# TRANSAMINASES

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Braunshtein and his coworkers (1) in their now classical investigations discovered the process of transamination in animal tissues and plants, and laid the foundation for an understanding of the enzymatic mechanisms by which this process takes place. They were led by their experiments to postulate the existence of two transaminating enzymes which they called glutamic and aspartic aminopherases, respectively. The glutamic enzyme reversibly transferred the amino group of *l*-glutamic acid to the keto acids of each of a considerable number of amino acids. Similarly, the aspartic enzyme was specific for *l*-aspartic acid as the primary donor of an amino group. Later, Cohen (2) took up the investigation of transamination, using a specific analytical method for the estimation of glutamic acid. While he confirmed the main features of the process of transamination as outlined by the Russian workers, he disagreed with them on the score both of enzyme specificity and the number of enzymes involved. Apart from alanine and glutamic and aspartic acids, Cohen found no other naturally occurring amino acid which underwent transamination at a significant rate. Furthermore, he was led to believe that glutamic and aspartic acids were dealt with by only one enzyme. He concluded that this enzyme was responsible for transamination between the pairs, glutamate-pyruvate and glutamate-oxalacetate.

Much of the confusion and difficulty which have attended the study of transamination has been due either to inadequate analytical procedures or to procedures too laborious to permit the rapid testing of activity which is an essential preliminary to any successful enzyme study. In the present communication simple and rapid methods are described for the study of transamination. With the use of these methods we have been able to prosecute expeditiously the isolation of two transaminating enzymes from pig heart, each of which has been brought to a stage at which the bulk, if not all, of the protein present has transaminase activity. One of the enzymes catalyzes the reaction
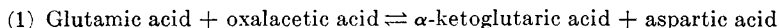
(1) Glutamic acid + oxalacetic acid $\rightleftharpoons$ $\alpha$-ketoglutaric acid + aspartic acid

and will be referred to as the aspartic-glutamic transaminase. The other enzyme catalyzes the reaction

(2) Glutamic acid + pyruvic acid $\rightleftharpoons$ $\alpha$-ketoglutaric acid + alanine
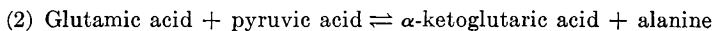
and will be referred to as the alanine-glutamic transaminase. Thus both transaminases share glutamic acid or its keto acid as an obligatory member of a transaminating pair. They differ only in their specificity for the second member of the pair. We have found no evidence for the existence of the glutamic acid and aspartic aminopherases with the specificities postulated by Braunshteïn and Kritsman (1). Furthermore, neither of the two enzymes which we have isolated can catalyze transamination between the pair, aspartic acid-pyruvic acid. This reaction is only possible in the presence of the two enzymes and with glutamic or $\alpha$-ketoglutaric acid as catalyst. In other words, the reaction between aspartic acid and alanine is not direct but is rather a consequence of the two transaminating systems coming into equilibrium with one another.

## Aspartic-Glutamic Transaminase

*Assay of the Enzyme*—The action of the aspartic-glutamic enzyme may be followed by determining the formation or disappearance either of oxalacetic acid or of $\alpha$-ketoglutaric acid. The formation or disappearance of oxalacetic acid can be followed either manometrically or spectrophotometrically. Finally, $\alpha$-ketoglutaric acid can be readily oxidized to succinic acid by $H_2O_2$ and then estimated manometrically by measuring the uptake of oxygen in the presence of the specific succinic oxidase.

Regardless of which method is selected for assay of the enzyme, the relation between the amount of chemical change and the amount of enzyme is not linear, except at the beginning of the reaction. The transamination reaction is reversible and its rate becomes progressively slower as the equilibrium point is approached. This difficulty can be by passed (a) by working with such an excess of substrate that a measurable amount of chemical change does not alter the initial concentrations appreciably, (b) by selecting for estimation an amount of enzyme which lies on that portion of the enzyme-concentration curve which is more or less linear, and (c) by carrying out the estimation of the enzyme over a relatively short period of time. By observing these precautions, the assay of transaminase can be made both accurate and consistent.

*Manometric Estimation of Oxalacetic Acid*—Ostern (3) has described a method of determining oxalacetic acid as $CO_2$ which depends upon the decarboxylation of the keto acid in the presence of a concentrated aqueous solution of aniline adjusted to pH 4.6. If one prefers to work at 38°, the error arising from the decomposition of oxalacetic acid during the equi-

libration period can be eliminated by carrying out the transaminating reaction in which oxalacetic acid is formed, in the manometer cup, and then tipping the aniline reagent into the cup from a side arm. The $CO_2$ evolved represents both the $CO_2$ formed by decarboxylation of oxalacetic acid and the $CO_2$ initially present in the reaction mixture as bicarbonate. The $CO_2$ evolved from the control without transaminating enzyme should be equal to the bicarbonate $CO_2$ initially present in the reaction mixtures. The control without the enzyme is preferred, since the enzyme solutions used are extremely dilute and the volume of enzyme solutions added is very small ($< 0.1$ cc.). Thus, in practice no error is involved in neglecting the bicarbonate $CO_2$ present in the enzyme solution.

The following mixture was set up in a Warburg manometer cup: 0.50 cc. of 0.2 M phosphate buffer of pH 7.3, 1.0 cc. of 0.2 M aspartate, 0.50 cc. of 0.2 M α-ketoglutarate (in one of the side arms), and 0.1 cc. or less of enzyme solution. After temperature equilibration was attained, the taps were closed and the α-ketoglutarate solution was tipped into the cup from the side arm. At the end of 10 minutes, 0.5 cc. of aniline citrate reagent[1] was tipped in from the second side arm or from the center well. Evolution of $CO_2$ was complete within 10 minutes of the addition of the reagent.

Fig. 1 (Curve A) shows the relation between the amount of oxalacetic acid formed under the conditions mentioned above and the amount of enzyme. We have arbitrarily defined as a unit of transaminase activity an amount of enzyme which produces an amount of oxalacetic acid equivalent to 100 c.mm. of $CO_2$ in 10 minutes at 38°. It appears that the best range for assay lies between 0.2 and 1.5 units. If the amount of oxalacetic acid formed is known, the amount of enzyme in transaminase units can be read directly from the curve.

For a given concentration of transaminase the amount of oxalacetic acid formed depends markedly upon the initial concentrations of aspartate and α-ketoglutarate. Thus Curve B of Fig. 1 describes the relation when the concentrations of substrates were increased 2.5 times those obtaining for the standard curve, A. The importance of adhering rigorously to fixed initial concentrations of substrates becomes obvious.

*Spectrophotometric Estimation of Oxalacetic Acid*—The light absorption curves of α-ketoglutaric, pyruvic, and oxalacetic acids are shown in Fig. 2. In the transformation of 1 micromole per cc. of α-ketoglutaric acid to glutamic acid and of 1 micromole of aspartic acid to oxalacetic acid at pH 7.3, the density reading at 280 mμ increases by 0.438 when the light path is 10 mm. Neither aspartic nor glutamic acid absorbs measurably at this

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[1] The aniline citrate reagent was prepared by mixing equal parts of aniline and a solution of citric acid made up by dissolving 50 gm. of citric acid in 50 cc. of water.
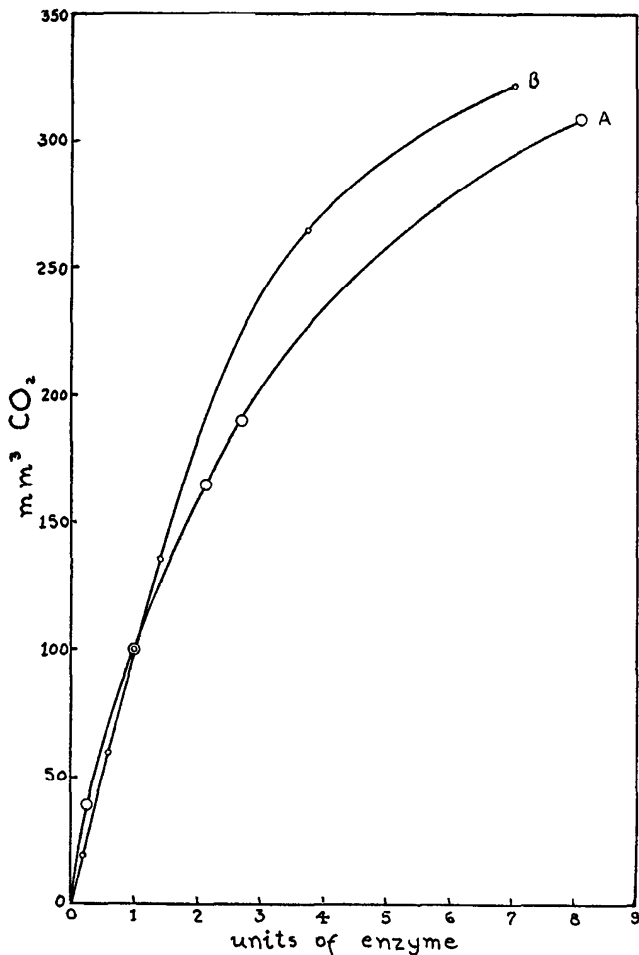
Fig. 1. Oxalacetic acid formation as a function of the concentration of aspartic-glutamic transaminase. The initial concentration of reactants in the experiments which make up the points of Curve A were 1.0 cc. of 0.2 M aspartate, 0.5 cc. of 0.2 M α-ketoglutarate, and 0.5 cc. of 0.2 M phosphate buffer of pH 7.3. The experiment was begun after temperature equilibration in the bath at 38° by tipping in the aspartate solution from a side arm. After 10 minutes 0.4 cc. of aniline citrate reagent was tipped in from the center pot and the $CO_2$ evolution measured. In the experiments which make up the points of Curve B the initial concentrations of substrate were 2.5 M higher than in the corresponding experiments of Curve A.

wave-length. The value 0.438 represents the difference between the absorption of oxalacetic acid and that of α-ketoglutaric acid. Fig. 3 shows the course of the reaction when oxalacetic acid either is formed or dis-

appears. The spectrophotometric method[2] makes it possible for the first time to follow the process of transamination almost continuously. The reaction under the conditions outlined in the legend of Fig. 3 is linear for about the first 10 minutes and then progressively decreases to zero as
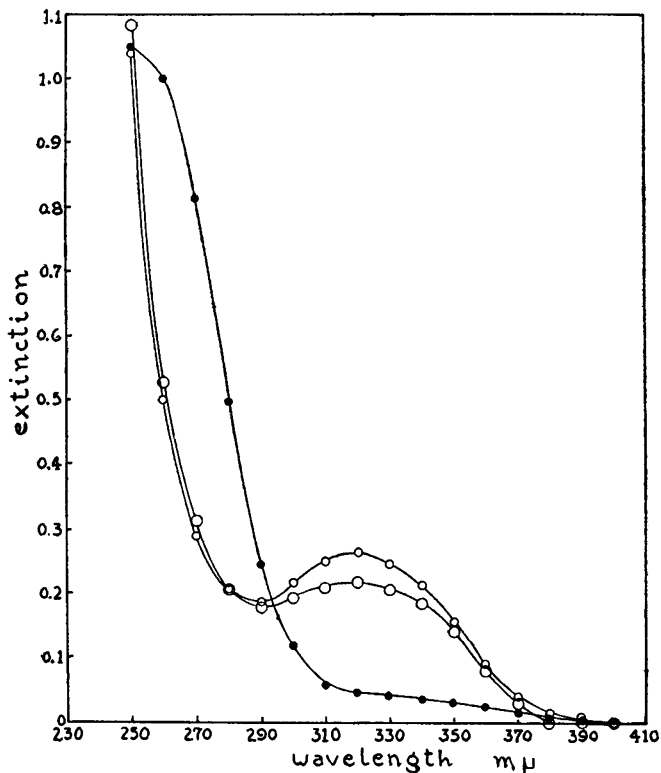


FIG. 2. Light absorption spectra of pyruvate, oxalacetate, and $\alpha$-ketoglutarate. The ordinates represent extinctions ($\log I_0/I$ in a 1 cm. cell) at pH 7.3 and for the following concentrations of keto acid, oxalacetate ($\bullet$) 1 micromole per cc., $\alpha$-ketoglutarate($\circ$) 10 micromoles per cc., pyruvate (O) 10 micromoles per cc.

the equilibrium point is approached. Oxalacetic acid decomposes spontaneously at an appreciable rate under the conditions of pH and tempera-

[2] The main drawback we have found to the more extensive use of the spectrophotometric method is the lack of provision for temperature control in the present model of the Beckman spectrophotometer. However, the National Technical Laboratories are in process of producing a unit for temperature control of the compartment housing the absorption cells. Given adequate temperature control, the spectrophotometric method would certainly be the method of choice for following the kinetics of aspartic-glutamic transaminase.

ture of the spectrophotometric test. It therefore becomes necessary either to correct for the spontaneous decomposition by running a control containing oxalacetic and all other components except the enzyme, or to insure the rapid attainment of the end-point within a few minutes after
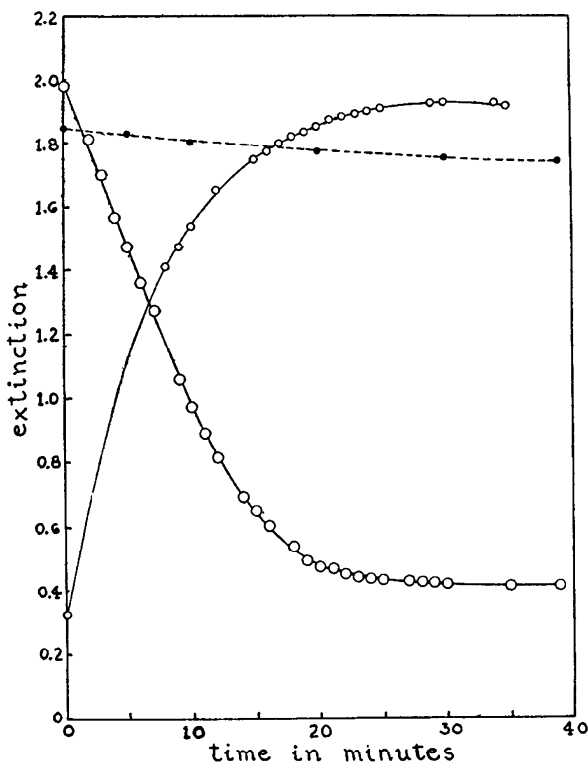


FIG. 3. The formation and disappearance of oxalacetate followed spectrophotometrically at 280 mμ in the glutamic-aspartic transaminase system. The curve O represents the formation of oxalacetate. The reaction mixture consisted of aspartate (10 micromoles per cc.), phosphate buffer of pH 7.3 (0.033 M), enzyme, and α-ketoglutarate (10 micromoles per cc.) which was added at time zero. The length of the absorption cell was 1 cm. The curve O represents the disappearance of oxalacetate. The reaction mixture consisted of glutamate (8 micromoles per cc.), phosphate buffer of pH 7.3 (0.033 M), enzyme, and oxalacetate (3.7 micromoles per cc.) which was added at time zero. The curve ● represents the decomposition of oxalacetate (3.7 micromoles per cc.) at pH 7.3 in the presence of 0.033 M phosphate buffer.

the run has started by using larger amounts of enzyme. In the latter case the error due to the instability of oxalacetic acid can be neglected.

*Manometric Estimation of α-Ketoglutaric Acid*—The sample of α-ketoglutarate should not exceed 2 cc. and should not contain more than 30

micromoles. The sample under test (previously boiled to inactivate any catalase, if this is present) is introduced into a test-tube and 0.2 cc. of 3 per cent $H_2O_2$ is added. The oxidation is allowed to proceed for 10 minutes at room temperature. 1 drop of paraffin oil is added (the use of capryl alcohol is to be avoided because it inhibits the succinic enzyme), and then a drop of a concentrated solution of catalase. When the foaming has stopped, the tube is heated in a boiling water bath for 3 minutes. Then a 2 cc. aliquot is transferred to a Warburg manometer cup containing 0.2 cc. of 6 N sodium hydroxide in the center well, 0.5 cc. of a concentrated preparation of succinic dehydrogenase in the side arm, and 0.5 cc. of phosphate buffer of pH 7.3 in the main compartment of the cup. After temperature equilibration, the succinic enzyme is tipped into the main compartment and the oxygen uptake is recorded when no further change ensues (usually 1 to 2 hours). 1 atom of oxygen is equivalent to 1 molecule

TABLE I

*Estimation of α-Ketoglutaric Acid*

| α-Ketoglutaric acid added | Oxygen uptake, observed | Oxygen uptake, theory |
|---|---|---|
| *micromoles* | *c.mm.* | *c.mm.* |
| 10 | 104 | 112 |
| 13.3 | 150 | 149 |
| 16 | 186 | 179 |
| 20 | 235 | 224 |
| 20 | 228 | 224 |
| 26.5 | 286 | 297 |
| 30 | 336 | 336 |

of α-ketoglutaric acid. Table I shows the results of some typical estimations of known samples of α-ketoglutaric acid by the above manometric method.

*Preparation of Reagents*—The following preparations were used: succinic enzyme (4), catalase (5, 6), lithium pyruvate (7), α-ketoglutaric acid (8), and oxalacetic acid (9).

*Preparation of Enzyme*—Pig heart is the richest and most convenient source of glutamic-aspartic enzyme that we have found. The enzyme is stable indefinitely when the fresh hearts are kept frozen on dry ice and also in solution at all stages of purification. We have used as our index of purity the ratio of light extinction at 280 m$\mu$ in a 1 cm. cell to the number of enzyme units per cc. (10). The conditions for measuring enzyme units are given in the legend for Curve A, Fig. 1.

*Preparation of Aspartic-Glutamic Transaminase*—Pig hearts (10.2 kilos) were minced finely and mixed with 4 volumes of water. The suspen-

sion was stirred for 30 minutes and then filtered through several layers of fine gauze. The residue was pressed out and washed with 2 liters of water. The combined filtrates (42.4 liters) contained 14.3 units per cc., purity index 0.42, total number of units 608,000. Ammonium sulfate (30 gm. per 100 cc.) was added and the precipitate filtered and kept for the preparation of the alanine-glutamic enzyme. The filtrate was treated again with ammonium sulfate (40 gm. per 100 cc.) and the precipitate was filtered through fluted papers. The papers were stirred in 3 liters of water and the mixture was filtered through layers of fine gauze. The residue was pressed out and washed several times with 0.4 liter of water. The combined filtrates had a volume of 5 liters, of which 1.6 liters represented the volume of the ammonium sulfate precipitate. The neutralized solution contained 18.8 units per cc.,[3] purity index 0.85, total number of units 94,000.

TABLE II

*Determination of Optimum Amount of Gel to Be Added*

| Volume of gel added per volume of enzyme solution | Enzyme units* per cc. corrected for dilution with gel | Purity index* |
|---|---|---|
| 0 | 70 | 0.25 |
| 0.5 | 70 | 0.097 |
| 1.0 | 56 | 0.086 |
| 1.5 | 52 | 0.081 |
| 2.0 | 48 | 0.096 |

\* As defined in the text.

Ammonium sulfate (1.2 kilos) was stirred slowly into the enzyme solution to make the final concentration 46 gm. per 100 cc. The precipitate was filtered off through fluted papers and dissolved by the addition of 500 cc. of water. The solution (640 cc.) containing 105 units per cc., purity index 0.45, total number of units 67,000, was heated to 60° in a boiling water bath and then filtered through fluted papers. The filtration is usually rather slow, requiring at least 12 hours for completion. The filtrate (500 cc.) contained 90 units per cc., purity index 0.25, total number of units 45,000. The enzyme solution was then dialyzed for 6 hours against running tap water in a Visking sausage casing, final volume 620 cc., 72 units per cc., purity index 0.25.

[3] In testing dilute solutions of the enzyme containing considerable amounts of ammonium sulfate, the estimation may be in error owing to the inhibition of the enzyme activity by ammonium sulfate. This inhibition becomes apparent when the estimated number of units per cc. increases as the volume of the sample used for the test becomes smaller. Dialysis for 5 hours against running water suffices to reduce the level of ammonium sulfate below the inhibitory level.

The dialyzed solution was then treated with tricalcium phosphate gel (11). A trial run to determine the optimum amount of gel to be added was carried out as indicated in Table II. Hence the enzyme solution was mixed with 1.5 volumes of tricalcium phosphate gel. The supernatant fluid after removal of the gel by centrifugation was treated with ammonium sulfate (70 gm. per 100 cc.). The precipitate was filtered through fluted papers and dissolved by addition of 100 cc. of water. The concentrated solution of the enzyme was then fractionated by stepwise addition of ammonium sulfate into five fractions (Table III). Fraction IVa, con-

TABLE III

*Fractionation of Concentrated Enzyme Solution*

| Fraction No. | Volume | Units per cc. | Purity index |
|---|---|---|---|
| | *cc.* | | |
| Ia | 11.4 | 5 | 4.38 |
| IIa | 12 | 15 | 1.18 |
| IIIa | 25 | 94 | 0.25 |
| IVa | 24 | 650 | 0.048 |
| Va | 20.8 | 77 | 0.143 |

TABLE IV

*Refractionation of Fraction IVa*

| Fraction No. | Volume | Units per cc. | Purity index |
|---|---|---|---|
| | *cc.* | | |
| Ib | 10 | 32 | 0.265 |
| IIb | 10 | 156 | 0.135 |
| IIIb | 10 | 89.5 | 0.108 |
| IVb | 10 | 1060 | 0.043 |
| Vb | 10 | 36 | 0.153 |

taining 15,600 units with a purity index of 0.048, was again fractionated into five fractions with ammonium sulfate (Table IV).

Fraction IVb, containing 10,600 units of purity index 0.043, was found in the Tiselius apparatus at pH 7.4 to contain three components with the following mobilities: 0.94, 2.87, and 4.90 $\times$ 10$^{-5}$ sq. cm. gm.$^{-1}$ sec.$^{-1}$. These were separated, and activity was found to be associated with both the 4.90 and 2.87 components of Fraction IVb which, respectively, accounted for 45 and 35 per cent of the total protein. The 0.94 component was found to be inactive. It should be pointed out that by using another and less satisfactory method of purification a different electrophoretic picture was obtained at the stage of purity index 0.043. In this case the ratio of the two components with velocities of 2.87 and 4.90 respec-

tively was 7:1 instead of 1.3:1 as above. Apparently the relative proportions of these two active components are determined by the method of purification. After electrophoretic separation of the 0.94 component the preparation of the enzyme has a purity index of 0.037, and appears homogeneous in the ultracentrifuge. The fact that the aspartic enzyme occurs in two forms of equal catalytic activity is not unique. The *l*-amino acid oxidase has recently been shown to occur in the form either of a unit with a molecular weight of 120,000 or one of 480,000; these forms have identical catalytic properties (12).

The first aqueous extract in the above preparation was found to contain 608,000 units. Only 94,000 units were recovered after the solution was fully saturated with ammonium sulfate. The first precipitate (30 gm. per 100 cc.) contained only insignificant amounts of the enzyme. There is always some loss associated with the precipitation of an enzyme from a dilute solution by full saturation with ammonium sulfate, but in this instance 85 per cent of the initial activity is lost. We are inclined to the interpretation that the estimate of the amount of the enzyme in the first extract is fictitiously high. The basis for this interpretation is the change in the purity index before (0.42) and after (0.85) precipitation by full saturation with ammonium sulfate. One would expect the precipitated enzyme to be purer rather than less than half as pure as it was in the original extract. This discrepancy has yet to be explained.

The initial amount of enzyme per 2 kilos of dry weight of pig heart muscle is about 750,000 units, allowing for the volume of the insoluble residue after making the first aqueous extract of the minced muscle. This amount of aspartic transaminase corresponds to 31 gm. or 1.6 per cent of the dry weight of muscle. If the estimate of the number of enzyme units in the first extract is too high, as suggested above, the estimate of the concentration of transaminase in the intact muscle would have to be lowered proportionately.

*Constants of Aspartic Transaminase*—At the stage of purity index 0.037, 1 mg. of enzyme is equivalent to 24 units. Since 1 unit represents an amount of oxalacetic acid equivalent to 100 c.mm. of $CO_2$ formed per 10 minutes at 38°, the $Q_{CO_2}$ (the $CO_2$ equivalent formed per hour per mg. of dry weight of enzyme) is 14,400. On the basis of a molecular weight of 60,000 for glutamic-aspartic transaminase, calculated from the diffusion and ultracentrifuge data of Dr. D. Moore, each molecule of enzyme would catalyze about 640 transamination reactions per minute. Finally, the enzyme at the stage of purity index 0.037 in a concentration of 1 mg. per cc. in a 1 cm. cell has an extinction of 1.07 at 280 m$\mu$.

*Specificity of Enzyme*—The glutamic-aspartic transaminase is highly specific for the two naturally occurring dicarboxylic amino acids. No

other amino acid has been found to be active as a donor of amino groups to α-ketoglutaric acid. Alanine, leucine, serine, and methionine cannot replace aspartic acid. Glutamine and pyrrolidinecarboxylic acid cannot replace glutamic acid. Mesoxalic acid can replace oxalacetic acid, but the rate of transamination in the presence of mesoxalic acid is only a small fraction of that in the presence of oxalacetic acid. Similarly cysteic acid can replace aspartic acid, though again very feebly.

### Alanine-Glutamic Transaminase

*Assay of Enzyme*—The activity of the alanine-glutamic enzyme may be followed by determining the formation or disappearance either of α-ketoglutaric acid or of pyruvic acid. The manometric estimation of α-ketoglutaric acid has been described above.

*Colorimetric Estimation of Pyruvic Acid*—Straub's method (13) consists of condensing pyruvic acid with salicylaldehyde in strongly alkaline solution to form a colored substance with a light absorption peak at 440 mμ in the visible spectrum. Neither α-ketoglutaric acid nor oxalacetic acid reacts with the reagent under these conditions. 1 cc. of the pyruvic acid solution containing not more than 5 micromoles was mixed in order with 1.0 cc. of KOH solution (100 gm. plus 60 cc. of water) and exactly 0.5 cc. of salicylaldehyde solution (2 per cent in alcohol). After 10 minutes incubation at 38°, the solution was made up to 25 cc. with water and the extinction at 440 mμ was determined against a blank with all additions except pyruvic acid. Fig. 4 shows that a linear relation obtains for the range of concentrations investigated.

For estimation of the activity of the enzyme a reaction mixture was set up consisting of 0.5 cc. of 0.2 M phosphate buffer of pH 7.3, 0.2 cc. of 0.2 M α-ketoglutarate, and enzyme solution in a volume not exceeding 0.2 cc. The mixture was warmed in a water bath at 38° for 5 minutes. Then 0.2 cc. of M *dl*-alanine solution (also kept at 38°) was introduced and the transamination reaction allowed to proceed for 10 minutes, at which time the KOH solution was added. From then on the procedure is identical with that described for the estimation of pyruvic acid. A blank with all additions except that of enzyme was included and the extinction of the experimental at 440 mμ was measured against this reagent blank. Since the color of the salicylaldehyde-pyruvic acid condensation product is not stable, depending on factors which are not easily reproduced from one set of estimations to another, a pyruvic acid standard was run through the estimation procedure simultaneously with the experimentals. Fig. 5 shows the relation between the amount of enzyme and the amount of pyruvic acid formed. An approximately linear relation obtains when the enzyme is added in an amount which does not produce more than 5

micromoles of pyruvic acid in 10 minutes under the conditions of the test. We have arbitrarily defined as a unit of glutamic-alanine activity an amount of enzyme which forms 4.47 micromoles of pyruvic acid per 10 minutes. This apparently odd quantity is equivalent to 100 c.mm. of $CO_2$ and thus the unit of activity is identical with that of the glutamic-aspartic enzyme.

*Preparation of Enzyme*—The unit of activity and the index of purity for the glutamic-alanine enzyme are defined in the same way as for the
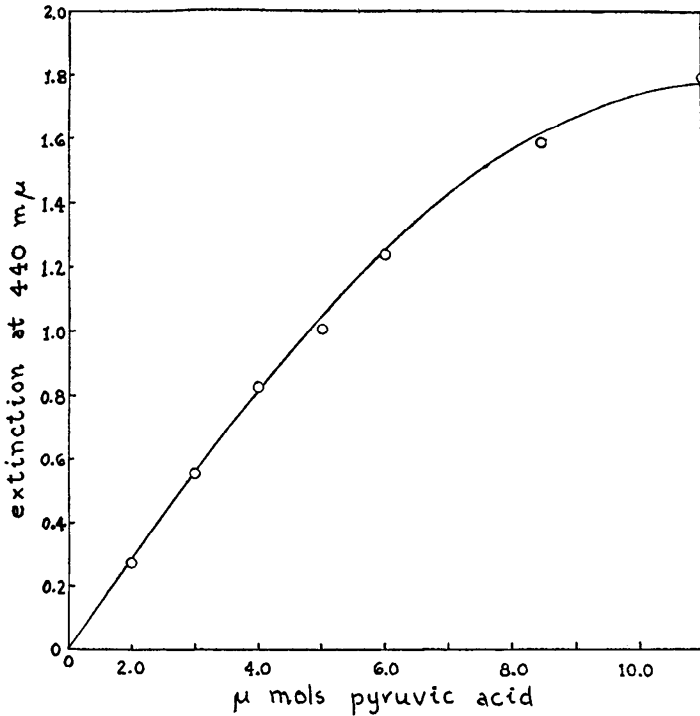


FIG. 4. Relationship between the light absorption at 440 m$\mu$ and the amount of pyruvic acid. Increasing amounts of lithium pyruvate were mixed with salicylaldehyde in alkaline solution, as described in the text. The density readings were made in a 1 cm. cuvette at 440 m$\mu$.

other transaminase. The glutamic-alanine enzyme is comparatively less stable in pig heart and occurs in lower concentrations. The precipitate formed by addition of 30 gm. of ammonium sulfate per 100 cc. in the first step in the purification of the glutamic-aspartic enzyme serves as the starting point of the preparation.

*Preparation of Alanine-Glutamic Transaminase*—Fresh or frozen pig hearts (6.8 kilos) were minced finely and mixed with 4 volumes of water.

After being stirred for 30 minutes, the suspension was filtered through layers of fine gauze. The filtrate (27 liters) contained 5 units per cc., purity index 0.94, total number of units 137,000. Ammonium sulfate was added (30 gm. per 100 cc.) and the precipitate was filtered off through fluted papers. The papers were stirred in 1 liter of water and the mixture was filtered through layers of fine gauze. The residue of filter paper was pressed out and washed several times with water until the washings were
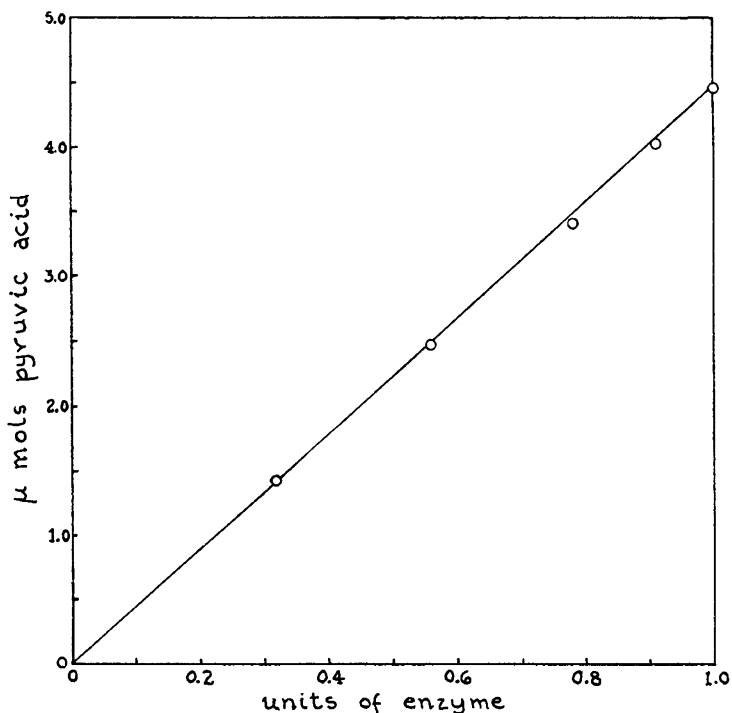


Fig. 5. Pyruvic acid formation as a function of the concentration of alanine-glutamic transaminase. The initial concentrations of reactants were 0.2 cc. of 0.2 M α-ketoglutarate, 0.2 cc. of M alanine, and 0.5 cc. of 0.2 M phosphate buffer of pH 7.3. Total volume 1 cc., reaction time 10 minutes, 38°.

colorless. The filtrate (1.11 liters) contained 72 units per cc., purity index 0.32, total number of units 80,000.[4]

The above filtrate was neutralized to pH 7 with 6 N sodium hydroxide and rapidly heated to 60° in a bath of boiling water. The coagulum was removed by filtration or centrifugation and washed thoroughly with water. The turbid filtrate or supernatant was mixed with ammonium sulfate

[4] *Cf.* foot-note 3.

(30 gm. per 100 cc.). The precipitate was centrifuged down and redissolved by addition of 200 cc. of water. The enzyme solution is now sufficiently concentrated to permit of fractionation by stepwise addition of ammonium sulfate. The solid reagent is added slowly with vigorous stirring until a precipitate is formed. The precipitate is centrifuged down and the supernatant fluid is again treated with ammonium sulfate. In this manner three fractions were obtained, only the first of which (Fraction Ia) contained any appreciable amount of activity, volume 116 cc., 390 units per cc., purity index 0.22, total number of units 45,000.

The solution of Fraction Ia was dialyzed for 4 hours against running tap water and then for 12 hours at 1° against distilled water. The bulky

TABLE V

*Refractionation of Fraction Ia*

| Fraction No. | Volume | Units per cc. | Purity index | Total No. of units |
|:---:|:---:|:---:|:---:|:---:|
| | *cc.* | | | |
| Ib | 40 | 296 | 0.101 | 11,800 |
| IIb | 18 | 480 | 0.087 | 8,600 |

TABLE VI

*Refractionation of Fraction Ib*

| Fraction No. | Volume | Units per cc. | Purity index | Total No. of units |
|:---:|:---:|:---:|:---:|:---:|
| | *cc.* | | | |
| $Ib_1$ | 17 | 290 | 0.153 | 4900 |
| $Ib_2$ | 17 | 270 | 0.071 | 4600 |
| $Ib_3$ | 17 | 88 | 0.107 | 1500 |

precipitate which formed on dialysis was centrifuged off and washed. The combined supernatant fluids (168 cc.) contained 208 units per cc. of purity index 0.098, total number of units 35,000.

Stepwise fractionation with ammonium sulfate was now repeated. Four fractions were obtained, Fractions Ib, IIb, IIIb, and IVb, only two of which were active, viz., Fractions Ib and IIb (Table V). Fraction Ib was refractionated into three fractions with the results indicated in Table VI. Fractions $Ib_2$ and IIb were combined and refractionated into six fractions with ammonium sulfate (Table VII). Fractions IIc and IIIc were mixed and refractionated with ammonium sulfate into five fractions (Table VIII). Fractions IId and IVd were separately fractionated into three fractions each. The last two fractions of IId and the first fraction of IVd were combined with Fraction IIIb, and the resulting solution was

fractionated with ammonium sulfate for the fifth and final time into three fractions (Table IX). We have been unable to advance the purity of the enzyme beyond the stage of purity index 0.020 by chemical fractionation procedures. While the preparation of the enzyme at this stage appears homogeneous in the ultracentrifuge, two components can be seen

TABLE VII

*Combination and Refractionation of Fractions Ib₂ and IIb*

| Fraction No. | Volume | Units per cc. | Purity index | Total No. of units |
|---|---|---|---|---|
| | *cc.* | | | |
| Ic | 6 | 372 | 0.052 | 2200 |
| IIc | 12 | 670 | 0.029 | 8000 |
| IIIc | 11 | 576 | 0.039 | 6400 |
| IVc | 11 | 85 | 0.195 | 940 |
| Vc | 11 | 0 | ∞ | 0 |
| VIc | 11 | 0 | ∞ | 0 |

TABLE VIII

*Refractionation of Combined Fractions IIc and IIIc*

| Fraction No. | Volume | Units per cc. | Purity index | Total No. of units |
|---|---|---|---|---|
| | *cc.* | | | |
| Id | 4 | 216 | 0.067 | 865 |
| IId | 4 | 712 | 0.0316 | 2850 |
| IIId | 4 | 950 | 0.0213 | 3800 |
| IVd | 4 | 684 | 0.0344 | 2740 |
| Vd | 4 | 70 | 0.25 | 280 |

TABLE IX

*Fractionation of Combined Fractions of Fractions IId and IVd*

| Fraction No. | Volume | Units per cc. | Purity index | Total No. of units |
|---|---|---|---|---|
| | *cc.* | | | |
| Ie | 3 | 567 | 0.020 | 1700 |
| IIe | 5 | 980 | 0.020 | 4900 |
| IIIe | 4.5 | 194 | 0.045 | 875 |

in the Tiselius apparatus. An appreciable amount of the smaller and faster moving component can be separated after 12 to 24 hours from the other component. The purity index of this electrophoretically homogeneous component was found to be 0.008. The second component was inactive.

The initial yield of enzyme from 1.36 kilos of dry weight of minced heart muscle is 171,000 units. Since 107 units are equivalent to 1 mg.,

the amount of alanine enzyme present initially is 1.6 gm. or about 0.12 per cent of the dry weight of heart muscle.
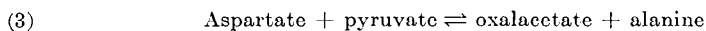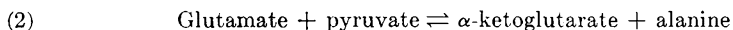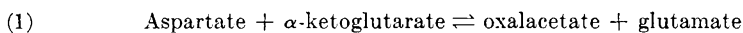
*Constants of Alanine-Glutamic Enzyme*—At the stage of purity index 0.008, 1 mg. of enzyme is equivalent to 107 units ($Q_{CO_2}$ of 64,000). The activity per unit weight of the glutamic-alanine enzyme is therefore about 4.5 times that of the glutamic-aspartic enzyme under comparable conditions. Assuming a molecular weight of 180,000, each molecule of the glutamic-alanine enzyme would catalyze 8600 transamination reactions per minute at 38° under the conditions of the standard test. This turnover number is some 13 times greater than that of the glutamic-aspartic enzyme. 1 mg. of homogeneous enzyme per cc. in a 1 cm. cell has an extinction at 280 m$\mu$ of 0.86.

*Specificity of Enzyme*—In the reaction between alanine and $\alpha$-ketoglutaric acid, alanine can be replaced by $\alpha$-aminobutyric acid, though the velocity is only a small fraction of that with alanine. N-Monomethylalanine, phenylalanine, valine, serine, methionine, leucine, $\alpha$-aminovaleric acid, cysteic acid, and *d*-alanine cannot replace *l*-alanine. In the reaction between glutamic acid and pyruvic acid the latter can be replaced by $\alpha$-ketobutyric acid and mesoxalic acid, while the former cannot be replaced by cysteic acid, glycyl cysteine, glutathione, pyrrolidonecarboxylic acid, acetylglutamic acid, leucine, methionine, glutamine, tyrosine, threonine, $\alpha$-aminocaproic acid, lysine, phenylalanine, cystine, valine, and hydroxyproline.

*Equilibrium Constants*—In the reactions between $\alpha$-ketoglutarate and aspartate or alanine, an equilibrium is established, as was clearly shown by Kritsman (14) and Cohen (15). Using enzymes of the highest purity level, we have confirmed the approximate positions of the equilibria which Cohen reported for the two transaminating reactions, $K = 0.3$ for the reaction shown below in reaction (1), and $K = 1$ for the reaction shown in reaction (2). In the calculation of the equilibrium constant, the error in estimating any one of the four reactants is squared. Since the errors of estimation lie within the range of 5 to 10 per cent the uncertainty in evaluating the equilibrium constant is 25 to 100 per cent. With the present methods of estimation there is little point in assigning other than tentative values for the equilibrium constants of the two transaminating reactions.

*Prosthetic Group of Transaminases*—Braunshtein and Kritsman (16) have reported that the so called aspartic aminopherase contains a dissociable coenzyme, and they have reported on the preparation of this coenzyme from yeast. We have been unable to find any evidence that a transaminase exists in animal tissues which is specific for aspartic acid and not for glutamic acid. It is our impression that the so called aspartic enzyme is merely a mixture of the two transaminases with glutamic or $\alpha$-keto-

glutaric acid as "catalyst." Thus reaction (3) between aspartate and pyruvate is the summation of reactions (1) and (2):

(1)  Aspartate + α-ketoglutarate ⇌ oxalacetate + glutamate

(2)  Glutamate + pyruvate ⇌ α-ketoglutarate + alanine

(3)  Aspartate + pyruvate ⇌ oxalacetate + alanine

The mode of preparation and the properties of the coenzyme which Braunshteïn and Kritsman have isolated from yeast strongly suggest that it is glutamic acid. In other words, the coenzyme effect can be explained entirely in terms of reactions (1) and (2).

Although no evidence has been found that either of the two transaminases becomes inactivated during the purification procedure as the result of the loss of a dissociable coenzyme, we have been able to show that both enzymes contain a substance, some of whose properties are known to be shared by a derivative of pyridoxal. But first it will be necessary to digress to a consideration of the properties of dihydroxyphenylalanine or dopa carboxylase[5] of pig kidney. This carboxylase on purification was found to be inactive unless supplemented with a coenzyme found in boiled extracts of heart and liver. Gale and Epps (18) in their investigations of the bacterial enzymes which decarboxylate amino acids showed that the lysine and tyrosine carboxylases contained a dissociable coenzyme which they called codecarboxylase. They studied the distribution of codecarboxylase, finding that it occurred universally in animals and bacteria. Furthermore, they isolated the coenzyme in highly purified form from yeast. A sample of codecarboxylase prepared from yeast by Gale's

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[5] Dopa carboxylase, which was first discovered by Holz *et al.* (17), hitherto has not been obtained in purified and concentrated form. Our method of preparation is as follows. Pig kidneys were finely minced and then extracted with 2 volumes of icecold 0.8 per cent sodium chloride solution. After coarse particles were strained out through several layers of wide-meshed gauze, the extract was clarified by centrifugation. The highly opalescent supernatant fluid was then treated with ammonium sulfate (18 gm. per 100 cc.) and the precipitate discarded. Ammonium sulfate was again added (12 gm. per 100 cc.) and the precipitate which contained the enzyme was redissolved in water. The solution of the enzyme was then again submitted to fractional precipitation with ammonium sulfate as above. The solution of the twice fractionated enzyme was dialyzed against 0.002 M ammonia at 0° during 4 days. The solutions so dialyzed are virtually inactive in the absence of codecarboxylase. In addition to the coenzyme, reducing substances such as cysteine or glutathione are essential for reconstituting the activity of the split enzyme. The process of splitting off the coenzyme by dialysis against ammonia water is also attended with considerable irreversible loss of activity. The purification procedure should be carried out as quickly as possible, and always at 0°, to minimize the rate of destruction of the enzyme. The enzyme is most stable when stored as an ammonium sulfate precipitate.

method was found to replace boiled extracts of heart and kidney in restoring the activity of dopa carboxylase. More recently Gunsalus and his colleagues (19) have discovered that pyridoxal which has been subjected to phosphorylating agents, either inorganic or enzymatic, can replace Gale's coenzyme as codecarboxylase for various amino acids and carboxylases of bacterial origin. Through the kindness of Dr. Gunsalus and
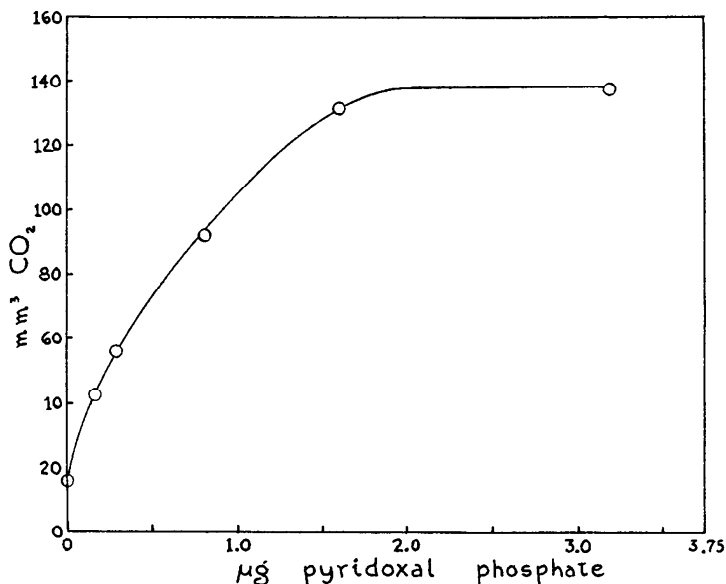


Fig. 6. Rate of decarboxylation of dihydrophenylalanine by dopa carboxylase as a function of the concentration of codecarboxylase. The reaction mixtures contained 0.8 cc. of 0.015 M dihydrophenylalanine, 0.5 cc. of 0.2 M phosphate buffer of pH 7.0, 1 mg. of cysteine, 1 cc. of dialyzed pig kidney enzyme, and varying amounts of synthetic codecarboxylase as prepared by Umbreit et al. (19). The gas space was filled with nitrogen. At time zero the substrate was tipped into the central compartment of the manometer cup from a side arm. After 15 minutes 0.4 cc. of 6 N sulfuric acid was tipped in from the center pot and the carbon dioxide evolution was then measured. The $CO_2$ evolution was corrected for the blank without enzyme. The reaction was carried out at 38°. The pyridoxal phosphate solution used was not pure, but the concentration in terms of the pure substance has been estimated by Dr. Gunsalus.

Dr. Folkers, we were able to test "pyridoxal phosphate" as coenzyme for dopa carboxylase. It was found to be fully equivalent to Gale's yeast coenzyme in its action on the split dopa enzyme (cf. Fig. 6).

The fact that extremely valuable information was obtained by using split d-amino acid oxidase as a test system for flavin dinucleotide suggested to us the use of the split dopa enzyme as a test system for codecarboxylase. Various enzyme preparations were tested for the presence of codecar-

boxylase, including the purest preparations of the two transaminases. The extremely high concentration of the codecarboxylase which was found in the solutions of the two transaminases compared to the negligible amounts found in other highly purified enzymes made it seem unlikely that the presence of codecarboxylase was fortuitous. The relationship between the activity of each of the two transaminating enzymes and the amount of codecarboxylase was investigated in different fractions and in the final homogeneous preparations. Table X shows that there is a very satisfactory correlation between the amount of transaminase and the amount of codecarboxylase in the case of the alanine enzyme. The proportionality is admittedly less exact in the case of the aspartic enzyme but there can be no doubt that there is a correlation. The codecar-

TABLE X

*Presence of Codecarboxylase in Preparations of Two Transaminases at Different Stages in Purification*

In testing for codecarboxylase activity the solution of the transaminase was first boiled and then added to the assay system without removing coagulated protein.

| Alanine enzyme | | Aspartic enzyme | |
|---|---|---|---|
| Purity index | Micrograms codecarboxylase / Enzyme units | Purity index | Micrograms codecarboxylase / Enzyme units |
| 0.22 | 0.0037 | 0.85 | 0.038 |
| 0.10 | 0.0035 | 0.55 | 0.016 |
| 0.09 | 0.0028 | 0.45 | 0.018 |
| 0.06 | 0.0042 | 0.42 | 0.021 |
| 0.034 | 0.0041 | 0.14 | 0.045 |
| 0.006 | 0.0025 | 0.11 | 0.032 |
| | | 0.041 | 0.023 |

boxylase associated with the aspartic enzyme is extremely difficult to separate from the coagulated protein and this unpleasant property may well explain some of the variations in the estimation. A crude solution of alanine transaminase after being boiled will show full codecarboxylase activity in the assay system only if the coagulated protein is added. The filtered solution is much less active. As the purification proceeds, the codecarboxylase shows less and less tendency to be retained by the protein coagulum, and at the highest purity level a protein-free solution can be obtained containing the original codecarboxylase activity of the enzyme. In the case of the aspartic enzyme, the tendency of the codecarboxylase to adhere to the coagulated protein is greater and persists from the first crude extracts to the highest purity level. At best a few per cent can be obtained in protein-free solution. The fact that the coagulated protein

shows codecarboxylase activity must mean that the codecarboxylase is at least slightly dissociable, and that it preferentially combines with the protein of dopa carboxylase. In other words, codecarboxylase has a stronger tendency to combine with the protein of dopa carboxylase than with the coagulated protein. However, the transfer may not be quantitative, and that may be at the root of the difficulty in getting better correlations between the amount of codecarboxylase and the amount of aspartic transaminase activity. It is of interest to point out that by boiling "pyridoxal phosphate" in the presence of coagulable protein, the synthetic coenzyme is found to be retained partially or completely in the coagulum to an extent, depending upon the nature and amount of protein added.

Both transaminases lose activity following prolonged dialysis against 0.002 M ammonia at 0°. Thus the alanine enzyme is completely inactive at the end of 3 days. The aspartic enzyme loses activity more slowly, reaching complete inactivation only after about 12 days. This loss in transaminase activity is paralleled by a loss in the codecarboxylase activity of the boiled enzyme. It is not possible to decide which is cause and which is effect; i.e., whether loss in transaminase activity is the result of loss of codecarboxylase or whether inactivation of the enzyme is followed by splitting off of the codecarboxylase. In any case, the two events occur simultaneously and we may regard these dialysis experiments as evidence for the view that codecarboxylase is a functional part of transaminase in the sense of being a coenzyme or prosthetic group.

It became important to know whether the "coenzymes" of the two transaminases are identical with pyridoxal phosphate. Both Gunsalus[6] and Baddiley and Gale (20) have already established the identity of yeast codecarboxylase with pyridoxal phosphate. When the coenzymes were heated for 1 hour at 100°, the per cent destruction of the codecarboxylase activity was found to be 57 for the coenzyme of aspartic transaminase, 65 for the coenzyme of alanine transaminase, and 54 for pyridoxal phosphate, when treated with 0.1 N sulfuric acid; when treated with 0.1 N sodium hydroxide or with neutral solution there was no destruction. There appears to be complete correspondence of the three coenzymes, at least in regard to their pH stability. When the preparations of the coenzyme contain protein as an impurity, it may appear that boiling in neutral solution leads to destruction of the coenzyme. Analysis has shown, however, that this apparent loss of activity is due to a reversible combination of the coenzyme with protein. Exposure to 0.1 N alkali for a few minutes at 100° suffices to regenerate most of the original activity.

The comparison of the three coenzymes was extended to a study of their action in stimulating the growth of Lactobacillus casei in a medium lacking in pyridoxine. Table XI shows that all three show growth-

promoting activity at about the same dilutions. Two complicating factors are encountered in testing solutions of coenzyme which contain significant amounts of protein. As pointed out above, the coenzyme shows a strong tendency to combine with protein in the medium when the solution is sterilized by autoclaving. The coenzyme is not available for growth when present in this bound form, but it may be regenerated by hydrolyzing for a short period in 0.1 N HCl at 100°. Some of the coenzyme is hydrolyzed during this period but, since pyridoxal is equivalent to its phosphoric ester as far as growth stimulation is concerned, the estimation is unaffected. When working with a protein-free solution of the coenzyme, no difference

TABLE XI

*Effect of "Coenzymes" of Two Transaminases on Growth of Lactobacillus casei in Pyridoxal-Free Synthetic Medium*
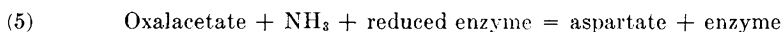
|  | Micrograms per test | Growth in 48 hrs. |
|---|---|---|
| | 0 | 0 |
| Pyridoxal | 0.02 | + |
| | 0.10 | ++++ |
| "     phosphate | 0.005 | 0 |
| | 0.05 | ++++ |
| "Coenzyme" of alanine-glutamic trans-aminase | 0.01 | + |
| | 0.05 | ++++ |
| "Coenzyme" of aspartic-glutamic trans-aminase | 0.005 | 0 |
| | 0.05 | ++++ |

The concentrations of the three coenzymes were determined by comparing their codecarboxylase activity against a standard pyridoxal phosphate solution. The "coenzymes" of the transaminases were prepared by treating the respective enzyme solutions at 100° for 1 hour in 0.1 N acid. The solution of the "coenzymes" was then filtered before testing. The growth medium was that described by Snell and Rannefeld (21).

in growth-stimulating effects is observed between untreated and acid-hydrolyzed solutions. A second complication which again is referrable to the presence of protein in the solution of coenzyme arises from the liberation of growth-stimulating substances by acid hydrolysis of protein. In the absence of pyridoxal these growth-stimulating substances have no influence on the growth of the test organism, but in the presence of pyridoxal they induce a rate of growth which is very much more rapid than in the presence of pyridoxal alone. Thus crude solutions of coenzyme which have been subjected to acid hydrolysis appear even more active than pyridoxal or its phosphoric ester in stimulating growth. However, with purified solutions of the coenzyme containing little or no protein, no significant difference can be discerned between the coenzymes isolated from the two transaminases and pyridoxal phosphate in so far as growth stimulation is concerned.
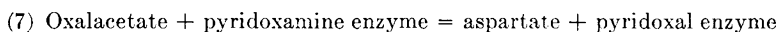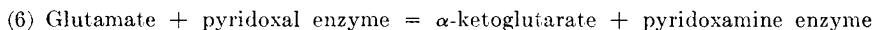
Some as yet unpublished experiments of Gunsalus and his colleagues[6] with *Streptococcus faecalis* have brought evidence from another direction that pyridoxal phosphate is the prosthetic group of transaminase. They grew the organism in a medium deficient in vitamin $B_6$ and showed that the suspensions of avitaminous bacteria were unable to catalyze the transaminations characteristic of the aspartic and alanine enzymes. However, following addition of pyridoxal phosphate the transaminating activity was completely restored to normal. Schlenk and Snell (22) previously tried to show the participation of pyridoxal in transaminating reactions by adding it to the tissues of vitamin $B_6$-deficient rats. However, their experiments were suggestive rather than conclusive.

*Mode of Action of Transaminase*—There are at least three possible mechanisms by which transamination reactions can take place. The reaction between glutamate and oxalacetate may be conceived of as an oxidation-reduction in which glutamate is oxidatively deaminated and oxalacetate simultaneously reductively aminated. According to this view, the process of transamination would be the summation of two separate reactions.

(4)      Glutamate + enzyme = $\alpha$-ketoglutarate + $NH_3$ + reduced enzyme

(5)      Oxalacetate + $NH_3$ + reduced enzyme = aspartate + enzyme

This mechanism can be excluded, since experiments with isotopic ammonia (*cf.* Shemin[7]) have shown conclusively that free ammonia is not formed during the process of transamination. Furthermore, there is no evidence that transaminase is capable of undergoing reversible oxidation and reduction.

Another mechanism was outlined by Braunshtein and Kritsman (1), according to which the enzyme catalyzed a Schiff base condensation between the amino acid and the keto acid of the transaminating pair. The Schiff base compound would then undergo rearrangement and dissociation into the transaminated pairs. This mechanism was in fact proposed by Herbst (23) to explain transamination reactions in non-biological systems. More recently Snell (24) found that pyridoxal heated with glutamic acid underwent transamination with formation of pyridoxamine and $\alpha$-ketoglutaric acid. This led him to postulate that the prosthetic group of transaminase is pyridoxal and that transaminase acted by alternately accepting and donating amino groups, as indicated in the equations below:

(6) Glutamate + pyridoxal enzyme = $\alpha$-ketoglutarate + pyridoxamine enzyme

(7) Oxalacetate + pyridoxamine enzyme = aspartate + pyridoxal enzyme

There is evidence from our own experiments and those of Gunsalus that the prosthetic group of transaminase is probably pyridoxal phosphate,

[6] Gunsalus, I. C., private communication.

[7] Shemin, D., private communication.

but we have been unable as yet to establish that cyclical amination and deamination of the prosthetic group take place during the process of enzymatic transamination.

It is a pleasure to acknowledge our debt to Miss Marion Blanchard and Dr. P. K. Stumpf for their generous help in studying the properties of the coenzymes obtained from the two transaminases.

## SUMMARY

The isolation and properties of two transaminating enzymes from pig heart are described. The aspartic-glutamic enzyme catalyzes the transfer of amino groups from oxalacetate to glutamate or from aspartate to $\alpha$-ketoglutarate. The alanine-glutamic enzyme catalyzes the transfer of amino groups from pyruvate to glutamate or from alanine to $\alpha$-ketoglutarate. Both of these enzymes have been purified to a point at which all the protein present has enzymatic activity. The catalytic constants at this purity level have been evaluated.

Rapid methods have been described for following the action and for determining the amounts of these enzymes. These methods depend upon the appearance or disappearance of oxalacetic acid, $\alpha$-ketoglutaric acid, and pyruvic acid.

Evidence has been presented which points to pyridoxal phosphate as the prosthetic group of both transaminating enzymes.

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