331. *Transamination. Part I. The Isolation of the Apoenzyme of Glutamic–Aspartic Transaminase from Pig Heart Muscle.*

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A procedure for the preparation, in quantity, of the apoenzyme of glutamic–aspartic transaminase from pig heart muscle is described. The final preparation had a higher specific activity than any previously reported for either the holoenzyme or the apoenzyme, and free-flow electrophoresis showed it to be, with respect to this technique, homogeneous. Convenient methods for following the transamination are described. Some properties of the apoenzyme are discussed.

In order to carry out chemical and kinetic studies on glutamic–aspartic transaminase and to investigate the binding between cofactor and apoenzyme we required a procedure for the preparation of relatively large quantities of apoenzyme in a homogeneous state. This paper describes such a procedure.

In the earliest studies of the purification of this enzyme, by Green, Leloir, and Nocito,[1] Schlenk and Fisher,[2] and O'Kane and Gunsalus,[3] yields and purification factors (11, 17, and 26, respectively) were relatively low and only O'Kane *et al.* achieved full resolution (*i.e.*, dissociation into apoenzyme and pyridoxal 5'-phosphate). Free-flow electrophoresis (phosphate buffer, pH 7·4) showed three components in the preparations of Green *et al.* and of Schlenk and Fisher. Both groups reported that activity was associated with two of the components.

Cammarata and Cohen [4] suggested that resolution results in partial, irreversible inactivation of the apoenzyme and described a procedure for the purification of the holoenzyme. The specific activity * (S.A.) of their final preparation, however, appears to be no greater than that of preparations obtained by the method of O'Kane and Gunsalus. Free-flow electrophoresis in phosphate (pH 7·4), and in barbiturate buffer (pH 8·6) showed the presence of three components in each preparation. It was reported, however, that, whereas in phosphate buffer two of the components contained transaminase activity, in barbiturate buffer only one was active.

An electrophoretically homogeneous preparation of the holoenzyme was obtained by Lis [5] who used zone-electrophoresis as the final step. However, comparison with our results (see below) indicates that the specific activity of this preparation (*ca.* twice that reported by Cammarata and Cohen [4]) is low and considerable inactivation must, consequently, have occurred in her purification. The most successful method for the preparation of the holoenzyme appears to be that described by Jenkins, Yphantis, and Sizer.[6] Free-flow electrophoresis showed their sample to contain 87% of the holoenzyme and comparison with our data indicates that no extensive inactivation occurs during their purification.

Detailed comparison, in terms of specific activities, of our preparation with those obtained by other workers is made, where possible, in the discussion section.

Experimental

Materials.—L-Aspartic and L-glutamic acids (Roche Products Ltd.) were chromatographically homogeneous: at 280 mμ, $\varepsilon = 0{\cdot}1$ and $0{\cdot}4$ respectively (phosphate buffer, 0·1M, pH 7·38). α-Oxo-glutaric acid, recrystallised four times from acetone–benzene, had m. p.

* We use this term to mean the catalytic activity, expressed in suitable, arbitrary, units, of any given preparation, per mg. by weight of protein.

[1] Green, Leloir, and Nocito, *J. Biol. Chem.*, 1945, **161**, 559.
[2] Schlenk and Fisher, *Arch. Biochem.*, 1947, **12**, 69.
[3] O'Kane and Gunsalus, *J. Biol. Chem.*, 1947, **170**, 425.
[4] Cammarata and Cohen, *J. Biol. Chem.*, 1951, **193**, 53.
[5] Lis, *Biochim. Biophys. Acta*, 1958, **28**, 191.
[6] Jenkins, Yphantis, and Sizer, *J. Biol. Chem.*, 1959, **234**, 51.

114°, and ε 28 at 280 mμ (phosphate buffer, 0·1M, pH 7·38). Oxaloacetic acid, recrystallised three times from boiling ethyl acetate, had m. p. 151° (decomp.). In tris(hydroxymethylamino)methane buffer (0·0667M) of pH 7·4 or 8·3, and in phosphate buffer (0·1M) of pH 7·38, ε = 625, 635, and 550, respectively, at 280 mμ; in borate buffer (0·03M) of pH 9·2, ε = 1730 at 270 mμ.

Pyridoxal 5′-phosphate (Roche Products Ltd.) had $\lambda_{max.}$ 325, 388 mμ (ε 2860, 5180) in phosphate buffer (0·1M, pH 7·38). Pyridoxamine 5′-phosphate (Californian Foundation for Biochemical Research), had $\lambda_{max.}$ 253, 326 mμ (ε 5250, 9800) in phosphate buffer (0·1M, pH 7·38). Calcium phosphate gel was prepared as described by Dixon and Webb.[7]

Pig's hearts were obtained from Wall's Bacon Factory and were either used immediately or stored at −20°. Fatty and vascular tissue was removed before use.

L-Glutamic decarboxylase was obtained from *E. coli* (NCTC 8548). The organisms were grown at 37° on a medium containing lab. lemco (1%), peptone (1%), sodium chloride (0·5%), and L-glutamic acid (0·5%). The cells were harvested after 18 hours' growth, and an acetone powder was prepared from them in the usual way. A standard solution of L-glutamic acid (10^{-5} mole) was completely decarboxylated in 15 min. (pH 5·0) by 0·5 c.c. of a 2% suspension of the acetone powder in water.

Assay Methods.—Two methods were used, both based on the fact that oxaloacetic acid has a higher absorption in the region 280 mμ than any of the other reaction components.

Method 1.* A freshly prepared solution (22 c.c.) of sodium aspartate, α-oxoglutaric acid, and phosphate buffer (0·1M, pH 7·38) was allowed to reach thermal equilibrium in a thermostat bath (25°). A solution (2 c.c.), also at 25°, of activated enzyme and phosphate buffer (0·1M, pH 7·38) was added to start the reaction. Aliquot parts (5 c.c.) were withdrawn at appropriate times (all within 10 min. from time zero), and run into standard flasks (10 c.c.) containing 30% trichloroacetic acid (0·5 c.c.). After addition of 0·5M-sodium hydroxide (sufficient to neutralise the trichloroacetic acid) and borate buffer (2 c.c.; 0·3M, pH 9·4), the contents of the flask were made up to the mark. The flasks were left for $\geqslant$10 min. (thus minimising errors due to decarboxylation of oxaloacetic acid), and the optical densities of the solutions at 270 mμ were determined in a 1 cm. cell using a Unicam spectrophotometer (S.P. 500). It was shown that the absorption of oxaloacetic acid in borate buffer obeys Beer's law up to concentrations of *ca.* 5×10^{-4}M, and also that the decarboxylation of oxaloacetic acid is negligibly slow under all relevant conditions.

The unit of enzyme activity is defined as that amount of enzyme which, under the standard conditions described above and with concentrations of sodium aspartate and α-oxoglutaric acid of, $8{\cdot}3 \times 10^{-3}$M and $2{\cdot}08 \times 10^{-3}$M, respectively, produces, in 10 min., an amount of oxaloacetic acid corresponding to an increase in the optical density of the final solution of 0·10. This increase in optical density corresponds to the production of $1{\cdot}40 \times 10^{-4}$M-oxaloacetic acid in the assay mixture, and is *ca.* 13% of the increase expected at complete reaction (calculated from the value of the equilibrium constant given below). Experiments showed that, under the above conditions, the reaction rate is independent of time up to *ca.* 20% reaction. The initial reaction rate (v'), therefore, can conveniently be found from the linear portion of a plot of optical density against time. Consequently, the amount of enzyme used in each experiment was chosen so that the increase in optical density at the longest time interval was less than 0·16 (*i.e.*, less than $2{\cdot}2 \times 10^{-4}$M-oxaloacetic acid produced).

The following experiment is illustrative. An enzyme solution (total protein 3·55 mg. c.c.$^{-1}$) was diluted (1 : 1000), and then assayed:

t (min.)	2·0	4·5	7·0	9·5
O.D. (270 mμ)	0·237	0·257	0·278	0·298

This gives $v' = 0{\cdot}0082$ O.D. unit min.$^{-1}$. The undiluted enzyme solution, therefore, contained 820 units c.c.$^{-1}$; specific activity 231.

It was established that, under the above assay conditions, v' is proportional to enzyme concentration (E), providing that this does not exceed *ca.* 0·1 unit c.c.$^{-1}$ (in the assay solution).†

* This is a modification of the method first introduced by Greenwood and Greenbaum.[8]

† At higher concentrations v' increases less rapidly than E. The functional dependence is, however, complicated and involves the substrate and the buffer concentration. This problem will be discussed in a later publication.

[7] Dixon and Webb, " The Enzymes," Longmans, Green and Co., London, 1958, p. 51.

[8] Greenwood and Greenbaum, *Biochim. Biophys. Acta*, 1953, **10**, 623.

Method 2. The mixture (substrates and enzyme in phosphate buffer of 0·1M, pH 7·38) was placed in a 1 cm. cell and the change in optical density recorded by using a Cary recording spectrophotometer. The control solution contained all the components except sodium aspartate. The reaction was initiated, as in method 1, by addition of the enzyme solution to a solution containing all the other components. For experiments of short duration, effective temperature control (25°) can be achieved by bringing the solutions to temperature in a thermostat-bath before initiation of the reaction. For experiments of longer duration a thermostatically-controlled cell-holder must be used. Readings could be obtained within 30 sec. of time zero. The initial reaction rate was found, as before, from the linear portion of the plot of optical density against time. A typical experiment (25°), with 10^{-2}M-sodium aspartate and $2·5 \times 10^{-3}$M-α-oxoglutaric acid, gave the data:

t (min.)	0·50	0·67	0·83	1·00	1·33	1·67	2·0	2·5	3·0
O.D. (280 mμ).....	0·042	0·056	0·071	0·085	0·111	0·135	0·156	0·184	0·208

The first five points lie on a straight line which passes through the origin; hence $v' = 0·084$ O.D. unit min.$^{-1}$.

To convert values of v' (optical density unit min.$^{-1}$) into values of v [mole l.$^{-1}$ (of oxaloacetic acid) min.$^{-1}$], the appropriate molar extinction coefficients (given above) of oxaloacetic acid and α-oxoglutaric acid must be used. The two assay methods gave, substantially, the same values of v. Table 1 illustrates with data obtained at 25° with sodium aspartate (10^{-2}M), α-oxoglutaric acid ($2·5 \times 10^{-3}$M), and phosphate buffer (0·1M, pH 7·38).

TABLE 1. *Comparison of assay methods.*

Enzyme solution (c.c.)	0·1	0·05	0·025	0·01	0·0025
$10^4 v$ { method 1	3·86	2·64	1·84	1·06	0·33
$10^4 v$ { method 2	3·78	2·55	1·72	1·00	0·32

Determination of Protein Concentration.—Routine protein determinations were made by measuring the absorption of buffered solutions (0·1M-phosphate, pH 7·38) against a control of the buffer solution, at 280 mμ in 1 cm. cells. The relation between absorbancy and dry weight (R = mg. c.c.$^{-1}$ O.D. unit^{-1}) was determined for samples at different stages in the purification procedure by drying to constant weight (at 100°) solutions of known absorbancy which had been exhaustively dialysed:

S.A. (unit mg.$^{-1}$)	275	420	610	700
R	1·04	0·86	0·79	0·74

Reactivation of Apoenzyme.—Preparations of the apoenzyme (*ca.* 1 unit c.c.$^{-1}$) were activated (25°; 0·1M-phosphate buffer, pH 7·38) by addition of pyridoxal 5′-phosphate ($7·5 \times 10^{-5}$M) or of pyridoxamine 5′-phosphate ($3·75 \times 10^{-4}$M). It was established that (*a*) complete activation occurred within 30 min., (*b*) the level of activity reached was independent of which cofactor was used, (*c*) no loss of activity occurred on subsequent dilution or change of pH, and (*d*) the level of activity obtained by activating concentrated solutions and then diluting them was the same as that obtained by activation after dilution.

Resolution of Holoenzyme.—Loss of cofactor is virtually complete if the holoenzyme is heated at 60° for 50 min. in 1·0M-phosphate buffer at pH 6·0. Since the enzyme is thermally more stable in concentrated solution, the resolution step was normally carried out (after dialysis) on the concentrated solution obtained after the first ammonium sulphate treatment (*i.e.*, from step 2 of the procedure given below). Experiments showed that all the activity present in the original solution could be restored, after resolution, by activation with pyridoxal 5′-phosphate as described above.

Estimation of L-*Glutamic Acid.*—The following procedure was used to estimate L-glutamic acid in mixtures containing oxaloacetic acid, α-oxoglutaric acid, L-aspartic acid, and transaminase. An aliquot part (5 c.c.) was run into 30% trichloroacetic acid (0·5 c.c.), and the mixture set aside for 5 min. After neutralisation with 0·5M-sodium hydroxide, sufficient acetate buffer (0·5M, pH 4·5) was added to bring the volume to 10 c.c. An aliquot part (5 c.c.) of the resulting solution was placed in a Warburg flask containing 0·5 c.c. of an active, cell-free

solution of L-glutamic decarboxylase in the side-arm. When thermal equilibrium (25°) was reached the reaction was started; evolution of carbon dioxide was measured for about 1 hr. With highly active L-glutamic decarboxylase nearly all the L-glutamic acid was decomposed within a few minutes, but it was necessary to take readings for longer in order to correct for the slow decarboxylation of oxaloacetic acid. Extrapolation to zero time of the final, linear portion of the plot of volume of gas evolved against time gives the volume of carbon dioxide produced from the L-glutamic acid (see Fig. 1).

Purification of the Apoenzyme.—Ammonium sulphate fractionations were carried out by slow addition of the solid to cooled solutions. Ethanol fractionations were carried out with careful temperature control (below 0°) and efficient stirring. Untreated Visking tubing was used for dialysis of solutions obtained from the early steps. For dialysis of solutions of the highly purified enzyme, Visking tubing was left for 24 hr., first, in 50% aqueous ethanol, secondly, in a solution containing EDTA (10^{-4}M) and glutathione (10^{-4}M).

Step 1. Sliced muscle was homogenized with water (weight ratio 1 : 5) in an M.S.E. Ato-Mix at maximum speed for 2 min. The suspension was stirred at 0° for 1 hr. and then centrifuged.

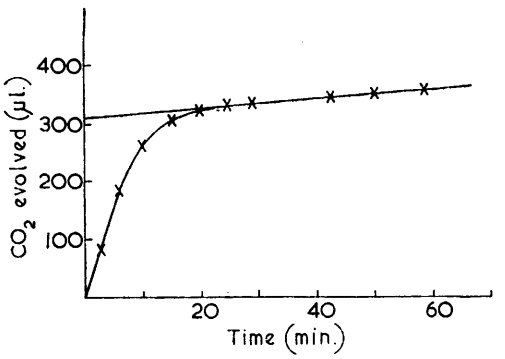


FIG. 1. *Decarboxylation of L-glutamic acid.*

Step 2. Ammonium sulphate (to 90% saturation; all percentages refer to 25°) was added to the solution and the precipitate removed, then dissolved in the *minimum* quantity of water and dialysed against running tap water until all the ammonium sulphate had been removed.

Step 3. Phosphate buffer (to M, pH 6·0) was added and the solution heated with occasional shaking for 50 min. in a water-bath at 60°. The precipitate was removed. The filtrate was used directly for step 4 (*i.e.*, without dialysis). Resolution of the enzyme was, at this point, virtually complete.

Step 4. The protein was fractionally precipitated with ammonium sulphate. The fraction 0—30% was discarded and the fractions 30—35, 35—40, and 40—45% were collected, dissolved in the minimum volume of water, dialysed, and then assayed. Fractions with S.A. > 75 were combined.

Step 5. Step 4 was repeated. The fraction 0—50% was discarded and the fractions 50—52·5, 52·5—57·5, and 57·5—60% were collected and treated as above. Fractions with S.A. > 100 were combined.

Step 6. Calcium phosphate gel was added to the combined active fractions from (5) and the whole was left for 5 min. before centrifugation. Only inactive protein is to be absorbed in this step and it is necessary to carry out pilot experiments to determine the optimum volume of gel. In general, the gel absorbed *ca.* 10 mg. of protein per c.c. The enzyme concentration in the supernatant layer must not, for stability reasons, be allowed to fall below 600 units c.c.$^{-1}$.

Step 7. Step 4 was repeated, fractions (2·5%) between 50 and 60% saturation being collected, and treated as before. Fractions with S.A. > 420 were combined. If none of the fractions was sufficiently active, steps (6) and (7) were repeated.

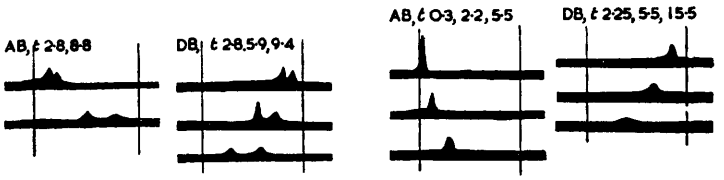
Step 8. The combined active fractions (0°) from step 7 were treated with ethanol at −20° in the presence of ammonium sulphate (0·3%). Preparations with S.A. > 420 contain only two protein components (as judged by free-flow electrophoresis), and these are precipitated at widely separated ethanol–water ratios. The fraction precipitated by an equal volume of ethanol was discarded. Addition of another volume of ethanol precipitated the apoenzyme. It was

centrifuged off, dissolved in the minimum of ice-cold water, and dialysed. The final solution was clear and colourless.

Table 2 illustrates the progress of purification. Except for step 8, all the activity present in the original solution can be accounted for. Step 8 results in considerable loss of activity (*ca* 50%).

Electrophoresis.—A preparative cell of the type described by Kekwick *et al.*[9] was used in a Tiselius electrophoresis apparatus (Hilger and Watts Ltd., Model M 470). The protein solutions (final concns. 0·7—1·0%) were exhaustively dialysed (48 hr.) in Visking tubing (treated as previously described) against two changes of the appropriate buffer (1 l.). Experiments were carried out at 4°. Table 3 summarises the results obtained. The Schlieren pictures obtained with the last two preparations of Table 3 are shown in Fig. 2.

FIG. 2. *Electrophoresis in phosphate buffer* (μ 0·1, pH 6·4).



First two diagrams, material of specific activity 530. *Last two diagrams, material of specific activity* 700. AB *and* DB = *ascending and descending boundary respectively.* t = *time in hours.*

Electrophoresis in maleate buffer resulted in considerable loss of enzymic activity. This buffer, therefore, does not seem to be suitable for preparative electrophoresis of the apoenzyme. In phosphate buffer (pH 6·4) the two components (of preparations with SA > 420) are well

TABLE 2. *Purification of glutamic–aspartic transaminase.*

Step	Volume (c.c.)	Enzyme (units c.c.$^{-1}$)	Protein (mg. c.c.$^{-1}$)	Specific activity (units mg.$^{-1}$)	Purifn. factor	Yield (%)
1	64,350	34·5	9·20	3·8	[1·0]	[100]
2	14,040	158	25·80	6·1	1·6	100
3	13,140	169	9·30	18·2	4·8	100
4	630	2890	33·7	86·0	22·6	82
5	230	7080	65·5	108	28·4	73
6, 805 c.c. gel	767	1420	7·8	182	48·0	49
7, 51·5—55·5%	109	6340	30·2	210	55·5	31
6, repeat, 139 c.c. gel	211	2208	8·05	274	72·0	21
7, repeat, 51·25—57·5%	73	5958	14·19	420	110	19·5
8, 50—60% ethanol	22	6140	8·77	700	184	6

TABLE 3. *Electrophoresis of glutamic–aspartic transaminase preparations.**

S.A. prep.	Buffer	Current (mA)	No. of compts.	S.A. LC	S.A. TC
290	Barbiturate, 0·1M, pH 8·6	12	3	I	A
330	Acetate, 0·2M, pH 5·9	10	3	—	—
540	Maleate, 0·03M, pH 6·4	10	2	I	A
480	Phosphate, 0·1μ, pH 6·4	10	2	I	A
530	Phosphate, 0·1μ, pH 6·4	8	2	0	730
700	Phosphate, 0·1μ, pH 6·4	8	1	700	

* S.A. = specific activity, LC = leading component, TC = tail component, I = inactive, A = active.

separated and little loss of activity occurs. Electrophoresis in this buffer can, therefore, be used on a preparative scale as an alternative to step 8 (ethanol fractionation) of the purification given above. With a preparation of specific activity 530, fractions of the following specific activities were obtained (on successive reversal of current), LC 730, 670, 730, 690, mean 705;

[9] Kekwick, Lyttleton, Brewer, and Dreblow, *Biochem. J.*, 1951, **49**, 253.

TC 0, 77, 36, 25, mean 34. Except for the first fractions removed, some contamination of one component by the other is difficult to avoid: the figures for the tail component suggest that separation is, overall, about 95% efficient.

Properties of the Apoenzyme.—Dilute solutions (0·1%) lose activity in a few hours. Concentrated solutions (>1%) are stable for a few weeks at 0° and then slowly lose activity. Activity can be partially restored by addition of glutathione (10^{-4}M). Consistently, storage with glutathione (10^{-4}M) increases the stability. Higher concentrations appear to be inhibitory.

The effect of dialysis against chelating agents was investigated. The loss of activity (*ca.* 3%) on dialysis against EDTA (10^{-4}M) in solutions of phosphate buffer (0·1M, pH 7·4; glutathione 10^{-4}M) was the same as in an experiment without EDTA. Prolonged dialysis (48 hr., 3 changes) against a mixture of *o*-phenanthroline (10^{-3}M) and sodium salicylate (10^{-3}M) in phosphate buffer (0·1M, pH 7·4; glutathione 10^{-4}M) resulted in the same loss of activity (*ca.* 40%) as in the control experiment.

Cyanide ion, as previously reported,[10] inhibits transaminase. The following solutions were prepared and left for 60 min.: (A) pyridoxal 5′-phosphate (3×10^{-4}M), phosphate buffer (0·1M, pH 7·4), enzyme. (B) as A, plus 3×10^{-4}M-potassium cyanide added after 30 min. Assay (by method 2) of A and B, and of A to which cyanide (3×10^{-4}M) had been added immediately before assay (A′), gave the following values of v' (OD unit min.$^{-1}$), 0·219, 0·058, 0·049 for A, A′, and B respectively. The rate obtained after activation of the apoenzyme in the presence of cyanide ion is, therefore, substantially the same as when cyanide is added in the same concentration to the fully activated enzyme.

TABLE 4. *Equilibrium constant for the transamination reaction.*

Concns. (10^{-3}M) of substrates	Method 1	Method 2	Method 3	Concns. (10^{-3}M) of substrates	Method 1	Method 2	Method 3
1·25, (A, K)	—	5·9	—	1·25, (O, G)	—	5·4	—
2·5, (A, K)	—	5·7	—	5·0, (O, G)	—	6·6	5·9
5·0, (A, K)	—	6·1	6·8	10·0, (O, G)	6·8	7·9	5·4
10·0, (A, K) ...	6·9	7·6	6·9	20·0, (O, G)	6·5	5·1	5·7
20·0, (A, K)	7·3	7·5	6·2				

Equilibrium Constant for Transaminase.—The apparent equilibrium constant, K_e, for the reaction,

$$\underset{(G)}{\text{glutamic acid}} + \underset{(O)}{\text{oxaloacetic acid}} \rightleftharpoons \underset{(A)}{\text{aspartic acid}} + \underset{(K)}{\alpha\text{-oxoglutaric acid}}$$

$K_e = [C_A C_K]/[C_O C_G]$, was determined at 25°, in phosphate buffer (0·1M, pH 7·38), for a highly purified enzyme preparation. Enzyme, sufficient to bring the system to chemical equilibrium within a few minutes, was added to equimolar mixtures of substrates in phosphate buffer. Analysis for oxaloacetic acid was made either by withdrawing aliquot parts and determining the absorption at 270 mμ in borate buffer at pH 9·4 (method 1), or directly in a Cary recording spectrophotometer (method 2). Appropriate controls were used in each case and all operations were conducted sufficiently quickly for corrections required for decomposition of oxaloacetic acid to be very small. Glutamic acid was estimated manometrically as described above (method 3). The results are shown in Table 4. The mean value of K_e was 6·5 (at 25°).

DISCUSSION

It has been established that the assay methods correctly measure the initial rate of the enzyme-catalysed reaction between L-aspartate and α-oxoglutaric acid. At high enzyme concentrations, method (2) (in which a direct-recording spectrophotometer is used) must be employed. A similar technique has been described by Cammarata and Cohen [4] and by Sizer and his co-workers.[6] The latter express their results in terms of a pseudo-rate coefficient derived by assuming the reaction to be a reversible first-order process. However, such a procedure is arbitrary * and appears to have no advantage, especially since v'

* One advantage claimed is that no use is made of the value of the extinction coefficient of oxaloacetic acid (ε). However, to find v (the initial reaction rate expressed in mole l.$^{-1}$ time^{-1}) from any set of data obtained by measuring optical density changes, the appropriate value of ε must be used.

[10] Bonavita and Scardi, *Experientia*, 1958, **14**, 7.

(the initial reaction rate expressed in O.D. unit time^{-1}) can easily be found as described above.

The holoenzyme has been resolved by heating it at pH 6·0. All the activity originally present can be restored by addition of pyridoxal 5′-phosphate or by pyridoxamine 5′-phosphate. The suggestion by Cammarata and Cohen [4] that resolution results in some irreversible loss of activity is therefore unfounded.

Except in the final (ethanol) fractionation, no loss of total activity occurs in any step of the purification procedure (*i.e.*, the total activity present in the fraction selected for the next stage and in the discarded fractions is equal to the activity before fractionation). Electrophoresis (phosphate buffer, pH 6·4) shows that, after step 7, the preparation contains two proteins. Electrophoretic separation gives the apoenzyme with a specific activity of *ca.* 700 units mg.$^{-1}$. No loss of total activity occurs during this process. Ethanol fractionation also separates the two proteins; precipitation occurs at two well-separated, well-defined ethanol–water ratios. The specific activity of the apoenzyme obtained in this way is also *ca.* 700 units mg.$^{-1}$, and the preparation is homogeneous with respect to electrophoresis in phosphate buffer at pH 6·4 (Fig. 2). Therefore, although considerable loss of total activity (*ca.* 50%) occurs in ethanol fractionation, the apoenzyme obtained is not, apparently, contaminated with inactivated enzyme.

Some previous workers [4] have reported that electrophoresis of impure glutamic–aspartic transaminase in phosphate buffer showed the presence of two components with transaminase activity. However, no experimental details of how this was established were given. Our experience is not consistent with this. Electrophoresis of any preparation with a specific activity greater than *ca.* 420 units mg.$^{-1}$, either in phosphate or in maleate buffer, showed only two components, of which one was active and the other inactive. Our results do not indicate, therefore, any abnormal behaviour of the apoenzyme in phosphate buffer.

It is important to compare the maximum value of the specific activity obtained by us with the values reported by other workers. Detailed comparison is, however, difficult since, with this enzyme, the reaction rate varies in a complicated way with enzyme, substrate, and hydrogen-ion concentration, and also depends on the nature of the buffer used. Our best preparation (specific activity *ca.* 700 units mg.$^{-1}$) gives an initial reaction rate (R), under the stated standard conditions, of $97{\cdot}2 \times 10^{-6}$ mole per min. per mg. of protein (at 25°). For the first four preparations reported (refs. 1—4), the corresponding figures are, for our conditions, *ca.* 5, 8, 10, and 12×10^{-6} respectively. The comparison is not very precise, since different substrate concentrations were used. However, the figures show that these preparations contained much inactive protein and this is consistent with the low purification factors reported (*ca.* 11, 17, 26, 14, respectively, compared with 184 for the present work), and with the observed electrophoretic behaviour. From the data given by Lis [5] it can be calculated that her homogeneous preparation of the holoenzyme would give, under our conditions, an initial reaction rate (R) in the range $25—35 \times 10^{-6}$ mole min.$^{-1}$ mg.$^{-1}$. It may be, therefore, that her preparation suffered some irreversible inactivation during purification.

Comparison with the data of Sizer *et al.*[6] is harder since the standard conditions used by these workers differ in temperature, pH, and buffer from ours. Our preparation gives, at 37° and under our assay conditions, a value $R = 181 \times 10^{-6}$ mole min.$^{-1}$ mg.$^{-1}$. Calculation shows that the preparation of Sizer *et al.*, under the conditions described by them, gave $R = 158 \times 10^{-6}$ moles min.$^{-1}$ mg.$^{-1}$ (at 37°; ε_{280} oxaloacetic acid = 630 in tris-buffer of pH 8·4). We have tried to make a better comparison by measuring reaction rates with the same enzyme sample under both conditions of reaction. The results indicate that the specific activity of our sample is slightly greater.* This is consistent

* Some ambiguity remains, however, since, in our hands, for reactions in this buffer, the results obtained by using the direct spectrophotometric method (method 2) are different from those obtained when aliquot parts are diluted with borate buffer (method 1).

with the report of Sizer *et al.* that their preparation contained *ca.* 13% of inactive protein.

Concentrated solutions of the apoenzyme are stable at 0° for some weeks in the presence of glutathione. The stabilising effect of this compound may be due to its ability to preserve thiol groups in the reduced form, although transaminase does not appear to contain a catalytically functional thiol group.[11]

No evidence has been found for the involvement of a metal cation in the catalytic activity of the enzyme. Dialysis against buffered solutions of EDTA, or of *o*-phenanthroline (effective chelating agent for Fe^{2+}) and sodium salicylate (effective for Fe^{3+}), gave no greater loss of activity than dialysis against buffer alone. This is consistent with the results of other workers: apart from some experiments on a transaminase of plant origin,[12] attempts to show that enzymic transamination involves a catalytically functional metal cation have yielded negative results.

Contrary to the claim of Bonavita and Scardi [10] the formation of the holoenzyme is not impeded by the presence of cyanide ions. No evidence is produced from experiments concerned with inhibition by cyanide ions that pyridoxal 5'-phosphate is attached to the protein through the aldehyde group.

The mean value of the equilibrium constant ($K_e = 6{\cdot}5$, at 25°) for the overall reaction obtained with a highly purified enzyme sample is in excellent agreement with the mean value ($K_e = 6{\cdot}75$) found by Krebs [13] using a crude preparation from horse heart. Braunstein's suggestion [11] that the position of equilibrium depends on the purity of the enzyme sample used appears, therefore, to be unfounded.

We thank the Medical Research Council and the Nuffield Foundation for grants to one of us (B. E. C. B.). Purchase of the electrophoresis equipment by the Nuffield Foundation is also gratefully acknowledged. We also thank Drs. E. M. Thain and K. G. Oldham for much valuable discussion, and Professors Sir Christopher Ingold, E. D. Hughes, and R. S. Nyholm for their interest.

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[*Received, September 27th*, 1960.]

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