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T4-Induced Activity Required for Specific Cleavage of a Bacteriophage Protein In Vitro

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We have examined the acid-soluble products formed during incubation of labeled substrate protein from T4-infected cells with unlabeled phage-infected cell extracts. If the substrate protein is prepared from cells infected with a T4 mutant blocked in cleavage of phage head precursor proteins, the products formed in vitro include a peptide indistinguishable by several criteria from one of the T4 internal peptides. Denatured as well as undenatured protein can serve as the substrate for the formation of this peptide. As expected, this peptide is not formed if protein from either uninfected cells or cells infected with wild-type T4 is used as substrate. The formation of this peptide in vitro is dependent on a factor present in extracts of phage-infected cells but absent from extracts of uninfected cells.

Previous studies by Eddleman and Champe (4) have shown that bacteriophage T4 contains two internal peptides which are packaged together with the DNA in the head of the phage. These peptides, designated II and VII, have molecular weights of about 3,900 and 2,500, respectively, are composed predominantly of acidic amino acids (2), and are soluble in 5% trichloroacetic acid. The formation of both peptides in T4-infected cells is dependent on the functioning of seven phage genes—20, 21, 22, 23, 24, 31, and 40—all of which participate in early steps of head assembly. To explain this fact, it was postulated that the peptides are derived from an acid-insoluble precursor(s) by a process coupled to head assembly. Pulse-labeling experiments confirmed the existence of a precursor (4).

More recently, studies in several laboratories (3, 5, 7, 9) have demonstrated that certain T4 proteins undergo a reduction in molecular weight during phage assembly. By using sodium dodecyl sulfate (SDS)-gel electrophoresis, Laemmli (9) identified two of these proteins as the products of T4 genes 23 and 24, the cleaved forms of which are structural subunits of the phage head and are, respectively, some 17 and 3% smaller than their precursors. A third protein is the precursor of an internal protein, and a fourth, the product of gene 22, appears to be degraded to such an extent that its fragments cannot be identified on gels (5, 6, 9). Laemmli showed that the occurrence of these cleavage reactions is also coupled to head assembly, having the same gene dependence as does the formation of the internal peptides. This suggests that the internal peptides may be fragments of one or another of the cleaved proteins. Other fragments have not been detected on gels, possibly because they are rapidly degraded to free amino acids. The survival of the internal peptides may be exceptional due to their entrapment in the phage head.

These modifications of phage proteins are apparently due to the action of proteolytic enzymes. However, the nature of the reactions, the determinants of the specificity, and the number and origin of the proteolytic activities are unclear. To elucidate the details of the cleavage process it would clearly be advantageous to study the reactions in cell-free extracts.

Since any mutation which blocks head assembly in vivo results in accumulation of the uncleaved precursor proteins, the protein from such mutant-infected cells could serve as a substrate to demonstrate in vitro cleavage. The assumption underlying this approach is that the dependence of cleavage on head assembly observed in vivo would be obviated in disrupted cells. One assay for cleavage would be the occurrence of molecular weight reductions of the precursor proteins, as determined by SDS-gel electrophoresis, during incubation of the substrate with a suitable source of proteolytic factor. This method has the limitation, however, that the cleaved proteins, still of considerable size, can be easily characterized only with regard to molecular weight and only within the

limit of resolution of the SDS gel. It would thus be difficult to prove by this method that in vitro cleavage produces a protein identical to that formed in vivo. Alternatively, the formation of small peptide fragments, presumed to be cleaved from the precursors, could be assayed. The internal peptides, which as suggested above may represent two of these fragments, can be characterized, as will be shown, by parameters more specific than molecular weight. This approach thus has the advantage that it is more easily possible to establish the identity of the in vitro- and in vivo-formed products. In this report we describe experiments demonstrating the in vitro formation of one of the T4 internal peptides from its precursor and the dependence of this reaction on a factor present in T4-infected cells but not in uninfected cells.

MATERIALS AND METHODS

Phage and bacteria. Phage amber mutants are designated in the text according to the phage gene that is defective, e.g., (21^-), (e^-23^-). The particular mutants used are *am*H29 (21^-), obtained from Robert S. Edgar, and the double mutant *am*H26*am*H11 (e^-23^-), obtained from Michael Showe. Both were derived from the commonly used wild-type strain T4D. In addition, *am*111 (21^-) derived from T2L, was obtained from Richard Russell. *Escherichia coli* strain BB was used as the restrictive host and strain CR63 as the permissive host for amber mutants. Strain BB was used as the host for growth of wild-type phage.

Media. Tryptone broth (1% Difco Bacto-tryptone, 0.5% NaCl) was used for the growth of unlabeled bacteria. In experiments involving the labeling with ^{14}C- or ^{3}H-amino acids and for the growth of all phage stocks, minimal M9 medium, as described by Adams (1), was used.

Radioisotopes. Radioactively labeled amino acids were obtained from Schwarz/Mann. The most commonly employed amino acids were L-[^{3}H]lysine (generally labeled) with a specific activity of 7,000 Ci/mol and L-[^{14}C]lysine (uniformly labeled) with a specific activity of 300 Ci/mol. The isotopes were added directly to cultures without dilution of specific activity.

Preparation of internal peptides. In general, the labeling and purification procedures described by Eddleman and Champe (4) were used. For the preparation of [^{3}H]- or [^{14}C]lysine-labeled phage, 250 or 50 μCi, respectively, was added at 12 min after infection to 100 ml of T4D- (or T2L)-infected *E. coli* BB growing with aeration at 37 C. The infected cells were lysed with chloroform at 30 min after infection, and the phage was purified by two cycles of low- (5,000 $\times$ *g*, 10 min) and high- (25,000 $\times$ *g*, 60 min) speed centrifugation, suspending the pellets slowly in cold M9 to avoid ghosting. After the final centrifugation, the phage pellet was extracted with 2 ml of 10% dichloroacetic acid (DCA). DCA is used in preference to trichloroacetic acid because the internal peptides, especially peptide II, appear to be more reproducibly extracted by the former. The labeled components in the resulting extract, after removal of the precipitated protein and DNA by centrifugation, consist essentially of the two internal peptides II and VII and free lysine. When necessary, the peptides can be separated from each other and from unlabeled components by chromatography on Dowex 50 and Sephadex G50 as described below.

Unlabeled peptides, used as carrier in certain experiments requiring lyophilization of labeled peptides, were prepared in the same manner from 1 g of purified T4D. When required, column fractions containing the peptides can be identified by the ninhydrin reaction.

Preparation of labeled substrate protein. Cells of strain BB infected with *am*H29 (21^-) and superinfected with the same phage at 12 min to induce lysis inhibition were labeled with [^{14}C]lysine (300 Ci/mol, 1.0 μCi/ml) beginning at 10 min after infection (37 C). At this specific activity the isotope is almost totally incorporated during the first few minutes. At 30 min after infection, the cells were collected by centrifugation (10,000 $\times$ *g*, 10 min) at 4 C and lysed by suspension in chloroform-saturated water (1.0 ml for every 10.0 ml of culture). In one experiment (see Fig. 2) this concentrated lysate was used, without further treatment, as the substrate in the in vitro reaction. In all other experiments, the lysate was denatured by adding DCA to a final concentration of 10%. The resulting precipitate was washed by centrifugation at least twice with water to free it of acid-soluble label and residual DCA and finally was dispersed by sonication to a uniform suspension in 0.1 M Tris-hydrochloride, pH 7.8 (1.0 ml for every 10.0 ml of culture).

Preparation of cell extract. A 30-liter culture of strain BB was grown in broth at 37 C to 5 $\times$ 10^8 cells/ml, infected with the double mutant *am*H26*am*H11 (e^-23^-) at a multiplicity of five phage per bacterium, and superinfected at the same multiplicity at 15 min. (The use of an e^- [lysozyme] mutant allows the cells to be harvested without lysis.) The cells were harvested at 2 h after infection, and the cell paste was divided into 2.0-g samples which were kept frozen until needed. For each independent experiment, a 2.0-g sample of cells was thawed immediately before use and resuspended in 10.0 ml of 0.05 M Tris-hydrochloride, pH 7.5, containing 0.0014 M 2-mercaptoethanol. The cells were disrupted by extrusion through a French pressure cell, followed by sonication for 1 min at 4 C. The protein concentration of such extracts, as estimated by the Folin-Ciocalteau reaction with bovine serum albumen as standard, is approximately 12 mg/ml.

In one experiment in which extracts of infected and uninfected cells are compared (see Fig. 9), one-half of a 2-liter culture of cells was infected, and the other half was harvested at the time of infection. Each pellet was resuspended in 5.0 ml of buffer, and the cells were disrupted as described above.

Incubation of substrate protein with cell extract. The standard incubation mixture contains 0.5 ml of ^{14}C-labeled substrate protein (corresponding to a 5.0-ml culture), 0.5 ml of cell extract, each pre-

pared as described above, and 1.0 ml of 0.1 M Tris-hydrochloride, pH 7.8. The final pH of the mixture is 7.5 to 7.6. After incubation for 6 h at 37 C, DCA was added to a final concentration of 10% and the resulting precipitate was removed by centrifugation. This yields sufficient acid-soluble ^{14}C-labeled material for one two-step chromatographic analysis of the products. In experiments requiring additional analyses, the volumes of the components were increased proportionately.

Column chromatography: (i) Sephadex G50. The DCA-soluble products of the incubation (in a volume of 2 ml) were applied together with ^{3}H-labeled marker peptides II and VII to a column (2.5 by 39 cm) of Sephadex G50 (fine beads) which had been equilibrated with 0.05 M ammonium bicarbonate. Elution was performed with the same buffer at a flow rate of about 0.3 ml/min. The void volumn of the column was determined by using the high molecular weight marker Blue Dextran (Pharmacia Fine Chemicals, Inc.). For a standardly used fraction volume of 2.0 ml, this marker elutes at fraction 30, just before the emergence of any ^{14}C material. Another visible marker, ϵ-dinitrophenyl (DNP)-lysine, which elutes after all ^{14}C material, was added to all applied samples to mark the end of the run. Half of each fraction was counted and, if further analysis was required, the remaining half, in the regions of interest, was pooled and lyophilized with about 20 μg of unlabeled internal peptides added as carrier.

(ii) Dowex 50. The lyophilized material obtained from the pooled fractions eluting from Sephadex was redissolved in 1.0 ml of 5% trichloroacetic acid and applied to a column (0.6 by 120 cm) of Dowex 50-X2 water jacketed at 60 C. Elution was performed with a gradient of pyridine-acetic acid buffers as previously described (4). Samples of 4.0 ml were collected and counted.

Isotope counting. Samples to be assayed for ^{14}C or ^{3}H, or both, were evaporated to dryness in scintillation vials under heat lamps. The material was redissolved in 0.5 ml of water and, after addition of 10 ml of Aquasol (New England Nuclear Corp.), the samples were counted in an Intertechnique SL-30 liquid scintillation counter. The counting rates in the two channels were corrected for channel overlap when necessary.

RESULTS

Stability of internal peptides to degradation by a cell extract. To detect in vitro formation of the internal peptides, the peptides themselves must be stable upon incubation with cell extracts. Otherwise, even if they are formed, they may be too short-lived to be observed. The experiment shown in Fig. 1 demonstrates that both peptides are, in fact, stable under conditions which allow extensive proteolysis to occur. In this experiment, ^{14}C-labeled peptides II and VII were incubated with an unlabeled extract of T4-infected cells. Also present in the incubation mixture was ^{3}H-labeled protein from infected cells. After incubation the material soluble in 10% DCA was chromatographed on Dowex 50. It is seen that most of the ^{14}C label is chromatographically unchanged after 6 h of incubation, during which time the ^{3}H-labeled protein has undergone extensive degradation to an acid-soluble form.

In vitro formation of peptide II*. In an attempt to demonstrate in vitro formation of the T4 internal peptides, a lysate of 21^{-}-infected cells labeled with [^{14}C]lysine (an amino acid present in both peptides) was incubated with an unlabeled crude extract of $23^{-}e^{-}$-infected cells, which serves as the source of the proteolytic factor. The resulting ^{14}C-labeled acid-soluble reaction products were fractionated by gel-filtration through Sephadex G50 together with ^{3}H-labeled marker peptides as shown in Fig. 2a. It is seen that a peak of ^{14}C material elutes with marker peptide II. Rechromatography of this pooled material on Dowex 50-X2 shows that a major portion of it again elutes with the marker peptide (Fig. 2d). We will hereafter define the in vitro formed acid-soluble reaction product which elutes with peptide II from both Sephadex G50 and Dowex 50-X2 as II*—the asterisk indicating the possibility that it is not identical to II.

The ^{14}C-labeled material which elutes from G50 with marker peptide VII shows no discernible peak in this region and re-chromatography on Dowex 50-X2 shows it to be composed of several components (Fig. 2e), none of which is

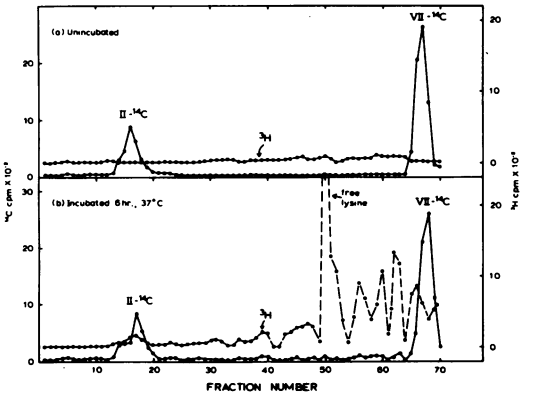

FIG. 1. *Stability of internal peptides to degradation by a cell extract. Purified T4 internal peptides II and VII labeled with* [^{14}C]*lysine and protein from* 21^{-}*-infected cells labeled with* [^{3}H]*lysine were incubated with an unlabeled extract of* $e^{-}23^{-}$*-infected cells prepared as described in Materials and Methods. The figure shows a comparison of the elution profiles from Dowex 50-X2 of the acid-soluble material present before and after incubation.*

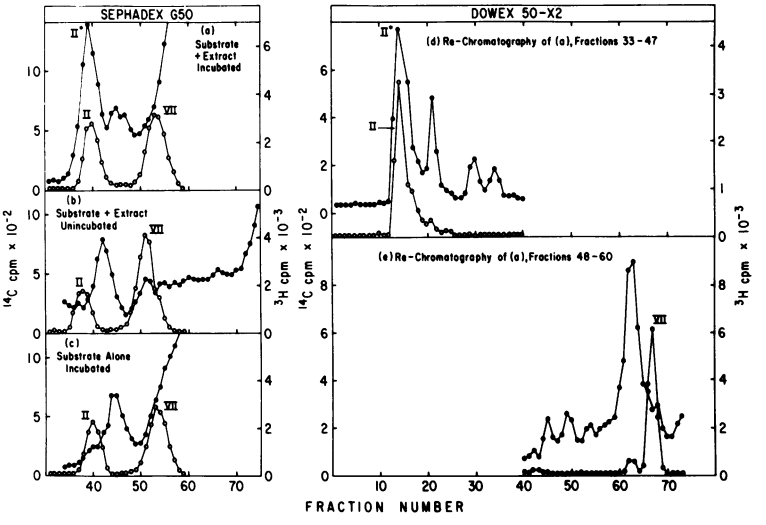

FIG. 2. *In vitro formation of peptide II* from undenatured substrate protein. A lysate of 21⁻-infected cells labeled with [^{14}C]lysine was incubated with an extract of e⁻23⁻-infected cells as described in Materials and Methods. The reaction products soluble in 10% DCA were chromatographed on Sephadex G50 and re-chromatographed on Dowex 50-X2 as shown. [^{3}H]lysine-labeled internal peptides II and VII were added as markers. Symbols: ●, ^{14}C; O, ^{3}H.*

identical with VII. Repeated attempts to demonstrate the in vitro formation of a component corresponding to peptide VII have been unsuccessful.

The controls of this experiment (Fig. 2b, c) show that the incubation mixture does not initially contain II* and that its formation is dependent on the presence of the 23⁻e⁻ extract. It is seen, however, that considerable acid-soluble ^{14}C-material is present in the unincubated substrate and increases upon incubation of the substrate alone. To eliminate this high base line, which can sometimes obscure the presence of II*, the above experiment was repeated under identical conditions except that the 21⁻ lysate was treated with 10% DCA. The resulting precipitate, washed free of acid-soluble label and residual DCA as described in more detail in Materials and Methods, was used as the substrate in the incubation. The results, given in Fig. 3, show that again a component satisfying the definition of II* is formed and, as in the case of the undenatured substrate, no component corresponding to peptide VII can be detected. The controls of this experiment (Fig. 3b, c), as compared with those of the previous experiment (Fig. 2b, c), show much reduced ^{14}C-material present initially in the unincubated substrate or arising from incubation of the substrate alone. For this reason, all of the following experiments were performed using denatured substrate. This has the advantage of insuring that all proteolytic activity is derived only from the unlabeled extract, but has the disadvantage that the physical state of the substrate is not completely controllable.

Figure 4 shows the time course of II* formation. It is mostly complete by 2 h, reaching a plateau at about 5 h, although the liberation of total acid-soluble material continues until 24 h, by which time about 28% of the total radioactivity of the substrate is solubilized. In the following experiments we have chosen an incubation period of 6 h, by which time about 15% of the total radioactivity is solubilized. The amount of labeled material recoverable as II* at this time is generally around 0.2 to 0.3% of the total incubated ^{14}C-labeled material. The amount of II* formed in vitro ranges, for various preparations, from 20 to 60% of the amount of peptide II formed in vivo by identically labeled T4 wild-type infected cells.

We have not yet studied the dependence of II* formation on the concentration of cell extract, although rough measurements show that under the in vitro conditions standardly employed, the yield of II* is not limited by the amount of extract.

The relation of II* to II. The fact that II* chromatographs with peptide II both on Sephadex G50 and Dowex 50-X2 shows that the two peptides at least have similar molecular weights and ionic properties. However, because there must be a multitude of peptides produced

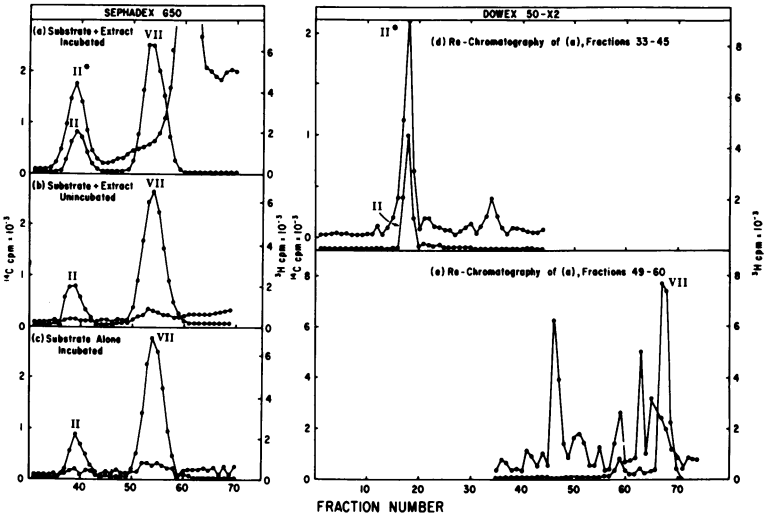

FIG. 3. *In vitro formation of peptide II* from denatured substrate protein. The experiment is identical to that described in Fig. 2 except that acid-denatured protein rather than an untreated lysate was used as the substrate.*

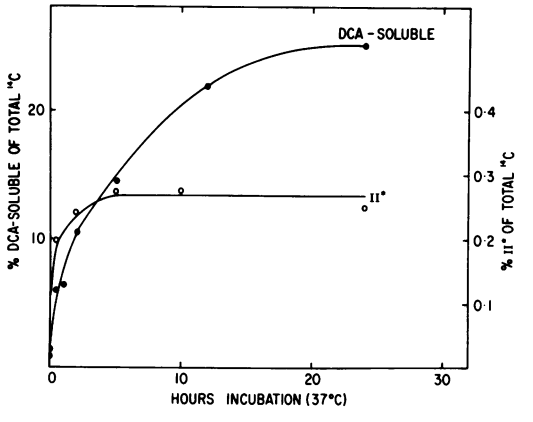

FIG. 4. *Time course of II* formation relative to release of DCA-soluble material. Samples of an incubation mixture identical to that used in the experiment of Fig. 3 were removed at the indicated times and precipitated with 10% DCA. The soluble material was assayed for total ¹⁴C and II*.*

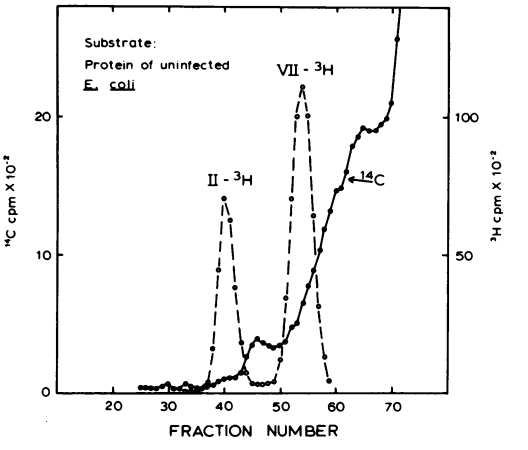

FIG. 5. *Analysis on Sephadex G50 of the acid-soluble products formed in vitro using [¹⁴C]lysine-labeled protein from uninfected E. coli as substrate.*

during in vitro incubation, the identity of II* with II must be established more rigorously.

One kind of evidence for the relatedness of II* and II would be a demonstration that II* is not formed when material from which the precursor of II* is expected to be absent is used as substrate. Thus, the experiment of Fig. 5 shows that II* is not formed when protein from uninfected *E. coli* is used as substrate. Similarly, we would not expect to detect II* formation when using protein from pulse-labeled T4D wild-type (am^+) infected cells as substrate because, in this case, the labeled peptide precursor would already have been cleaved in vivo and the peptides would be discarded in the acid-soluble wash of the protein substrate. The experiment of Fig. 6 confirms this expectation. We have thus shown that II* is derived from a phage-induced protein which disappears under conditions which allow in vivo cleavage to occur.

It should be pointed out that although chromatography on Sephadex alone is sufficient to show the absence of II* in the experiments of Fig. 5 and 6, in other experiments, material, which varies in amount from one experiment to

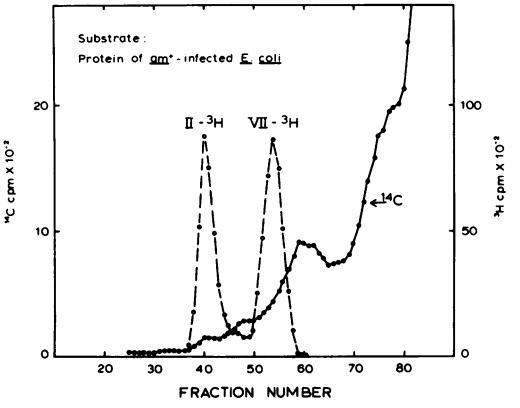

FIG. 6. *Analysis on Sephadex G50 of the acid-soluble products formed in vitro using [^{14}C]lysine-labeled protein from T4D wild-type infected cells as substrate, prepared as described in Materials and Methods.*

another, is often found to elute with the marker peptide II on Sephadex but not upon re-chromatography on Dowex. Thus, although the absence of II* can sometimes be determined on Sephadex alone, determination of its presence requires re-chromatography.

A definitive test of the relatedness of II* and II would be afforded by a comparison of their amino acid compositions. To prepare a sufficient quantity of II* for such analysis, however, is not practical. As an alternative, we have studied the specificity of labeling of II* with some of the amino acids known to be present in or absent from II. Accordingly, we have found that II* is labeled by [^{14}C]proline (present in II) but not by [^{14}C]valine, -isoleucine, or -alanine, which are absent from II (2).

Another test of similarity would be a comparison of fragments of II* and II produced by specific proteolytic enzymes. Unfortunately, neither II nor II* are significantly hydrolyzed by trypsin or chymotrypsin (itself another indication of similarity), but Pronase does yield fragments of II larger than free amino acids. Figure 7 shows a chromatographic comparison of the Pronase fragments of [^{3}H]lysine-labeled II and [^{14}C]lysine-labeled II* from which it is seen that the major fragments of both are similar.

Sternberg and Champe (11) have previously shown that phage T2 produces a peptide II^{2} similar (although not identical) in amino acid composition to peptide II^{4} (produced by T4), but which elutes from Dowex 50 some 10 fractions earlier than II^{4}. (Note: superscripts will be used only when necessary to distinguish the species variants of this peptide; when omitted, the peptide is of T4 origin.) This chromatographic species difference between the in vivo-formed peptides II^{2} and II^{4} should thus be reflected in the properties of the in vitro-formed peptides if, in fact, the in vivo- and in vitro-formed peptides are related. The experiment of Fig. 8 shows that this is the case. If protein from T2 (21^{-})-infected cells is used as the substrate (Fig. 8c, d), the in vitro-formed peptide II*^{2} elutes coincident with marker peptide II^{2} and is displaced from marker peptide II^{4}. In the control experiment (Fig. 8a, b) in which substrate protein from T4 (21^{-})-infected cells was incubated with the same cell extract, the in vitro-formed component elutes with marker peptide II^{4}.

In this experiment an extract of T4 (23^{-}e^{-})-infected cells was used as the source of the proteolytic factor. The experiment has been repeated by using an extract of T2 (23^{-})-infected cells with the identical result. This indicates that the difference between II^{2} and II^{4} is not due to a proteolytic factor that differs in specificity between T2- and T4-infected cells.

Origin of the proteolytic factor required for II* formation. Having established with reason-

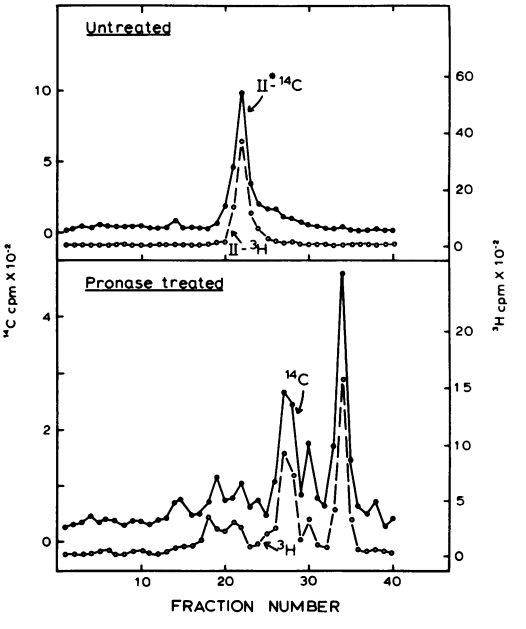

FIG. 7. *Chromatographic comparison of Pronase digestion products of [^{14}C]lysine-labeled II* and [^{3}H]lysine-labeled internal peptide II on Dowex 50. II* was obtained from a prior two-step Sephadex and Dowex purification. The labeled peptides were incubated with 50 μg of Pronase per ml in 0.1 M Tris-hydrochloride, pH 8.0, for 10 h at 37 C.*

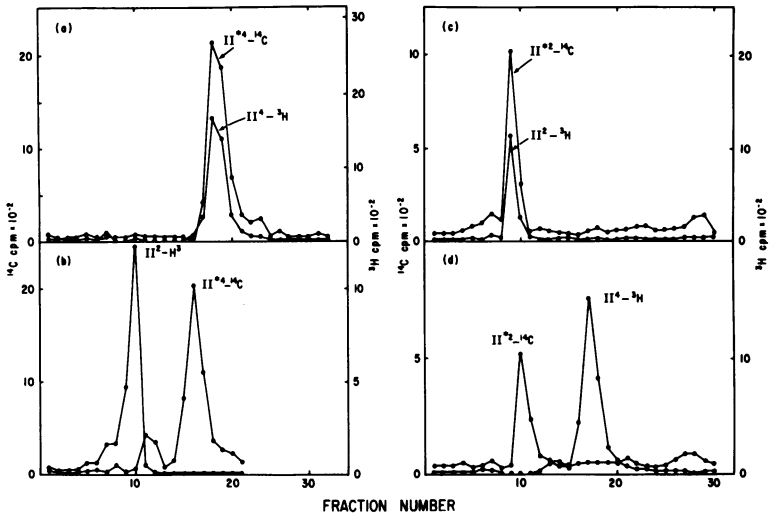

FIG. 8. *Species specificity of II*.* [^{14}C]*lysine-labeled substrate protein from T4D(21$^-$)-infected cells and T2L(21$^-$)-infected cells were separately incubated with an extract of T4D(e$^-$23$^-$)-infected cells. At the end of the incubation period, the acid-soluble extract of each sample was divided into two parts, to one of which was added* ^{3}*H-labeled marker peptide II^2 and to the other* ^{3}*H-labeled marker peptide II^4. The four samples were prefractionated on Sephadex G50. The fractions corresponding to the peak of the* ^{3}*H-marker were pooled and re-chromatographed on Dowex 50-X2 as shown.*

able certainty that the in vitro-formed peptide II* is very similar to, if not identical with, the in vivo-formed internal peptide II, we can now inquire whether the proteolytic factor responsible for II* formation is a host enzyme or is phage induced. The experiment shown in Fig. 9 was performed by incubating, under identical conditions, [^{14}C]lysine-labeled protein from T4 (21$^-$)-infected cells with (A) an infected cell extract and (B) an uninfected cell extract. The left panel shows the superimposed elution profiles from Sephadex G50 of the acid-soluble material formed in the two cases. The elution of the marker peptides (dashed lines) was identical for the two fractionations, only one set of which is shown. The ordinate scale is logarithmic in order to show that the release by infected and uninfected cell extracts of low molecular weight material, comprising the preponderance of the soluble counts liberated, is nearly identical. In both cases, a component is formed which elutes with marker peptide II on Sephadex, but rechromatography of this material on Dowex 50 shows no component identifiable as II* in the case of the uninfected extract. This strongly suggests that a phage-induced function is required for the formation of II*.

In addition, it is noted in Fig. 9 that a significantly greater amount of peptide material of intermediate size range (around fractions 50 and 65) is released by an infected cell extract than by an uninfected cell extract. Whether these peptides are fragments of other T4 proteins known to be cleaved in vivo and whether the same proteolytic factor is involved as that which produces II* remains to be determined.

DISCUSSION

We have demonstrated the in vitro formation from a protein precursor of a peptide, designated II*, which is indistinguishable from the in vivo-formed T4 internal peptide II. Protein prepared from 21$^-$-infected cells, in which peptide formation is blocked in vivo, can serve as a substrate for II* formation, but protein from T4 wild-type infected cells, in which the peptide precursor is cleaved in vivo, cannot. The in vitro reaction requires a factor present in T4-infected cells but absent from uninfected cells. Although other possibilities can be conceived, it is likely that this factor is a proteolytic enzyme. Experiments using specific protease inhibitors may help to confirm this and identify the kind of protease involved.

The most definitive evidence for the relatedness of II* and II is the fact that the in vitro-formed peptide has the same T2-T4 chromatographic species specificity as the in vivo-formed peptide. To account for the amino acid composition difference and the size difference between II^2 and II^4, Sternberg and Champe (11) suggested that these species-specific forms

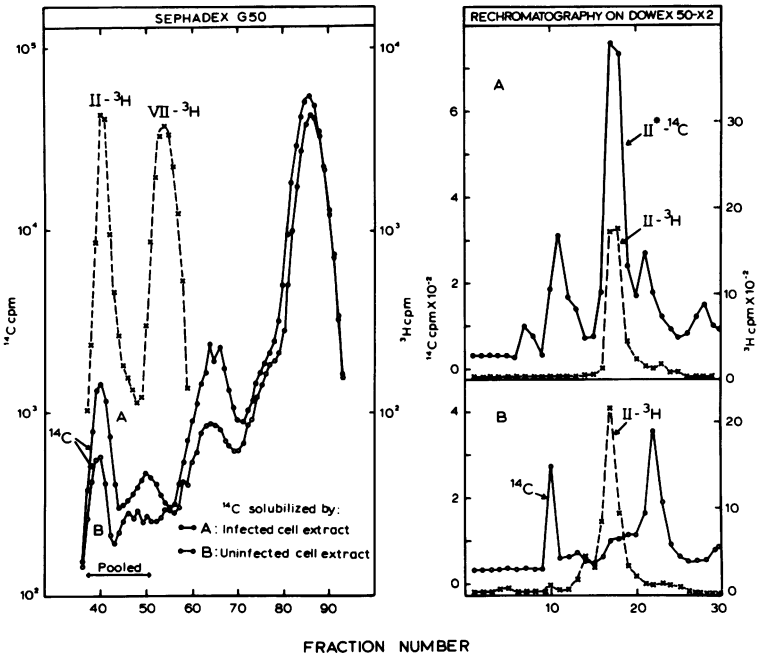

FIG. 9. *Comparison of infected and uninfected cell extracts as source of proteolytic factor. [^{14}C]lysine-labeled protein from 21$^-$-infected cells was incubated with: A, an extract of e$^-$23$^-$-infected cells, and B, an extract of uninfected E. coli. The panel on the left shows two superimposed Sephadex analyses, each of which was run with ^3H-labeled marker peptides. Only one set of the markers is shown since their elution was identical in the two runs. The panels on the right show the re-chromatography on Dowex 50 of the material eluting with marker peptide II from Sephadex G50. Small differences in the Dowex elution profiles are probably due to the relative efficacy of the prior separations on Sephadex.*

of the peptide are cleaved from their respective precursors at different sites. This in turn might result from minor differences in the amino acid sequence of the T2 and T4 precursors or, alternatively, from differing specificities of the cleaving enzymes. Our finding that either a T2- or T4-infected cell extract can produce both II*[2] and II*[4] argues that the species specificity is not due to a proteolytic factor which differs in specificity between T2- and T4-infected cells, but instead reflects a difference in their precursors.

Although cleavage implies the action of an endoprotease, we have used the term loosely. The formation of peptide II from its precursor could conceivably be due to an exoprotease, an endoprotease, or both. In either case, some structural feature of the precursor must limit the extent of exoproteolysis or define the sites of endoproteolytic cleavage. The finding that denatured or undenatured protein can serve equally as the substrate for II* formation argues that the tertiary structure is not the determining factor. The composition of II[4] has been shown to be $asp_{16}glu_{10}lys_3\ ser_2\ pro_1\ gly_1$ (2). A region of such strongly acidic character could conceivably limit the progress of an exoprotease or be protected from endoproteolytic cleavage. Peptide II is, in fact, resistant to hydrolysis by trypsin in spite of the fact that it contains several lysine residues.

Our failure to detect the in vitro formation of a peptide corresponding to internal peptide VII implies that proteolysis in vitro does not in all cases occur with the same specificity or the same relative efficiency as it does in vivo. Although we have shown that peptide VII is stable upon incubation with infected cell extracts (Fig. 1), it is conceivable that, unlike the free peptide, the region of the intact precursor corresponding to VII could undergo additional cleavages in vitro, thus giving rise to fragments not identifiable as VII.

The dependence in vivo of the known T4 protein cleavage reactions on head assembly is not understood. One explanation could be that the cleavage sites of the subunits become exposed only when the subunit is incorporated into a proper multimeric structure. Alternatively, the proteolytic enzyme may not be freely

diffusable but, in the intact cell, may be localized to sites at which assembly occurs. That a specific cleavage occurs in vitro when using denatured substrate protein argues that the latter possibility may be correct in this case.

The dependence of II* formation on a factor present in phage-infected cells but not detectable in uninfected cells suggests that this factor is an enzyme specified by a phage gene. It is not precluded, however, that the responsible factor is a host enzyme which becomes activated upon phage infection. It is also possible that, even if a phage-specified enzyme is involved, a host factor may, in addition, be required. Korant has reported that the cleavage of poliovirus capsid proteins appears to occur in several steps—some under host control and some under viral control (8).

Early preliminary experiments indicated that extracts of cells infected with a 23^- mutant are more active in II* formation than extracts prepared from cells infected with wild-type phage, and for this reason 23^--infected cells were standardly used as the source of the extract. This difference is being further investigated. If true, it would suggest that phage head maturation, which is blocked in 23^- mutants, results in inactivation or entrapment of the proteolytic factor.

The ability to effect the formation of one of the T4 internal peptides in vitro should now allow identification of the T4 genes controlling the peptide precursor and the proteolytic factor. Showe and Onorato (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 206, 1972) have reported that the in vitro degradation of the T4 gene 22 product (P22) specifically requires the T4 gene 21 product. They have also found (10) that both internal peptides II and VII prepared in our laboratory inhibit the precipitin reaction between P22 and anti-P22 serum, indicating that P22 may be the precursor of both internal peptides. The preponderance of negatively charged amino acids in these peptides, however, suggests the possibility that their interaction with antibody may not be specific, and the conclusion requires more direct evidence before it is firm. In any case, the suggestion that P22 may be an internal peptide precursor is consistent with the findings of Sternberg and Champe (11) who showed that the genetic determinant for the T2-T4 species difference of peptide II resides in the gene 20-22 region of the T4 genome. If, in fact, the gene 21-dependent degradation of P22 described by Showe and Onorato is the same proteolytic reaction which gives rise to II* in the present studies, we should be able to confirm this by using substrates and extracts prepared from cells infected with the appropriate mutants. Experiments in this direction are in progress.

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LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, New York.
2. Champe, S. P., and H. Eddleman. 1967. Polypeptides associated with morphogenic defects in bacteriophage T4, p. 55-70. *In* J. S. Colter and W. Paranchych (ed.), The molecular biology of viruses. Academic Press Inc., New York.
3. Dickson, R. C., S. L. Barnes, and F. A. Eiserling. 1970. Structural proteins of bacteriophate T4. J. Mol. Biol. **53**:461-474.
4. Eddleman, H., and S. P. Champe. 1966. Components in T4-infected cells associated with phage assembly. Virology **30**:471-481.
5. Hosoda, J., and R. Cone. 1970. Analysis of T4 proteins. I. Conversion of precursor proteins into lower molecular weight peptides during normal capsid formation. Proc. Nat. Acad. Sci. U.S.A. **66**:1275-1281.
6. Hosoda, J., and C. Levinthal. 1968. Protein synthesis in *Escherichia coli* infected with bacteriophate T4D. Virology **34**:709-727.
7. Kellenberger, E., and C. Kellenberger-van der Kamp. 1970. On a modification of the gene product P23 according to its use as subunit of either normal capsids of phage T4 or of polyheads. FEBS Lett. **8**:140-144.
8. Korant, B. D. 1972. Cleavage of viral precursor proteins in vivo and in vitro. J. Virol. **10**:751-759.
9. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
10. Showe, M. K., and L. W. Black. 1973. Assembly core of bacteriophage T4: an intermediate in head formation. Nature N. Biol. **242**:70-75.
11. Sternberg, N., and S. P. Champe. 1969. Genetic determinant of an internal peptide of bacteriophage T4. J. Mol. Biol. **46**:377-392.