SUMMARY

N-acetylseryltyrosine was isolated from the chymotryptic and peptic digests of TMV-protein. The structure of the acetylpeptide purified by ion-exchange chromatography, was studied by the FDNB method, hydrazinolysis and the use of carboxypeptidase. For the characterization of the acyl group in the peptide, hydrazinolysis was also used. The location of the acyl peptide in the protein was discussed.

REFERENCES

1 H. FRAENKEL-CONRAT AND B. SINGER, *J. Am. Chem. Soc.*, 76 (1954) 180.
2 H. FRAENKEL-CONRAT, in *Ciba Foundation Symposium on Chemical Structure of Proteins*, Edited by G. E. W. WOLSTENHOLME AND M. P. CAMERON, J. and A. Churchill, Ltd., London 1953, p. 102.
3 G. SCHRAMM AND G. BRAUNITZER, *Z. Naturforsch.*, 8b (1953) 61.
4 G. SCHRAMM, G. BRAUNITZER AND J. W. SCHNEIDER, *Nature*, 176 (1955) 456.
5 G. SCHRAMM, J. W. SCHNEIDER AND F. A. ANDERER, *Z. Naturforsch.*, 11b (1956) 12.
6 G. BRAUNITZER, *Biochim. Biophys. Acta*, 19 (1956) 574.
7 G. BRAUNITZER, *Naturwissenschaften*, 42 (1955) 371.
8 G. SCHRAMM AND F. A. ANDERER, *Naturwissenschaften*, 42 (1955) 74.
9 H. FRAENKEL-CONRAT, *Virology*, 4 (1957) 1.
10 M. SREENIVASNYA AND N. W. PIRIE, *Biochem. J.*, 32 (1938) 1708.
11 K. NARITA, S. FUJIWARA AND S. MURASAWA, *Bull. Chem. Soc. Japan*, in the press.
12 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
13 F. SANGER, *Biochem. J.*, 39 (1945) 507.
14 A. L. LEVY, in *Methods of Biochemical Analysis*, Vol. 2, Interscience Publishers, New York, 1955, p. 360.
15 S. AKABORI, K. OHNO AND K. NARITA, *Bull. Chem. Soc. Japan*, 25 (1952) 214.
16 S. AKABORI, K. OHNO, T. IKENAKA, Y. OKADA, H. HANAFUSA, I. HARUNA, A. TSUGITA, K. SUGAE AND T. MATSUSHIMA, *Bull. Chem. Soc. Japan*, 29 (1956) 507.
17 C.-I. NIU AND H. FRAENKEL-CONRAT, *J. Am. Chem. Soc.*, 77 (1955) 5882.
18 J. I. HARRIS, in *Methods of Biochemical Analysis*, Vol. 2, Interscience Publishers, New York, 1955, p. 397.
19 K. SATAKE AND T. SEKI, *J. Japan Chem.* (Kagaku no Ryoiki), 4 (1950) 557; *C.A.*, 45 (1951) 4604.
20 H. N. RYDON AND P. W. G. SMITH, *Nature*, 169 (1952) 922.
21 R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press Inc., New York, 1955, p. 157.
22 E. J. CONWAY AND M. DOWNEY, *Biochem. J.*, 47 (1950) iv; E. J. CONWAY, *Microdiffusion Analysis and Volumetric Error*, 3rd Ed., Crosby Lockwood, London, 1950, p. 246.

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PURIFICATION OF GLUTAMIC-ASPARTIC TRANSAMINASE

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Several methods for the partial purification of glutamic-aspartic transaminase from heart muscle have been developed[1-4], the most recent being that by CAMMARATA AND COHEN in 1951. These purification schemes are based on the classical methods of fractional precipitation with salts and organic solvents. During the past few years, however, new and more effective techniques for the fractionation of proteins have been developed. The remarkable success with which they have been applied to the purification of other enzymes seemed to make it worth while to try a new approach

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to the problem of the purification of transaminase. A preliminary note on the use of carboxymethyl cellulose for the purification of this enzyme was published recently by SOBER AND PETERSON[6]. The following report describes the preparation of a highly purified holotransaminase by the use of ion-exchange chromatography and zone electrophoresis.

MATERIALS AND METHODS

The L-glutamic and L-aspartic acids were obtained from Merck and Co., the oxalacetic acid and the pyridoxal-5-phosphate used as coenzyme from California Foundation for Chemical Research, Los Angeles, U.S.A. and the α-ketoglutaric acid from Sigma Chemical Company, St. Louis, U.S.A.

Measurement of transaminase activity. The spectrophotometric method according to CAMMARATA AND COHEN[6] was employed. The substrate concentrations used in the assay cell were 3.12 mmoles/ml and the temperature was 25°. Unless otherwise stated, no synthetic coenzyme was added.

The reaction was followed by observing the formation of oxalacetic acid. Density readings were taken at intervals of 2 min for 6–8 min. The rate of transamination remained constant during this period.

One enzyme unit is defined as that amount of enzyme which will give an increase in optical density of 0.001 unit/min under the conditions specified above.

Ion-exchange chromatography. The carboxymethyl (CM) cellulose used for the column chromatography was prepared according to PETERSON AND SOBER[7] from a cellulose powder supplied by Mo and Domsjö AB, Sweden. This exchanger contained 0.45 mequiv. of carboxyl groups per g. The powder was homogenized in a Waring blendor and the smallest particles were discarded by decantation. The CMC was then suspended in 0.01 *M* acetate buffer, pH 5.4, poured into the column to the desired height and equilibrated with the same buffer. The protein solution, adjusted to the same pH and buffer concentration, was applied to the column, and elutions were made by stepwise increases in the concentration of buffer. The effluent was automatically collected in fractions and the fractions were examined for their protein content by measuring their extinction at 280 mμ in 1 cm cells in a Beckman DU spectrophotometer. Their transaminase activity was determined according to the method described above.

Zone electrophoresis with cellulose powder. The apparatus used and procedure followed were those described by PORATH[8]. The protein solution was concentrated to 2 ml and applied to the top of the column (80 × 1.8 cm) and washed into the column with 3–5 ml of buffer, ionic strength 0.03, pH 7.4. The top of the column was connected to the cathode, the bottom to the anode and a current of 20 mA (1300 V) was applied for 30 hours. After the completion of the run the column was eluted at a flow rate of about 20 ml/h. Fractions of 3 ml each were collected and examined as described above.

Precipitation with acetone was used as a preliminary purification step. The precipitation was carried out in the cold room (+4°). The protein solutions were chilled to this temperature and acetone concentrations were computed on a volume basis; it was assumed that the volumes of acetone and protein solutions were additive. The precipitates were left for half an hour to equilibrate with the solution and centrifuged at 4°.

Recovery of the enzyme from the effluent fractions. At two phases of the purification scheme one is confronted with the necessity of recovering the enzyme from relatively dilute, salt-containing solutions. One way of working up such solutions is to remove the buffer salts by dialysis and concentrate by lyophilization. Sometimes it was observed, however, that this procedure led to considerable inactivation of the enzyme. This treatment had particularly detrimental effects on the highly purified material obtained after zone electrophoresis. In the attempt to recover the enzyme without inactivation, precipitation with acetone was tried and proved to be a most suitable method for this purpose. The operations were performed as follows:

(a) Recovery of the enzyme from the solution after chromatography on ion-exchange column.

The enzyme was precipitated by addition of 2 volumes of acetone, and the precipitate was centrifuged and taken up in water (about 10% of the original volume of the solution). While a part of the precipitate was insoluble, the transaminase was readily dissolved and the recovery of activity was 90%. The concentrated, salt-free solution of the enzyme thus obtained was lyophilized or used directly for electrophoresis.

(b) Recovery of the enzyme after electrophoresis.

The pH of the solution was adjusted to 5.4 in order to facilitate the precipitation of the enzyme and prevent the precipitation of phosphate from the buffer. The precipitated enzyme was recovered by centrifugation and freeze-dried.

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Method of purification. One kg of pig heart that had been removed and packed in ice immediately after slaughtering the animal, was minced, suspended in 2 l of distilled water (chilled to 4°), and extracted for 4 hours at 4°. The extract was centrifuged in the cold at 3000 r.p.m., the residue washed with water and the combined extracts were filtered (activity = 80 units/mg of protein). The pH of the clear, dark red filtrate was then adjusted to pH 5.4 with 0.1 *M* acetic acid. Sometimes a slight precipitation was observed at this point. This precipitate did not contain any transaminase activity and was subsequently discarded with the first acetone precipitate. Acetone was added to a final concentration of 33%.

Procedure

Precipitate PTD I	*Solution*
Discarded	Acetone was added to 50%.
Solution	*Precipitate PTA I* (activity = 150 units/mg protein).
Discarded	Suspended in 1,000 ml water and centrifuged.
Precipitate	*Solution*
Discarded	pH adjusted to 5.4; acetone was added to a concentration of 28%.
Precipitate PTD II	*Solution*
Discarded	Acetone to a final concentration of 60%.
Solution	*Precipitate PTA II* (activity = 250 units/mg protein).
Discarded	Suspended in 500 ml water and centrifuged.
Precipitate	*Solution*
Discarded	Acetate buffer, pH 5.4 was added to give a concentration of 0.01 *M* and the solution was applied to the CM cellulose column.

The enzyme was recovered from the fraction eluted from the column with 0.06 *M* buffer (activity = 2,000 units/mg protein) and further purified by zone electrophoresis in a cellulose powder column (final activity = 4,000 units/mg protein).

RESULTS AND DISCUSSION

Precipitation with acetone proved to be a very suitable method for removing much of the accompanying impurities prior to applying the material to the ion-exchange column. Transaminase seems to be quite insensitive to denaturation in the presence of even high acetone concentrations and at temperatures as high as 10–15°: it retains full activity for some hours even at room temperature in 30% acetone. The acetone remaining in the precipitate can be dialysed away without loss of activity.

The recoveries and degrees of purification obtained by the acetone fractionation are shown in Table I.

TABLE I

	% *Total protein*	% *Total activity*	*Purification factor*
PTA I	45–50	80	2
PTA II	20	60	3

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By far the highest one-step purification was obtained, however, by ion-exchange chromatography on CM cellulose. Fig. 1 shows a part of the elution curve from an experiment with 800 mg PTA II on a column (30·2.2 cm) with a total volume of 115 ml and a volume of 25 ml of adsorbent. The cellulose was equilibrated with 0.01 *M* acetate buffer at pH 5.4. The adsorption was carried out at this molarity and the elution was started with 0.06 *M* buffer, followed by 0.10 *M* and 0.40 *M* buffer, finishing with 0.1 *M* NaOH. Fractions were collected at 5-min intervals, giving 3 ml volumes. Practically all activity was eluted at the first elution step with 0.06 *M* buffer.

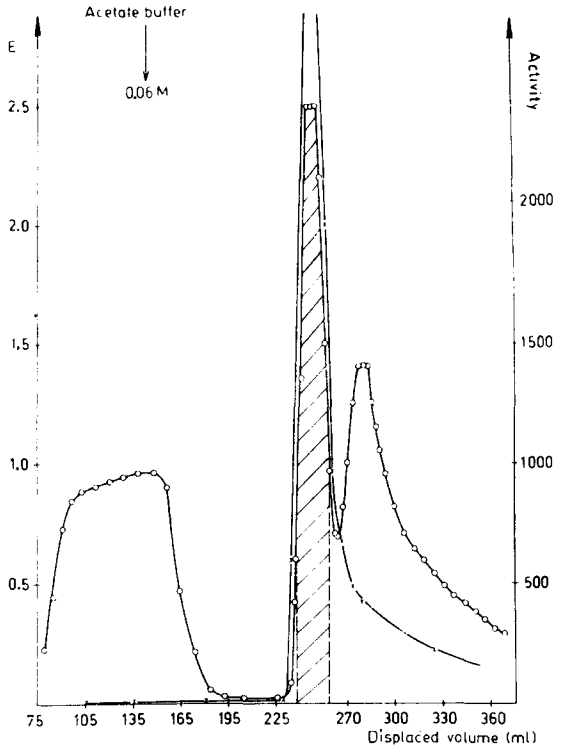

Fig. 1. Part of the elution diagram obtained with 800 mg of PTA II on a column 30 × 2.2 cm with CM cellulose. The arrow indicates the start of elution at the top of the column with 0.06 *M*, acetate buffer, pH 5.4. O–O–O–O extinction at 280 mμ; × -×-×-× transaminase activity expressed in units/ml.

Two zones were obtained in this range. The dashed area (see figure) accounts for 55% of the activity added to the column, but it contains only 5% of the protein material. This represents a 12-fold purification. It was found, however, that in order to obtain this high degree of purification, very rigorous adherence to the experimental conditions described above is required. In other experiments, when using larger columns or varying amounts of protein material, the results were, as a matter of fact, less satisfactory. While the chromatographic behaviour of the zone with transaminase activity was the same in all experiments, the overlapping of the two protein peaks in the 0.06 *M* elution range was increased, thus giving a lower specific activity and hence a lower degree of purification. The purification was, in such cases, 6- to 8-fold,

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and the material obtained was obviously heterogeneous. This inhomogeneity was confirmed by paper electrophoresis. At all values tested over the pH range 5.4–8.6, the presence of 3 spots was observed. As the best resolution was obtained at pH 7.4, zone electrophoresis on a cellulose powder column was performed at this pH with phosphate buffer, 0.03 *M*. The electropherogram obtained is shown in Fig. 2. Three major components are seen, the activity being associated with only one component, namely, the slowest moving one*. The ratio of activity units: optical density is constant throughout the peak and about twice as high as that for the starting material. For comparison the material purified according to the optimal conditions for ion-exchange chromatography (Fig. 1) was subjected to electrophoresis in the same buffer. The result is represented in Fig. 3. A comparison with Fig. 2 reveals the absence of the leading component and a shift in the relative percentage com-

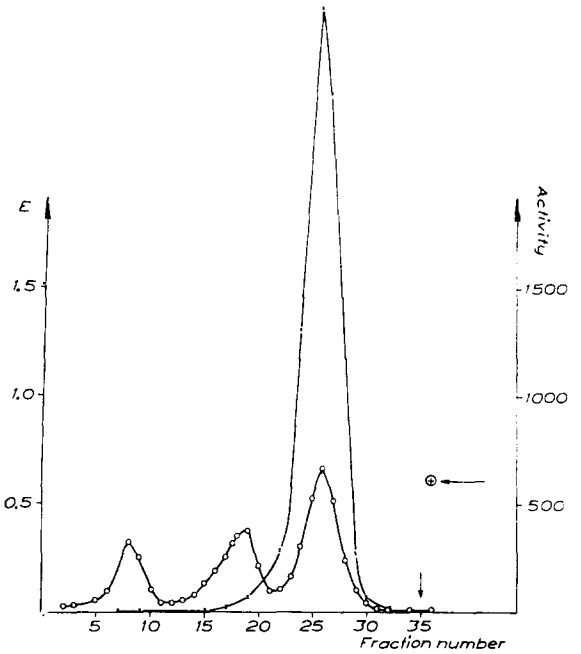

Fig. 2. Electropherogram of the active fraction obtained from ion-exchange chromatography. The electrophoretic run was made in phosphate buffer pH 7.4, ionic strength 0.03, on a 80 × 1.8 cm column for 30 hours at 20 mA. O–O–O–O extinction at 280 mμ; ×–×–×–× transaminase activity, expressed in units/ml.

position of the remaining components. As for the enzymic activity, however, no difference is found between the two experiments. Not only is the distribution the same, but even the ratio of activity units/optical density remains unchanged.

It is thus evident, that the ultimate purification, achieved by the combination of ion-exchange chromatography and zone electrophoresis, is the same in all cases, even in those in which the purification obtained by the chromatography step alone is not fully satisfactory.

* At the same pH as used here, previous workers[1,2] found the activity associated with two components. The reason for this discrepancy is not known; it may be due to differences in the starting material or to methodological differences between boundary and zone electrophoresis.

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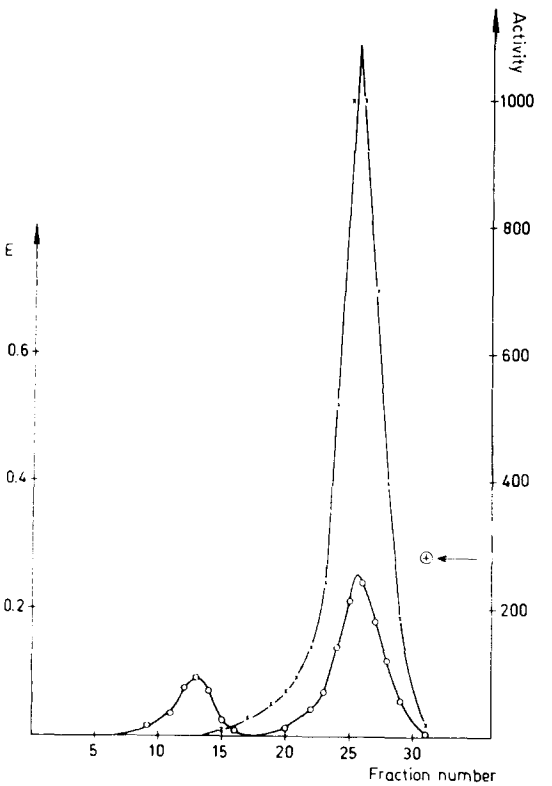

Fig. 3. Electropherogram of the material, corresponding to the dashed area in Fig. 1. The conditions of the run as in Fig. 2. O–O–O–O extinction at 280 mμ; ×–×–×–× transaminase activity, expressed in units/ml.

Activity and stability of the purified enzyme

The enzyme, recovered from the effluent fractions after electrophoresis and lyophilization, was obtained as a fine, water-soluble, slightly yellow powder. It yielded, when assayed under the conditions described earlier, an activity of 4000 units/mg protein. When assayed in the opposite direction, *i.e.* from right to left in the system:

$$\text{aspartic acid} + \alpha\text{-ketoglutaric acid} \rightleftharpoons \text{oxalacetic acid} + \text{glutamic acid}$$

as employed by CAMMARATA AND COHEN, it yielded an activity of 10,000 units/mg protein, which is twice the specific activity of the preparation reported by these authors. No difference in activity was observed on addition of synthetic coenzyme to the assay solution, indicating that the enzyme thus obtained is the holoenzyme.

The stability of the enzyme in the dry state was followed for only two weeks, but during this period no decline in activity was observed. The stability of solutions of the enzyme was studied at a number of different pH values. The solutions were 0.05 *M* in buffer and contained 0.65 mg protein/ml. For the activity determinations 10 μl of the solutions were added to the assay cell, giving a concentration of 2 γ enzyme per ml. The results are summarized in Table II.

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TABLE II

pH	Activity in units/ml of original solution after			
	0 h	½ h	36 h	72 h
4.5	2600	2600	2500	2550
5.4	2600	2600	2550	2500
7.4	2600	2600	2600	2450
8.6	2600	2600	2400	2000
9.2	2600	2400	1900	1500

In order to get some idea of the electrophoretical homogeneity of the purified enzyme, we submitted 200 γ of the sample to paper electrophoresis. The experiments were performed at pH 5.4, 7.4, and 8.6. In no case could more than one spot be detected on the developed electropherograms. Since 10 γ (or even less) of protein are still fairly visible on paper, this means that any impurities present would not account for more than 5% of the sample by this method. Some importance should be given to the observation of a perfectly constant (within the limits of experimental error) specific activity throughout the peak eluted from the electrophoresis column. Without being proof of the purity and homogeneity of the material, it offers, nevertheless, some evidence in its favour.

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SUMMARY

A scheme for the preparation of a highly purified glutamic-aspartic transaminase is reported. The methods employed are, precipitation with acetone, ion-exchange chromatography on carboxymethyl cellulose, and zone electrophoresis. A purification degree of 50 is achieved and the enzyme obtained is the holoenzyme. When studied in zone electrophoresis, the enzyme preparation appears to be a homogeneous protein, as judged from the constant specific activity across the zone.

REFERENCES

1 P. S. CAMMARATA AND P. P. COHEN, *J. Biol. Chem.*, 193 (1951) 53.
2 D. E. GREEN, L. F. LELOIR AND V. NOCITO, *J. Biol. Chem.*, 161 (1945) 559.
3 D. E. O'KANE AND I. C. GUNSALUS, *J. Biol. Chem.*, 170 (1947) 425.
4 F. SCHLENK AND A. FISHER, *Arch. Biochem.*, 12 (1947) 60.
5 H. A. SOBER AND E. A. PETERSON, *J. Am. Chem. Soc.*, 76 (1954) 1711.
6 P. S. CAMMARATA AND P. P. COHEN, *J. Biol. Chem.*, 193 (1951) 45.
7 E. A. PETERSON AND H. A. SOBER, *J. Am. Chem. Soc.*, 78 (1956) 751.
8 J. PORATH, *Biochim. Biophys. Acta*, 22 (1956) 151.

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