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THE PURIFICATION AND PHYSICOCHEMICAL PROPERTIES OF A LYTIC ENZYME INDUCED BY COLIPHAGE N20F'

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SUMMARY

1. The lytic enzymes induced by the infection of *Escherichia coli* by three mutants of bacteriophage N20F' have been isolated and purified by column chromatography. The lytic enzyme induced by each mutant has two pH optima, one at pH 5.5 and the other at pH 8.5. Relatively high concentrations of carboxylic acids and divalent cations enhance the pH 5.5 activity but inhibit at pH 8.5. However, in the presence of low concentrations of these chemicals the pH 5.5 activity is drastically reduced, while there is no effect on the pH 8.5 activity. A monomer–dimer transition has been observed for this protein. Under conditions of high carboxylic acid and divalent cation concentration monomers are the chief molecular species at both pH 5.5 and pH 8.5. Conversely, the enzymes exist predominantly as dimers when the concentrations of both these chemicals are lowered. These experiments indicate that monomers are involved in the enzymatic activity at pH 5.5 while dimers are responsible for the activity seen under alkaline conditions.

2. The amino acid content of the three mutant enzymes has been determined. End group analyses show that alanine is the N-terminal amino acid of the lytic enzymes induced by each mutant. However, the C-terminal amino acids are different. Differences in amino acid composition and C-terminal amino acids are also reflected in differences in the peptide "fingerprint maps" that were obtained. These results suggest that the three spontaneous mutants may arise by a "frame shift" mutation.

INTRODUCTION

The infection of host bacteria by bacteriophages leads to replication of the phage particles. After maturation the phage particles are released from the host cell by lysis of the bacterial cell wall. This action is produced by the presence of phage-induced enzymes which have been demonstrated in lysates of various phage-host systems and have been designated as lysozymes[1,2], muralysins[3], endolysins[4,5], lytic enzymes[6], or lysins[7].

In a previous[8] paper we reported on a lytic enzyme induced by the infection of *Escherichia coli* K12 by a newly isolated bacteriophage, N20F'. Three mutants of N20F' were studied, and it was shown that the clarity of the plaques formed could be correlated with the specific activities of the lytic enzymes that were induced by the various mutants. This paper describes the purification and the physicochemical properties of this phage-induced lytic enzyme.

MATERIALS AND METHODS

Purification of the phage-induced lytic enzyme

For the preparation of large lysates the bacteria were grown in 10-l batches with good aeration at 37° in a modification of FRASER medium[9] which consisted of the following: $NaH_2PO_4 \cdot H_2O$, 4.5 g; Na_2HPO_4, 10.5 g; NH_4Cl, 3.0 g; vitamin-free casamino acid, 15.0 g; glucose, 40.0 g; water to 1 l.

The *E. coli* cells were grown to a concentration of $2 \cdot 10^8$ cells/ml at 37° and infected at a multiplicity of infection of 0.005 (see ref. 10). Incubation was continued with aeration at 37° for about 5 h for the clear mutant (N20F'Cl) and 8 h for the turbid (N20F'Tu) and less turbid (N20F'Ltu) mutants. The lysates were then stored overnight at 4°.

The next day, 6,9-diamino-2-ethoxy acridine lactate (Aldrich Chemical Co.) was added to a final concentration of 0.20 mg/ml to precipitate the acidic protein and cell debris[10]. After filtration through several layers of cheesecloth, the lysate was centrifuged at 13 000 × *g* for 20 min in a Servall RC-2 centrifuge. The supernate was then dialyzed at 4° overnight against 15 vol. of distilled water.

All subsequent purification steps were carried out at 4°. The purification procedure involved the use of CM-cellulose ion exchanger (Sigma Chemical Co.) and G-75 Sephadex (Pharmacia Fine Chemical Co.). Coarse grade CM-cellulose was regenerated according to the method of PETERSON AND SOBER[11]. The cellulose was suspended in 0.001 M phosphate buffer (pH 7.0) and poured into columns measuring 80 cm in height and 4.5 cm in diameter. Sephadex G-75 was swollen in the same buffer. Air trapped in the particles was removed under vacuum. The Sephadex was poured into columns measuring 70 cm × 7 cm.

A dialysate of approx. 10 l containing about 2920 mg of protein was applied to a CM-cellulose column. The column was then percolated with 6 l of 0.001 M phosphate buffer (pH 7.0). Chromatography was continued using a discontinuous NaCl gradient. 6 l each of 0.05 M NaCl and 0.1 M NaCl in 0.001 M phosphate buffer (pH 7.0) were passed through the column. These fractions were discarded since they contained protein but no lytic activity. The enzymatic activity was eluted using 0.2 M NaCl in 0.001 M phosphate (pH 7.0). The flow rate was maintained at a maximum of 10 ml/min.

The fractions containing the lytic enzyme were lyophilized immediately to approximately a quarter the eluted volume. This was then applied to a G-75 Sephadex column equilibrated with 0.001 M phosphate buffer (pH 7.0). The enzymatic activity was eluted with the equilibrating buffer. The pooled fractions were lyophilized to about 100 ml, and then dialyzed overnight at 4° against distilled water. The dialysate was next clarified by centrifugation at 27 000 × *g* for 15 min and lyophilized to a final volume of 1–2 ml. The enzyme is stable in the final concentrated form but undergoes

rapid denaturation at any of the intermediate stages. For this reason the purification procedure should not be interrupted at any intermediate step.

Enzyme homogeneity

The purity of the enzyme preparations was determined by centrifugation in the analytical ultracentrifuge and by polyacrylamide gel electrophoresis. Disc gel electrophoresis was carried out with a Canalco Model 200 apparatus using the pH 8.9 and pH 4.3 polyacrylamide gel system of DAVIS[12]. The details of the sedimentation velocity runs are given below.

Enzyme assay

The assay for induced lytic enzyme was given in a previous report[8].

Protein concentration

Protein concentrations were determined according to the method of LOWRY *et al.*[13] and also by the biuret method[14].

Molecular weight determinations

The Beckman Spinco Model E analytical ultracentrifuge equipped with phase-plate Schlieren optics and rotor temperature indicator and control unit was used for both sedimentation velocity and ARCHIBALD approach to sedimentation equilibrium determinations[15–17]. All sedimentation velocity runs were done at 64 000 rev./min. The value of the initial concentration, c_0, was determined by means of a 12-mm synthetic-boundary cell operated at about 8000 rev./min. For the sedimentation equilibrium runs at 15 000 rev./min, a 12-mm cell was used with a 4° sector aluminum centerpiece. As soon as the rotor attained the desired operating speed, pictures were taken at 8-min intervals with appropriate angles of the Schlieren diaphragm. A titanium AN-H rotor was used in all experiments.

Measurements of the ultracentrifuge patterns were made using a Nikon Microcomparator.

Amino acid analysis

The amino acid analyses were performed according to the method of SPACKMAN, STEIN AND MOORE[18]. For amino acid composition determinations approx. 1 mg of protein was hydrolyzed in 6 M HCl for 18 and 72 h in evacuated sealed tubes. Hydrolysis was carried out at 110° in an air-circulating oven. The HCl was evaporated by lyophilization, and the dried samples were dissolved in 0.2 M citrate buffer (pH 2.2) and applied to the columns of a Beckman Spinco Auto Analyzer Model 120C. Columns contained Beckman resins PA-28 and PA-25. Cysteine and methionine were determined as cysteic acid and methionine sulfone, respectively, on performic acid oxidized samples. Performic acid oxidation was carried out at 0° for 4 h using the procedure described by HIRS[19].

Determination of the N-terminal amino acid

N-Terminal amino acids were determined by the method of HARSHMAN AND NAJJAR[20]. One μmole of [^{14}C]FDNB (1.61 mC/mmole, Volk Isotopes) was mixed with 0.10 μmole of protein dissolved in 0.3 ml of 0.1% triethylamine (pH 8.5–9.0). The

reaction mixture was allowed to stand at room temperature overnight in the dark. The mixture was then extracted several times with anhydrous ether following hydrolysis in 6 M HCl for 2 h at 100° and 18 h at 110° in a sealed evacuated tube. The ether fractions were combined, evaporated to dryness, and spotted on Whatman No. 1 chromatography paper with known DNP-amino acids. The chromatogram was developed in the first dimension using the solvent; toluene–pyridine–2-chloroethanol–ammonia (90:27:54:54, by vol.). 1.5 M phosphate buffer (pH 6) was used as the solvent for the second dimension[21]. The [^{14}C]dinitrophenyl end group was identified by radioautography.

DFP-treated leucine aminopeptidase (Worthington Biochemical Corp.) was also used to determine the N-terminal amino acid. The reaction mixture was incubated at 40° and contained per sample: approx. 0.10 μmole of performic acid-oxidized enzyme protein in 0.125 M barbital buffer (pH 8.5), 0.1 ml of 0.025 M $MgCl_2$, and activated leucine aminopeptidase[22]. The digestion was carried out using a leucine aminopeptidase to enzyme molar ratio of approx. 1:60. At the indicated times aliquots were withdrawn and added to an equal volume of 0.4 M citrate buffer (pH 2.2), and then analyzed on the amino acid analyzer. In order to free the short column samples of ammonia, 2 M NaOH was added to adjust the samples to pH 11, and the ammonia was removed by aspiration to dryness (S. MOORE, personal communication). The samples were next resuspended in 0.2 M citrate buffer, the pH adjusted to 2.2 with HCl, and then analyzed on the amino acid analyzer.

Determination of the C-terminal amino acid

The C-terminal amino acid was determined by the use of DFP-treated carboxypeptidase A (Worthington Biochemical Corp.). Approx. 0.10 μmole of performic acid-oxidized protein was suspended in 0.5 ml of 0.05 M Tris buffer (pH 7.5) containing 0.5 M NaCl. The carboxypeptidase was activated by diluting the enzyme in 10% LiCl. The digestion was carried out at 25° with a carboxypeptidase/enzyme molar ratio of approx. 1:30. The reaction was stopped by freezing the mixture. The entire reaction mixture, to which was added 1 ml 0.2 M citrate buffer (pH 2.2), was applied directly to the columns of the amino acid analyzer in order to determine quantitatively the residues that were released.

Tryptic digestion and peptide "fingerprinting"

Approx. 1 mg of performic acid-oxidized protein was suspended in 0.5 ml of 0.1 M ammonium bicarbonate (pH 8.6). 10 μg dialyzed salt-free trypsin (Mann Research Laboratories) was added to the sample and the mixture was incubated overnight at room temperature.

The trypsinized sample was lyophilized and suspended in 0.1 ml distilled water. This solution was applied to a Whatman 3 MM filter paper sheet in a 1-cm streak. The chromatogram was then subjected to descending chromatography using the upper layer of a mixture of butanol–acetic acid–water (4:1:5, by vol.) as the solvent. The paper was dried, and electrophoresis was performed according to the method of KATZ, DREYER AND ANFINSEN[23] in pyridine–acetic acid–water buffer (1:10:289, by vol.) at pH 3.5. Electrophoresis was at 40 V/cm using an amperage ranging between 1.5 and 3.0 mA/cm for 3 h. The chromatogram was then dried, sprayed with ninhydrin in butanol, and heated at 80° for 7 min.

RESULTS

Purification of the lytic activity

The purification of the lytic activity was comparatively simple. Large batches of phage lysates produced by N20F'Cl infection of *E. coli* W13a were applied to CM-cellulose columns. The columns were washed thoroughly with buffer and then eluted with a discontinuous gradient of NaCl. Both the pH 5.5 and 8.5 activities were eluted simultaneously by 0.2 M NaCl. These results are shown in Fig. 1. The lytic activity at pH 5.5 and 8.5 decreased rapidly if the volume of the eluate was not reduced as quickly

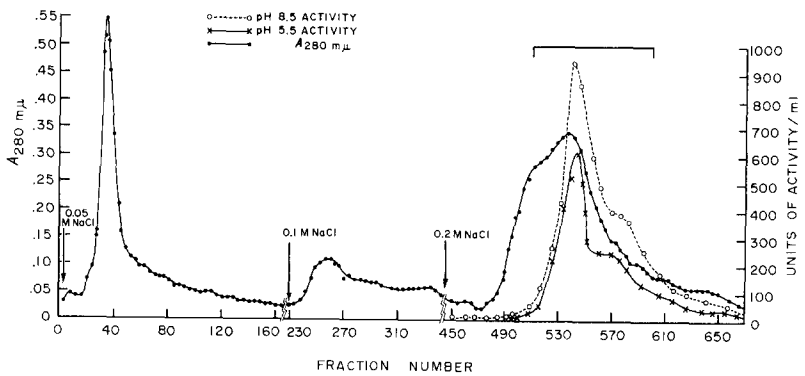


Fig. 1. Chromatography of N20F'Cl lytic enzyme on CM-cellulose. Experimental details are as described in MATERIALS AND METHODS. Elution was with a discontinuous gradient of NaCl in 0.001 M phosphate buffer (pH 7.0). 12-ml Fractions were collected after the start of elution with 0.05 M NaCl. Fractions 500–600 were pooled and lyophilized.

as possible. This was done by lyophilization overnight, which reduced the initial volume by approx. 60%. The extract was then applied to a G-75 Sephadex column equilibrated with 0.001 M phosphate buffer (pH 7.0). The same buffer was used to elute the lytic activity. Fig. 2 shows that the two pH activities eluted from Sephadex appear in the same fractions. The fractions containing activity were pooled and

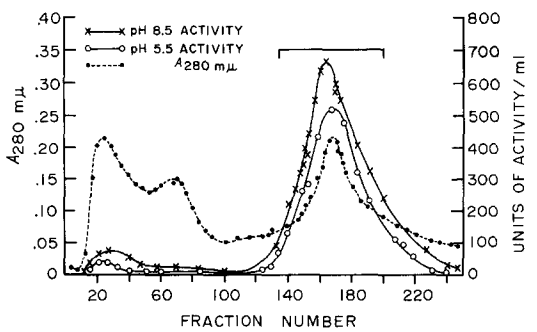


Fig. 2. Chromatography of N20F'Cl lytic enzyme on Sephadex G-75. The experimental procedure is described in MATERIALS AND METHODS. Elution was with a 0.001 M phosphate buffer (pH 7.0). Fractions 134–200 were pooled and lyophilized. The flow rate was maintained at approx. 2.5 ml/min.

TABLE I

PURIFICATION OF N20F'Cl LYTIC ENZYME
10 l of lysate yield approx. 10–12 mg protein.

Step	*Total units of activity*		*Specific activity (units of activity/ mg protein at 23°)*		*Recovery (%)*	
	pH 5.5	*pH 8.5*	*pH 5.5*	*pH 8.5*	*pH 5.5*	*pH 8.5*
1. Lysate	420 420	649 967	144	222	100	100
2. Elution from CM-cellulose using 0.2 M NaCl	330 221	507 153	10 570	16 234	78.5	78.0
3. After lyophilization of CM-eluate	170 000	272 769	3 935	7 618	40.43	41.9
4. After elution from Sephadex G-75 in 0.001 M phosphate buffer	140 895	206 040	16 666	20 200	31.5	31.7

lyophilized. The activities remain stable for months at 4° as long as the concentration of protein remained relatively high (approx. 4 mg/ml.).

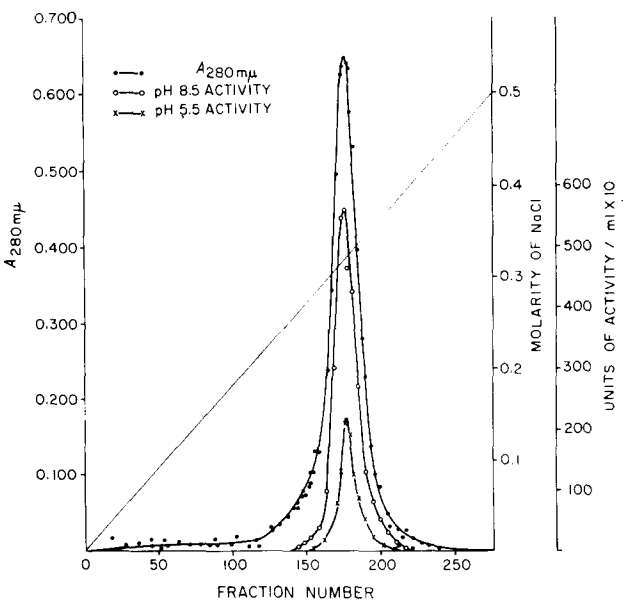
Table I summarizes the various steps involved in the purification and the yields that were obtained. The data show that there is an overall increase of approximately a 100-fold in the specific activity of the protein(s) responsible for the pH 5.5 and 8.5 activities. Table II illustrates that the activities of the purified N20F'Cl enzyme(s) are impaired in the presence of NaCl. This is particularly evident in the recovery of the activity in Step 3 where the NaCl used to elute the activity from Step 2 has been concentrated by lyophilization.

The lytic enzymes produced following N20F'Tu and N20F'Ltu infection of *E. coli* W13a were also purified according to the procedures set out in Table I. However, the degree of lysis of the batch cultures following infection by these two mutants was much less compared with N20F'Cl infections. In a N20F'Cl preparation the bacterial culture lysed completely at about 6 h after infection. In the case of N20F'Tu and N20F'Ltu, the media never cleared completely and samples were assayed at various times after infection to determine when to harvest the lysates. The highest specific activities of the purified enzymes were produced by N20F'Cl infection of the host bacteria. This is shown in Table III. The data presented in Table I and in Figs. 2 and 3

TABLE II

EFFECT OF NaCl ON THE LYTIC ACTIVITIES OF PURIFIED N20F'Cl ENZYME AT pH 8.5 AND 5.5
Assay conditions were as indicated in the standard assay but with the addition of the indicated amount of NaCl. The assays at pH 5.5 and 8.5 were done in 0.05 M Tris–maleate and 0.05 M Tris, respectively.

NaCl concn. (M)	*Remaining activity (%)*	
	pH 5.5	*pH 8.5*
No NaCl	100	100
0.025	80	92
0.050	19	59
0.10	18	16
0.20	7	1



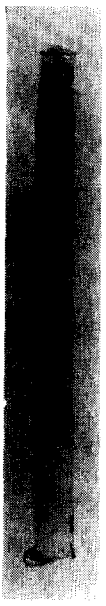


Fig. 3. Chromatography of purified N20F'Ltu lytic enzyme on SE-Sephadex C-25. The sample was applied to a SE-Sephadex-C25 column (45 cm × 2 cm) and elution was accomplished at 4° with a continuous linear gradient of NaCl (0–0.5 M) in 0.001 M phosphate (pH 7.0). The flow rate was 0.5 ml/min, and 2.95-ml fractions were collected. 73% of the initial activities were recovered, and there was a small loss in specific activities. This is probably due to the unstable nature of the enzyme in dilute solutions.

Fig. 4. Polyacrylamide gel electrophoresis of N20F'Cl lytic enzyme at pH 9.5. The gel was run under the conditions described by DAVIS[12]. The protein was stained with 0.5% Amido black in 7% acetic acid. The cathode is toward the top. 30 μg of protein was used per gel.

suggest that both lytic activities might be associated with the same protein. In order to test the concurrence of the pH 5.5 and 8.5 enzymatic activities, rechromatography of each purified lytic enzyme was carried out on SE-Sephadex. The results are shown in Fig. 3. Both enzymatic activities elute in the same fractions while the ultraviolet absorption reveals a single peak at 280 mμ. The homogeneity of the purified enzyme samples was further shown by polyacrylamide disc gel electrophoresis at pH's 9.5 and

TABLE III

SPECIFIC ACTIVITIES* OF THE PURIFIED LYTIC ENZYMES INDUCED BY THREE N20F' MUTANTS
The percent recovery of lytic activity at both pH's and at each step of the purification procedure was similar for all three mutant enzymes.

Bacterio-phage	*pH 8.5*	*pH 5.5*
N20F'Tu	4 028	2 434
N20F'Ltu	5 425	3 125
N20F'Cl	20 000	16 666

* Specific activity is expressed as the units of activity/mg protein. Lytic enzymes prepared by the procedure as described in MATERIALS AND METHODS.

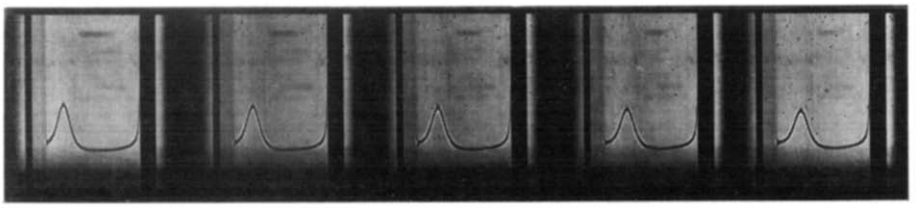
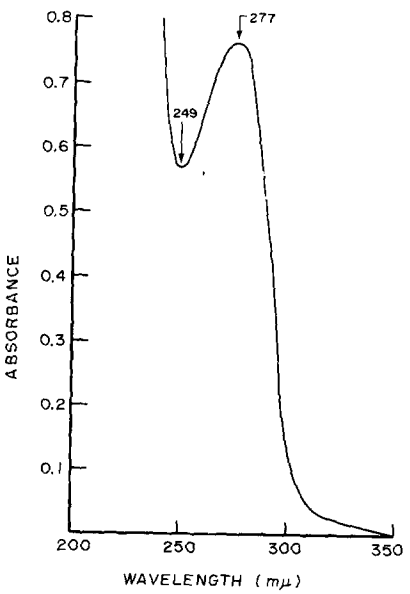


Fig. 5. Sedimentation velocity Schlieren patterns of N20F'Cl lytic enzyme. The enzyme was in 0.05 M Tris buffer (pH 8.5). Photographs were taken at 8-min intervals 32 min after the centrifuge reached the top speed of 64 000 rev./min. The bar angle was 50°. Sedimentation is from left to right.

4.3. In all cases a single protein band was obtained (Fig. 4). Sedimentation velocity experiments also reveal a single symmetrical peak. This is illustrated in Fig. 5. The ultraviolet absorption spectra of the enzyme from the three phages show a maximum at 277 mμ and a minimum of 249 mμ in 0.01 M Tris buffer (pH 8.5) (Fig. 6). There is good proportionality at both pH's 5.5 and 8.5 between the decrease in $A_{650\ m\mu}$ and the concentration of purified protein used in the assay (Fig. 7). This is also true for crude preparations of the lytic enzyme.

Suggestive evidence for the dimerization of enzyme subunits

The purification studies suggest that the two pH activities may be closely associated with a single protein structure. Polyacrylamide gel electrophoresis of the



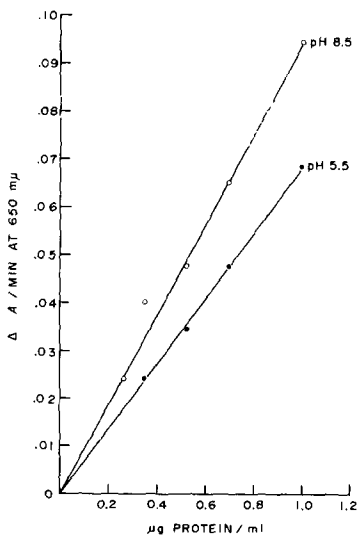


Fig. 6. Ultraviolet spectrum of purified N20F'Cl lytic enzyme. The N20F'Cl enzyme was in 0.05 M Tris (pH 8.5). A Cary Model 15 recording spectrophotometer was used in this determination.

Fig. 7. Lysis of chloroform-treated cells by purified N20F'Cl enzyme. The relationship between protein concentration of purified N20F'Cl lytic enzyme and the rate of decrease in $A_{650\ m\mu}$ of substrate cells in 0.05 M Tris (pH 8.5) and in 0.05 M Tris–maleate (pH 5.5).

TABLE IV

MOLECULAR WEIGHT DETERMINATIONS OF THE LYTIC ENZYMES FROM N20F′ BACTERIOPHAGE MUTANTS AT pH 8.5 AND 5.5

These determinations (approx. 1000) were done using approx. 7–10 mg protein per ml. The experimental procedure was that reported by SCHACHMAN[17].

Bacteriophage	*Mol. wt.$_m$**	*Mol. wt.$_b$***
	0.05 M Tris (pH 8.5)	
N20F′Cl	12 000	25 000
N20F′Ltu	11 500	22 000
N20F′Tu	10 000	23 000
	0.05 M Tris–maleate (pH 5.5)	
N20F′Cl	11 000	13 000
N20F′Ltu	11 500	12 000
N20F′Tu	12 500	12 500

* Molecular weight as determined from the meniscus of the cell in ARCHIBALD determination.

** Molecular weight as determined from the bottom of the cell.

purified protein under both acid and alkaline conditions gives a single band, which implies that the net charge on the protein molecules responsible for the enzymatic activities is the same. However, the evidence is still insufficient to state whether or not the double pH optima is due to a single enzyme.

Molecular weight determinations were performed on the purified protein obtained after infection of *E. coli* by the three N20F′ phage mutants. These determinations were done at the two pH optima using the same protein sample which was equilibrated overnight at 4° against the appropriate buffer. The ARCHIBALD method[15–17] was used throughout for all molecular weight determinations. The results of these determinations are given in Table IV.

The molecular weights at the meniscus for all three mutant enzymes at pH 8.5 in 0.05 M Tris fall within the range of 10 000–12 000 and are approximately half the value of the molecular weights determined at the cell bottom. This would indicate that at pH 8.5 the samples are composed of more than one component. However, similar determinations at pH 5.5 in 0.05 M Tris–maleate indicate that the molecular weights at the meniscus and at the bottom are roughly the same (11 000–13 000). These values are also similar to those obtained at the meniscus at pH 8.5. The observed data can be explained if we regard the sample as being an equilibrium mixture of monomers and dimers. Specific changes in the environmental conditions of the enzyme will lead to a shifting of the equilibrium in either direction. At pH 5.5 in 0.05 M Tris–maleate monomers exist and the conditions necessary for dimer formation are absent, while at pH 8.5 in 0.05 M Tris a mixture of monomers and dimers is present.

Sedimentation velocity studies were initiated in an attempt to determine the predominant molecular form at pH 8.5. Fig. 8 shows the results of these experiments with an N20F′Cl preparation. The sedimentation coefficients at both pH 8.5 and 5.5 appear to be independent of the protein concentrations used. The $s_{20,w}$ values in 0.05 M Tris–maleate at pH 5.5 and 8.5 are 1.99 and 1.90, respectively, indicating the

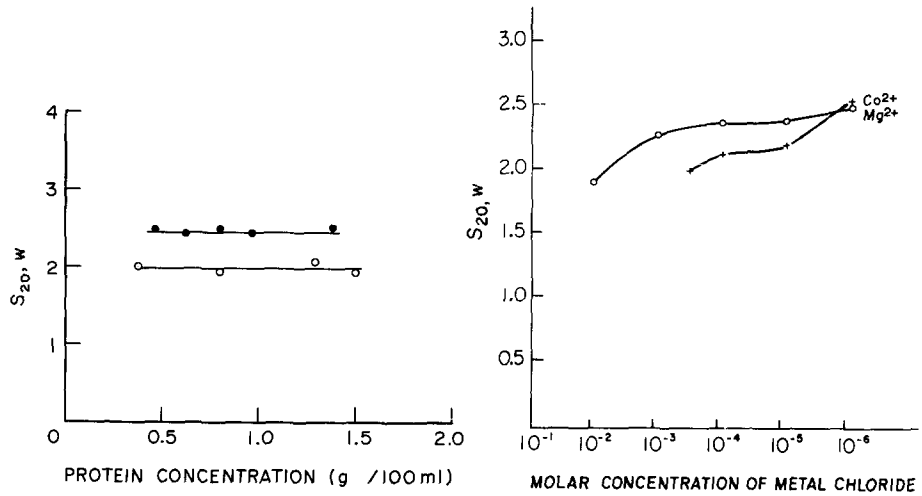


Fig. 8. Dependence of the sedimentation coefficient upon N20F'Cl lytic enzyme concentration in 0.05 M Tris–maleate (pH 5.5) and in 0.05 M Tris (pH 8.5). The same enzyme sample was used for determinations at both pH 5.5 and pH 8.5. The sample was equilibrated overnight at 4° against the appropriate buffer. ●—●, 0.05 M Tris (pH 8.5), $s_{20,w} = 2.45$; ○—○, 0.05 M Tris–maleate (pH 5.5), $s_{20,w} = 1.99$.

Fig. 9. The effect of divalent cations on the sedimentation coefficient of N20F'Cl lytic enzyme. The same sample was used for all $s_{20,w}$ determinations for each metal ion. Initial concentration of protein was approx. 8 mg/ml. The sample was dialyzed overnight at 4° against the appropriate buffer and then studied in the analytical ultracentrifuge. +—+, $CoCl_2$ + 0.01 M Tris buffer (pH 8.5); ○—○, $MgCl_2$ + 0.01 M Tris–maleate buffer (pH 5.5).

monomeric form. In 0.05 M Tris at pH 8.5 the $s_{20,w}$ value is 2.45 suggesting the predominance of dimers. The concentration of maleate affects the $s_{20,w}$ value at pH 5.5, since in 0.01 M Tris–maleate the $s_{20,w}$ is 2.48. Correspondingly, in low concentrations of Tris–maleate and Tris at pH 8.5 the $s_{20,w}$ values are 2.48 and 2.47. N20F'Tu and N20F'Ltu preparations also behave in a similar manner. These results indicate that the concentration of maleate is important in determining the molecular form of the enzyme.

In the previous report[8] the data (Table III) regarding the effect of carboxylic acids on the enzymatic activity at the two pH optima show that in 0.05 M maleic acid the enzymatic activity at pH 5.5 is optimal, while at pH 8.5 there is a sharp drop in activity. Conversely, in 0.01 M maleic acid the activity at pH 5.5 is 10% of that which was observed at the higher carboxylic acid concentration. Also, under these conditions of low concentrations of maleic acid (0.01 M) over 70% of the activity at pH 8.5 remains. Thus, the effect of carboxylic acids in determining both the molecular form of the enzyme and the enzymatic activity at pH 5.5 and 8.5 indicates that the pH 5.5 activity is associated with monomers while the pH 8.5 activity is dependent on the presence of dimers.

The effects of divalent cations on the molecular form of the lytic enzyme have also been studied. These results are given in Fig. 9. They show that increased divalent cation concentrations can produce the same effects as high concentrations of carboxylic acids. There is a gradual decrease in the s values as the concentration of divalent cations is increased, which reflects the conversion of dimers to monomers. These physical

studies support the earlier experiments, which show that at acid pH high divalent cation concentrations enhance the lytic activity but inhibit under alkaline conditions.

The apparent association of monomers into dimers and the opposite process of dimers forming monomers is reversible. The same protein sample at pH 8.5 in 0.05 M Tris buffer, which contains a mixture of monomers and dimers can be dialyzed against 0.05 M Tris–maleate (pH 5.5) overnight at 4° to give an apparently homogeneous system of monomers. If the sample is redialyzed against the above pH 8.5 buffer, a heterogeneous mixture of monomers and dimers will be formed. SMITH, WOOD AND CHARLWOOD[24] have shown that in an investigation of egg white lysozyme by the ARCHIBALD procedure at pH 6.6, the apparent molecular weight at the bottom of the solution column was consistently higher than at the meniscus. Other workers have reported similar results regarding the influence of pH on monomer–dimer transitions in the same enzyme[25,26].

Amino acid composition and preliminary attempts at sequence studies

The results of early experiments showed that there were differences in the specific activity of the lytic enzyme produced by the three N20F′ phage mutants. These differences in activities could be a reflection of possible differences in the primary

TABLE V

COMPOSITION OF N20F′Cl, N20F′Ltu AND N20F′Tu ENZYMES CALCULATED FROM AMINO ACID ANALYSES OF ACID HYDROLYSATES

Average means average of duplicate 18- and 72-h hydrolysates.

Amino acid	*Amino acid residues per molecule lytic enzyme***					
	N20F′Cl		*N20F′Ltu*		*N20F′tu*	
	Average	*Nearest integral number*	*Average*	*Nearest integral number*	*Average*	*Nearest integral number*
Lys	6.51	7	9.81	10	7.76	8
His	1.10	1	1.07	1	1.57	2
Arg	5.83	6	5.68	6	4.63	5
Asp	12.42	12	10.96	11	9.09	9
Thr*	6.30	6	5.02	5	3.83	4
Ser*	5.95	6	5.36	5	7.13	7
Glu	10.93	11	13.63	14	17.27	17
Pro	5.83	6	4.50	5	5.74	6
Gly	6.51	7	5.39	5	5.38	5
Ala	7.47	7	12.74	13	10.17	10
Val*	7.52	8	5.58	6	6.67	7
Met***	2.04	2	1.68	2	1.48	2
Ile*	9.00	9	5.72	6	8.74	9
Leu	5.44	5	4.89	5	4.99	5
Tyr	1.77	2	0.31	0	0.87	1
Phe	1.97	2	1.17	1	1.24	1
Cys***	0.15	0	0.31	0	0.37	0
Trp†	1.13	1	1.22	1	0.85	1

* Corrected for hydrolysis loss.
** The molecular weight was taken as 12 000.
*** Determined as methionine sulphone and cysteic acid on performic acid-oxidized samples.
† Determined spectrophotometrically[36].

TABLE VI

RELEASE OF AMINO ACIDS FROM THE C-TERMINAL ENDS OF N20F'Cl, N20F'Ltu AND N20F'Tu LYTIC ENZYMES* DURING DIGESTION WITH A 1:30 MOLAR RATIO OF CARBOXYPEPTIDASE A AT 25°

The experimental details are described in MATERIALS AND METHODS. These results are the averages of 3–4 determinations. In all cases the order of release of amino acids was consistent.

Bacteriophage lytic enzyme	*Amino acid released*	*μmole/μmole lytic enzyme*				
		15 min	*1 h*	*2 h*	*3 h*	*4 h*
N20F'Cl	Ile	0.28	0.37	0.49	0.62	0.68
	Val	0.28	0.32	0.35	0.36	0.37
	Ala	0.12	0.26	0.44	0.53	0.66
	Met	0.04	0.11	0.27	0.28	0.38
	Ser	Trace	0.04	0.12	0.13	0.17
	Thr	—	Trace	0.05	0.07	0.09
	Asp	—	—	0.02	0.03	0.06
		7 min	*1 h*	*3 h*	*5 h*	*7 h*
N20F'Ltu	Ala	0.41	0.60	0.75	0.86	0.90
	Leu	0.26	0.37	0.42	0.49	0.51
	Ile	0.24	0.33	0.42	0.48	0.50
	Val	0.15	0.28	0.38	0.44	0.43
	Ser	0.12	0.18	0.23	0.26	0.29
	Met	0.06	0.13	0.16	0.18	0.18
	Thr	0.06	0.08	0.13	0.15	0.17
		10 min	*15 min*	*1 h*	*4 h*	*7 h*
N20F'Tu	Ala	0.42	0.70	0.75	1.02	1.21
	Val	0.37	0.42	0.51	0.59	0.75
	Ile	0.33	0.39	0.48	0.57	0.66
	Leu	0.33	0.38	0.46	0.56	0.57
	Ser	0.09	0.30	0.31	0.40	0.50
	Thr	0.06	0.20	0.20	0.27	0.38
	Glu	0.02	0.13	0.14	0.19	0.27

* A molecular weight of 12 000 was used in these calculations.

amino acid sequence of the molecules produced. Table V gives the amino acid composition of the three mutant enzymes. The table shows that there are similarities as well as differences. All three enzymes lack cysteine and differ generally in the amounts of some of the amino acids present.

Table VI reveals the results of carboxypeptidase A digestion of the lytic enzyme derived from the three N20F' mutants. Fig. 10 shows a representative kinetic plot of the release of amino acids following the action of carboxypeptidase A on the N20F'Ltu enzyme. The data indicate that the C-terminal amino acids are different in the three mutant proteins. There is some uncertainty in the assignment of isoleucine or valine as the C-terminal amino acid of the N20F'Cl enzyme. Initially, both amino acids come off at approximately the same rate, but the amount of valine begins to level off after the first hour, whereas isoleucine continues to increase. Under these circumstances isoleucine has been tentatively designated the C-terminal amino acid. A possible C-terminal sequence of the three mutant enzymes which is consistent with the carboxypeptidase results is given below.

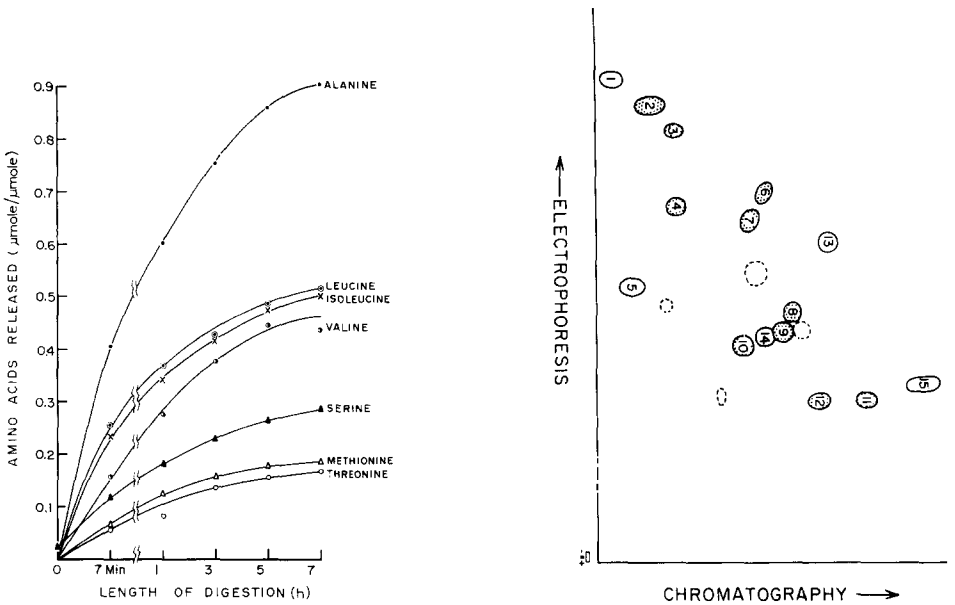


Fig. 10. The kinetics of amino acid release from N20F'Ltu lytic enzyme during digestion with carboxypeptidase A. The data were taken from Table VI.

Fig. 11. Schematic representation of the peptide pattern obtained from a tryptic digest of a performic acid-oxidized sample of N20F'Cl lytic enzyme. The hatched spots represent peptides common to all three mutant enzymes. ——, strongly ninhydrin positive; · · · ·, weakly ninhydrin positive. The digest was applied at the origin, which is represented by the small rectangle in the lower left corner.

N20F'Cl	–(Asp–Thr–Ser)–Met–Ala–(Val–Ile)
N20F'Ltu	–(Gly–Thr–Met)–Ser–Val–Ile–Leu–Ala
N20F'Tu	–(Glu–Thr)–Ser–Leu–Ile–Val–Ala

The N-terminal amino acid is alanine for all three mutant enzymes. It was isolated as the [^{14}C]dinitrophenyl derivative after hydrolysis of the [^{14}C]dinitrophenyl protein in 6 M HCl for 18 h. These results were confirmed by studies using leucine aminopeptidase, which show that the initial amino acid sequence at the N-terminal ends of the mutant enzymes are the same (Table VII).

Fig. 11 represents the peptide "fingerprint map" that was obtained after paper chromatography and electrophoresis of a trypsinized sample of the N20F'Cl protein. The number of peptides recorded agree well with the size of the subunit calculated from molecular weight and amino acid analysis data. Some differences were observed between the maps produced by different mutant enzymes. Peptides 1–12 are common to N20F'Cl and N20F'Ltu, while Peptides 2–4 and Peptides 6–12 are common to all three mutants. Peptides 14 and 15 Cl are present only in the N20F'Cl preparation. The differences in the fingerprint maps, together with the amino acid composition and C-terminal amino acid data, suggest that the differences in activity of the three mutant enzymes may be due to differences in the primary structure of the three types of molecules. Since these mutants were isolated as single-step variants of the parental

TABLE VII

RELEASE OF AMINO ACIDS FROM THE N-TERMINAL ENDS OF THE LYTIC ENZYMES* INDUCED BY THREE N20F' MUTANT BACTERIOPHAGES

The experimental procedure is described in MATERIALS AND METHODS. The number of μmoles of amino acids released by digestion of the N20F'Tu lytic enzyme is greater than in the other two digestions, since the specific activity of the leucine aminopeptidase used was higher than that for the N20F'Cl and N20F'Ltu digestions.

Bacteriophage lytic enzyme	*Amino acid released*	*μmole/μmole lytic enzyme*			
		30 min	*1 h*	*3 h*	*7 h*
N20F'Cl	Ala	0.13	0.16	0.22	0.49
	Ile	0.05	0.12	0.18	0.45
	Val	0.03	0.08	0.15	0.30
		1 h	*3 h*	*6 h*	*8.5 h*
N20F'Ltu	Ala	0.08	0.13	0.21	0.34
	Ile	0.06	0.12	0.20	0.30
	Val	0.04	0.11	0.17	0.19
		1 h	*3 h*	*6 h*	*9 h*
N20F'Tu	Ala	0.44	0.66	0.86	0.99
	Ile	0.32	0.41	0.50	0.71
	Val	0.22	0.38	0.48	0.54

* A molecular weight of 12 000 was used in these calculations.

N20F'Tu stock, it is thought that the mutation resulting in alterations in the C-terminal sequences reported above might be due to a single reading frame shift in each mutant phage genome specifying the lytic enzyme.

DISCUSSION

The present studies were concerned with the purification and physicochemical characterization of a phage-specific and phage-induced lytic enzyme. Both the crude lysates of N20F'-infected cells and the purified enzyme preparations exhibit two pH optima. Evidence is presented which supports the idea that a single protein is involved, *i.e.*, the pH optima are not a reflection of two independent enzymes. Similar observations have been reported for Ehrlich ascites ribonuclease[27], myosin ATPase[28], and other enzymes[29].

The relative activity of the lytic enzyme in lysates of N20F'-infected cells is roughly correlated with the plaque morphology of the various mutants isolated. Three such strains, N20F'Tu, N20F'Ltu, and N20F'Cl produce turbid, less turbid, and clear plaques, respectively. The specific activity of the lytic enzymes derived from host cells infected by these phage increases with increased plaque clarity.

Data derived from the sedimentation velocity studies of the purified protein and from molecular weight determination reveal that the lytic enzyme can exist in monomeric and dimeric states. The transition between these two molecular species is reversible. Dimers are the predominant molecular species at pH 8.5, in 0.05 M Tris, whereas at pH 5.5 in 0.05 M Tris–maleate, only monomers are present. The use of 0.05 M Tris–maleate at pH 8.5 prevents the dimerization of the monomers, but reduction in the

concentration of the buffer to 0.01 M at pH 8.5 permits dimerization to occur. The sedimentation coefficient in 0.01 M Tris–maleate at the acid pH is the same as the $s_{20,w}$ value in 0.05 M Tris at pH 8.5 indicating the presence of dimers. The effect of mono- and dicarboxylic acids indicate that high concentrations of carboxyl groups will tend to push the monomer–dimer equilibrium wholly in the direction of monomer formation. These data are interpreted to mean that in the absence of, or at low concentrations of, carboxyl groups, the enzyme exists as a heterogeneous mixture of monomers and dimers throughout the entire pH range, but at relatively high concentrations of maleate or other carboxylic acids, monomers are the only molecular species present. It is interesting that monomer–dimer transitions dependent on pH and on acetate buffer (at pH 4.7) have been reported for egg white lysozyme[24–26] and for hemoglobin[30,31], respectively. Further, the effect of maleate on inhibition of dimer formation has recently been reported for rabbit muscle aldolase by SIA AND HORECKER[32].

The following table summarizes the effects of various experimental conditions on the catalytic properties and the state of aggregation of the lytic enzyme at pH 5.5 and 8.5.

Experimental conditions	*pH 5.5*	*pH 8.5*
High carboxylic acid concn., 0.05 M	Monomers, active	Monomers, inactive
Low carboxylic acid concn., 0.01 M	Dimers, inactive	Dimers, active
EDTA and divalent metal ions	Activate	Inhibit

The effect of divalent cations on both the acid and alkaline activities of the lytic enzyme can also be interpreted in terms of the part that these ions play in determining the molecular form of the enzyme. The results show that high concentrations of cations cause a disaggregation of dimers to form monomers at both pH optima. Under these conditions the monomers would be active at pH 5.5 but inactive at pH 8.5. These considerations are supported by the experimental observations that divalent cations enhance the pH 5.5 activity but inhibit at pH 8.5. Thus it would appear that the splitting of the dimer is a complex phenomenon and might involve the binding of metal ions and carboxyl groups. The different chemical nature of this two reagents might also suggest multiple binding sites which are distinct from the catalytic loci. Similar results regarding the disaggregation of glutamate dehydrogenase in the presence of DPNH and divalent metal ions have been reported[33,34].

The experimental evidence indicates that the monomeric form accounts for the pH 5.5 activity while the dimeric form is involved at pH 8.5. This hypothesis suggests that the pH dependence of these two activities may involve one or more enzymatically active sites. The site that is active at pH 5.5 in the monomer could also be the region that participates in the formation of the dimer. If the dimer is formed by the interaction of the pH 5.5 active sites of two monomers, then changes in molecular conformation together with the ionization of different chemical groups at alkaline pH could result in another lytic activity. An alternative proposal could require the presence of two separate sites, one involving the monomer, and the other arising after dimer formation but without the involvement of active monomer sites. A single active site responsible for both activities could also be visualized if the same pH 5.5 site undergoes some

specific conformational changes after dimer formation which would now allow this site to be active under alkaline conditions. Heat denaturation studies indicate that the heat stability of the enzymatic site(s) involved at the two pH optima is different.

These proposals therefore suggest that different or altered active sites of the same lytic enzyme may perform the same type of enzymatic function. The enzymatic activity is dependent both on the molecular form of the enzyme, and on the H^+ concentration which governs the ionic state of the active site.

The results of the amino acid studies show that the enzymes from the three mutants differ in their primary amino acid sequences. The initial N-terminal amino acids in the three mutant enzymes are the same, but the amino acid sequence at the C-terminal end differs considerably. It seems likely that the three bacteriophage mutants could arise spontaneously by the addition or deletion of a base resulting in a reading frame shift of the genetic message[35]. However, the results could also be explained on the basis of point mutations (amber or ochre). The second explanation implies that point mutations occurring in distant parts of the gene would lead to changes in the number of amino acids in the final protein, *i.e.*, the amber or ochre mutation causing a termination of the genetic message. These changes would be expected to be reflected in molecular weight changes of the enzymes. Since the molecular weights and the amino acid composition of the three enzymes are similar, this would tend to argue against the above mechanism in favor of a frame shift mutation.

The results also indicate that the mutation occurs in the region of the gene which codes for the C-terminal portion of the protein. The differences in the amino acid sequence of the three mutant enzymes are also reflected in the different peptide "fingerprint maps" that are obtained. These alterations at the C-terminal ends of the three enzymes could therefore provide an explanation for differences in the specific activities that are observed.

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REFERENCES

1 T. C. MERIGAN AND W. J. DREYER, *Ann. N.Y. Acad. Sci.*, 103 (1963) 765.
2 G. STREISINGER, G. F. MUKAI, W. J. DREYER, B. MILLER AND S. HORIUCHI, *Cold Spring Harbor Symp. Quant. Biol.*, 16 (1961) 25.
3 J. S. MURPHY, *Virology*, 11 (1960) 510.
4 N. B. GROMAN AND G. SUZUKI, *J. Bacteriol.*, 84 (1962) 596.
5 F. JACOB AND C. R. FUERST, *J. Gen. Microbiol.*, 19 (1958) 518.
6 E. N. FOX AND M. K. WITTNER, *J. Bacteriol.*, 89 (1965) 496.
7 B. REUTER AND J. D. ORAM, *J. Gen. Microbiol.*, 32 (1963) 29.
8 W. MOO-PENN AND H. WIESMEYER, *Biochim. Biophys. Acta*, 178 (1969) 318.
9 D. FRASER, *J. Bacteriol.*, 61 (1951) 115.
10 A. TSUGITA, M. INOUYE, E. TERZAGHI AND G. STREISINGER, *J. Biol. Chem.*, 243 (1968) 391.
11 E. A. PETERSON AND H. A. SOBER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 5, Academic Press, New York, 1962, p. 3.
12 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
13 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.

14 S. ZAMENHOF, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 696.
15 W. J. ARCHIBALD, *J. Phys. Colloid Chem.*, 51 (1947) 1204.
16 S. M. KALINER AND G. KEGELES, *J. Phys. Chem.*, 59 (1955) 952.
17 H. K. SCHACHMAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 4, Academic Press, New York, 1957, p. 32.
18 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
19 C. H. W. HIRS, *J. Biol. Chem.*, 219 (1956) 611.
20 S. HARSHMAN AND V. A. NAJJAR, *Biochemistry*, 4 (1965) 2526.
21 H. FRAENKEL-CONRAT, H. HARRIS AND A. L. LEVEY, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 2, Interscience, New York, 1955, p. 359.
22 R. L. HILL, D. H. SPACKMAN, D. M. BROWN AND E. L. SMITH, in C. S. VESTLING, *Biochemical Preparations*, Vol. 6, Wiley, New York, 1958, p. 35.
23 A. KATZ, W. J. DREYER AND C. B. ANFINSEN, *J. Biol. Chem.*, 234 (1959) 2897.
24 D. B. SMITH, G. C. WOOD AND P. A. CHARLWOOD, *Can. J. Chem.*, 34 (1965) 364.
25 A. J. SOPHIANOPOULOS AND K. E. VAN HOLDE, *J. Biol. Chem.*, 236 (1961) PC 82.
26 A. J. SOPHIANOPOULOS AND K. E. VAN HOLDE, *J. Biol. Chem.*, 239 (1964) 2516.
27 G. A. LEVY AND C. A. MARSH, *Advan. Carbohydrate Chem.*, 14 (1959) 381.
28 J. S. COLTER, J. KUHN AND K. A. O. ELLEM, *Cancer Res.*, 21 (1961) 48.
29 S. J. LEVIN AND O. BODANSKY, *J. Biol. Chem.*, 241 (1966) 51.
30 R. BANERJEE AND L. SAGAERT, *Biochim. Biophys. Acta*, 140 (1967) 266.
31 E. CHINCONE AND G. A. GILBERT, *J. Biol. Chem.*, 240 (1965) 3866.
32 C. L. SIA AND B. L. HORECKER, *Biochem. Biophys. Res. Commun.*, 31 (1968) 731.
33 K. L. YIELDING, G. M. TOMKINS, M. W. BITENSKY AND N. TALAL, *Can. J. Biochem.*, 42 (1964) 727.
34 K. L. YIELDING, G. M. TOMKINS AND D. S. TRUNDLE, *Biochim. Biophys. Acta*, 77 (1963) 703.
35 F. H. C. CRICK, L. BARNETT, S. BRENNER AND R. J. WATTS-TOBIN, *Nature*, 192 (1961) 27.
36 T. F. SPANDE AND B. WITKOP, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 11, Academic Press, New York, 1967, p. 498.