidly that it is not detected. A high molecular weight protein appeared at the top of the gel, which could be an aggregate of P23 but only accounts for part of the label which disappears from P23*. Most of the label stays at the top of the gel. The molecular weight of this structure must therefore be greater than 300,000, for such a molecular weight is excluded from these gels (unpublished results of J. E. L. and J. V. Maizel). It is possible that this structure has a capsid-like shape. IP* and P24* are not resolved in gels.

This is because of the high salt concentration in these samples ('M9' growth medium) which impairs the resolution of the gel in the low molecular weight region.

Maturation of the Head

My experiments demonstrate that the assembly of the head of bacteriophage T4 is not a simple, straightforward self-assembly, because several structural proteins are chemically altered at some stage of assembly. The uncleaved precursor protein P23 can, however, be polymerized into single and multilayered polyheads and particles, if its cleavage is blocked as a result of mutation. Investigations of genes 2, 4, 13, 14, 16, 17, 49, 50, 64 and 65, which supposedly control late steps in head formation, are in progress. It is interesting that the cleavage of P23, P25, P24 and IP seems to be normal in cells infected with phage carrying mutations in these genes with the exception of genes 2, 50 and 64 (my unpublished results).

Why are these structural head components cleaved? The findings that IP gives rise to an internal 'IP*' which combines with an end of a DNA strand during the preparation of this manuscript I was informed that the alteration of P23 has also been observed by other workers: E. Kellenberger and C. Kellenberger-van der Kamp, FEBS Lett., 8, 3, 140 (1970); R. C. Dickson, S. L. Barnes and F. A. Eiserling, J. Mol. Biol. (in the press); and J. Hosoda and R. Cone, Proc. US Nat. Acad. Sci. (in the press). Received May 7, 1970.