Aminopherase.

by

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I.

D. M. NEEDHAM[1] was the first to observe that muscle tissue can decompose l (+) glutamic acid in such a way that the amino group remained in the amino-N fraction. BANGA and SZENT-GYÖRGYI[2] have discovered that glutamic acid reacts with oxaloacetic acid in the presence of muscle tissue. This reaction is so fast and complete that it could be used to trap oxaloacetic acid. The nature of the reaction was not investigated in either of these earlier investigations. It was BRAUNSTEIN and his associates'[3] merit to elucidate the mechanism of the very intensive intermediate metabolism of some natural aminoacids in the muscle. They have shown that a number of aminoacids are able to react with some ketoacids with the exchange of the amino and keto groups. According to these studies such reactions take place in many different organs, the most active among them being skeletal and heart muscle. The significance of these reactions is not yet clear.

No detailed information is available about these reactions from the point of enzyme chemistry. Neither the purification procedures elaborated by BRAUNSTEIN and KRITZMANN,[3] nor that described by COHEN,[4] could bring the question closer to its solution. Studies made on such preparations, assuming that they are purified, proved to be misleading.

We have undertaken the purification of such an enzyme in an attempt to study the mechanism of its action, whether it needs a coenzyme as suggested by BRAUNSTEIN. Moreover, the kinetics of such a reversible reaction between four substrates are of considerable interest from the point of enzyme-

substrate complex formation. Unfortunately, owing to external conditions, this work had to be abandoned at a rather early stage and therefore we decided to describe the purification of the enzyme along with some kinetic measurements.

II.

It is known from the work of BRAUNSTEIN that the following reactions occur most actively in the presence of muscle tissue:

(1) oxaloacetic acid + l(+) glutamic acid $\rightleftarrows$ l(—) aspartic acid + α-ketoglutaric acid.
(2) pyruvic acid + l (+) glutamic acid $\rightleftarrows$ l (+) alanine + α-ketoglutaric acid.

There are considerable difficulties connected with the measurement of reaction rates in reaction (1), owing to the relative instability of oxaloacetic acid in muscle tissue. Its reduction to malic acid, decarboxylation to pyruvic acid and condensation to citric acid lead to unreliable results unless the complete balance sheet is drawn up for all of the substances concerned. There are no such difficulties in determining the reaction rate in reaction (2). We have chosen this reaction as the subject of our studies.

In principle we accept the nomenclature advanced by BRAUNSTEIN and call the enzyme a m i n o p h e r a s e. As the aminopherase in question catalyzes only reaction (2), we think it advisable to call this enzyme g l u t a m i n o - p y r u v i c a m i n o p h e r a s e.

With the method, described below, we were able to purify glutamino-pyruvic aminopherase 550 times as compared with the original activity of the heart muscle tissue. The most active preparations are colorless. Different preparations obtained by somewhat altered procedures yielded the same maximal activity. Therefore, as attempts to purify the enzyme any farther have all failed, we believe that such preparations are practically pure.

We had no indication that the enzyme would require a coenzyme. The slight yellow colour invariably present in our preparations, shows no specific absorption bands and we do

not think its having anything to do with the enzymic activity.

Glutamino-pyruvic aminopherase brings about an equilibrium in reaction (2), there is less pyruvic acid than alanine in equilibrium.

We have determined two of the four enzyme-substrate dissociation constants, which are necessary to describe the reaction. The enzyme-alanine dissociation constant was found to be $1{,}5 \cdot 10^{-2}$ the enzyme-α-ketoglutaric acid dissociation constant: $1{,}7 \cdot 10^{-3}$. In spite of great deal of experimental work, we could not determine the similar values of the enzyme-glutamic acid and enzyme-pyruvic acid dissociation constants. This was mainly due to the lack of reliable and exact methods for the determination of other substrates than pyruvic acid.

III.

Methods.

Throughout this work the enzyme activity was determined by the amount of pyruvic acid formed or disappeared. STRAUB's salicilic aldehyde method[5] was used and we found it very satisfactory for the present purpose. None of the other three components gave any reaction with salicylic aldehyde, thus ensuring complete specificity. When muscle tissue, or a still impure enzyme was used, the protein was removed with the tungstate-sulfuric acid mixture (see STRAUB[5]). But at a rather low degree of purity, this deproteinization became superfluous as the amount of protein present in the test was negligible. In these cases 1 ml of the reaction mixture was pipetted into 1 ml of the concentrated KOH solution and 0,5 ml of the salicylic aldehyde reagent added. The protein originally present, was dissolved in the strong potash solution, without giving any opalescence. Nor did it give any colour with salicylic aldehyde.

We found that the method would give reliable results within 3% of error, if the following procedure was rigorously adhered to: The pyruvic acid solution (1 ml) was pipetted into a test tube, containing already 1 ml of the potash solution. 0,5 ml salicylic aldehyde solution was immediately added and the mixture thoroughly shaken up. Within a minute the test

tube was placed into a water bath of 38°, kept there exactly for 10 min., placed in an icebath for 10 minutes and the result read after this time. Determinations on pure pyruvic acid solutions have shown that in this case the extinction values (log I_0—log I) are directly proportional to the pyruvic acid concentration. The linear relationship will however break down if more than 0,3 mg pyruvic acid is taken for the determination. Should there be more pyruvic acid in solution, it should be correspondingly diluted. 0,1—0,3 mg pyruvic acid per ml is the ideal concentration to be taken for a determination. The readings were made with a Pulfrich photometer, using a cuvette of 1 mm thickness and the blue filter S47.

The following preparations were used as substrates: 1. l(+) glutamic acid. Its optical rotation was controlled in the presence of ten mols of HCl and was found to agree with the described value of $\alpha_D^{20} = +31°$. 2. Pyruvic acid was redistilled from the commercial product and a solution made up by acidimetric titration. 3. Alanine was used in the d, l form. As the d (—) form does not react at all, the concentration of l (+) alanine was taken only into consideration. 4. α-ketoglutaric acid was prepared synthetically from oxalosuccinic ester by the method of GABRIEL[6]. Its melting point was close to the theoretical value (111° instead of 112—113°). These preparations contained some impurity, which gave a colour with the salicylic aldehyde reagent. In the preparation mostly used in these studies, 1 mol/liter ketoglutaric acid gave a colour corresponding to 0,014 mol/liter pyruvic acid. The impurity, however, was not pyruvic acid, and corrections were taken for this value.

IV.

Test for the enzyme activity.

We have defined, as the glutamino-pyruvic aminopherase unit, the amount of enzyme, which formed 1 mg of pyruvic acid in 4 ml of the test solution within 15 minutes at pH 7,3 and 38°, when the initial concentration of l-alanine and α-ketoglutaric acid was 0,017 mol/liter each, the initial concentration of pyruvic acid and glutamic acid nil.

From 3—4 determinations with different quantities of an

unknown enzyme solution, this value is obtained by graphic interpolation. Fig. 1. shows such a determination. It is seen that the curve obtained is far from being linear. This is due to the fact that equilibrium is reached under these conditions if 2 mg pyruvic acid are formed.

The test solution was made up in the following way: Phosphate buffer of pH 7,3 to give a final concentration of 0,1 M, the enzyme solution and 0,5 ml of a 0,137 M l-alanine solution were placed in a test tube and filled up to 3.5 ml with dis-

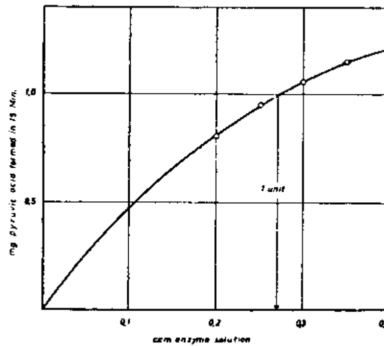

Fig. 1.

tilled water. The tube was then placed in a waterbath of 38°. The α-ketoglutaric acid solution (neutralized with NaOH) was placed in a test tube in the same waterbath and after 5 minutes, 0,5 ml of it was given to the test, mixed thoroughly and then left to stand in the waterbath for 15 minutes. It was found unnecessary to employ any shaking during the incubation. After 15 minutes the solution was either deproteinized by letting the required amount of sulfuric acid run into the tube, while still in the waterbath, or 1 ml of it were taken out and given immediately into 1 ml of potash solution. If there was too much pyruvic acid present, 1 ml of the test solution was taken out, mixed with 1 ml potash solution as before and then diluted with a twice diluted potash solution to reach a convenient pyruvic acid concentration. From this dilution 2 ml were pipetted into a test tube and mixed with 0,5 ml salicylic aldehyde for the determination.

V.

Purification of the enzyme.

Pig's hearts were minced with an ordinary meat mincer. 800 g of the mince were mixed with 800 ml of a 0,1 M acetate buffer of pH 3,8 and left to stand at room temperature for 24 hours. The mixture was then warmed up to 60° and kept at this temperature for 15 minutes. Then the fluid was squeezed through a thin cloth. If neutralized to pH 7 by the addition of a 10% NaOH solution, a precipitate was formed. This is centrifuged off, leaving a clear, pink solution (A).

To 1080 ml of solution (A) thus obtained, 242 g ammonium sulfate were added and the precipitate, which contained the enzyme, was filtered off through fluted filters. (Ammonium sulfate: 0,375 saturation.) The filter paper, together with the precipitate was suspended in 80 ml of distilled water. The enzyme solution was sucked off from the filter paper pulp and the residue was washed with 20 ml of distilled water. The solution and washing were combined and dialyzed against distilled water at 0° for 24 hours. After dialysis we obtained 149 ml of a clear brown solution (B).

130 ml of solution B were acidified by the addition of 6,5 ml of a 2 M acetate buffer of pH 4,6 and then mixed with 12 ml of an alumina Cγ suspension (280 mg Al_2O_3). The enzyme was adsorbed on the alumina gel and separated from the solution in the centrifuge. The precipitate was stirred up with 20 ml of a 0,1 M phosphate buffer of pH 7,3 and the solution was adjusted to pH 6,8 by the addition of a few drops of a dilute NaOH solution. The precipitate was eluted for a second time by adding 20 ml of a 0,1 M phosphate buffer of pH 6,8 to the gel. After thorough mixing it was centrifuged off again and this elution was combined with the first one. We obtained in this step 39 ml of enzyme solution (C). It is advisable to make a preliminary test in order to find the optimal amount of alumina gel, i. e. the amount of alumina necessary to bring down about 60—70% of the enzyme.

33 ml of the solution (C) obtained in the previous step, were refractionated with ammonium sulfate between 0,30 and 0,375 saturation values. Both precipitates settled well in the centrifuge. The fraction, which precipitated between 0,3 and

0,375 saturation, was dissolved in distilled water, giving 6 ml of a pale yellow solution. It contained the enzyme with maximal activity. (D).

The purified enzyme keeps well in solution, especially well in an ammonium sulfate solution. In some cases we found no loss of activity during a period of two months, if the enzyme was kept in a 0.15 saturated ammonium sulfate solution in the icechest.

In Table I. the numerical details of the purification procedure are summarized:

Table I.

Step	Enzyme units/ml	Total units	Enzyme unit/mg
A	4,1	4450	1,03
B	16,5	2150	4,17
C	28,4	937	12,6
D	103	618	28,6

It is seen that from the first solution 14% of the activity are retained in the last fraction and the activity per dry weight is 28 times higher. Actually the most successful step of the purification is the first one, in which at the very acid pH and high temperature, most of the muscle proteins are denatured and removed. We find that 1 g of fresh muscle tissue contains 10 units of glutamino-pyruvic aminopherase, i. e. about 0,05 units per mg protein (dry weight). Therefore in the present case the first step means a 20-fold increase in activity and the whole procedure a 570-fold purification.

In different preparations, the final value of the purified enzyme lay always between 28—29 units/mg.

Properties of the enzyme. Glutamino-pyruvic aminopherase shows a remarkable stability towards heat and a considerable resistance towards acid pH. The first point was already observed by BRAUNSTEIN. If kept at room temperature for half an hour, the enzyme is inactivated by pH 3 and pH 11. It is stable between these pH values. Alcohol, but not acetone destroys the enzyme even at 0°. Dialysis against distilled water does not diminish the activity of the enzyme. We have tested at different stages of the purification the effect of boiled muscle juice and of boiled enzyme solutions on the activity of the enzyme. We could never find any rise in the activity.

It is consequently plain that the enzyme does not have a dissociable prosthetic group.

Part of the enzyme can be removed by extracting the muscle with distilled water or salt solution, part of it will, however, remain in the residue even after prolonged washing. It is the heat treatment with — or without — the acid ph which brings the enzyme into solution.

The specificity of the enzyme was not studied extensively. We found that if alanine is replaced by aspartic acid in the

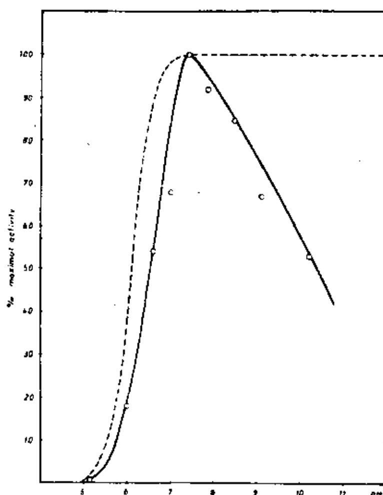

Fig. 2.

test, or aspartic acid and pyruvic acid are added to the enzyme, only traces of oxaloacetic acid are formed and no pyruvic acid disappears in the second case.

Fig. 2. shows the pH activity curve of the enzyme, when the reaction starts from alanine and ketoglutaric acid. The assimetry of the curve is an interesting feature. It is probable that on the acid side the activity is connected with the charge of the substrate, as shown by NORTHROP[7] for he pH-activity curves of pepsin and trypsin. The dotted line in Fig. 2. shows the percentage of negatively charged alanine ions as the function of pH. The enzyme seems to be able to react only with the negatively charged alanine ion and not with the positive ion or the zwitterion. It should be remembered, that the enzyme is not destroyed by even much lower pH values, than those at

which there is already no reaction. Preliminary experiments on the pH activity-curve of the enzyme for the reverse reaction show that this is shifted towards the alkaline side as compared with the curve of Fig. 2.

The experiments to determine the pH activity curve have been performed so as to get initial velocities. Therefore 2 units of enzyme were taken for the usual test solution of 4 ml. The reaction was started 5 minutes after the other ingredients were placed in the waterbath, by the addition of the ketoglutaric acid solution. It was stopped after 4 minutes by pipetting 1 ml of the solution into 1 ml of concentrated KOH solution. The buffers used were: glycine-HCl in the acid region, citrate-phosphate mixtures between pH 4,6—7,8 and glycine-NaOH on the alkaline side. All of them were present in 0,1 M final concentration. The pH values were controlled colorimetrically.

VI.

Kinetics of the reaction.

1. Equilibrium. The position of the equilibrium in the reversible reaction (2) was studied for the case of equal concentration of the two reacting substrates, under varyng enzyme concentration and varying initial concentration of the substrates. Table II. contains some of these results. It is seen that the equilibrium position does not depend on the concentration of the enzyme, nor on that of the substrate. From the mean value of 45,5% pyruvic acid in equilibrium, the equilibrium costant can be calculated:

$$K = \frac{C_{alanine} \cdot C_{ketoglutaric\ acid}}{C_{glutamic\ acid} \cdot C_{pyruvic\ acid}} = \frac{54,5^2}{45,5^2} = 1,43$$

2. Reaction velocity. The aim of kinetic investigations is the analysis of the mechanism of enzyme reactions. If the reactions, which take place during the enzyme action are set up correctly, we should be able to obtain an equation, which tells us the composition of the system at any given time. provided the initial concentrations of substrates and enzyme are given at a fixed pH and temperature.

There is no enzyme reaction for which such an equation

Table II.

Enzyme units/ml	Initial concentration mol/lit				pyruvic acid mol/lit found in equilibrium	% pvruvic acid in equilibrium
	l(+)glutamic acid	pruvic acid	l(+)alanine	α-ketoglutaric acid		
1,0	0,100	0,100	0	0	0,044	44,0
1,0	0,070	0,070	0	0	0,0309	44,1
1,0	0,040	0,040	0	0	0,0167	41,8
1,0	0,0200	0,0200	0	0	0,0094	47,0
1,0	0,0090	0,0090	0	0	0,00398	44,3
1,0	0,0050	0,0050	0	0	0,00241	48,2
1,0	0	0	0,0050	0,0050	0,00230	46,0
1,0	0,0025	0,0025	0	0	0,00122	48,8
1,0	0,0010	0,0010	0	0	0,00043	43
2,5	0,0060	0,0060	0	0	0,00278	46,4
0,82	0,0060	0,0060	0	0	0,00281	46,8
0,25	0,0060	0.0060	0	0	0,00276	46,0
					mean value:	45,5

is elaborated, owing no doubt to the complexity of the question. Several attempts were made earlier on kinetic studies of enzyme reactions, but most of them were connected with proving or disproving empirical laws. It was first realized by MICHAELIS and MENTEN[8] and later by WARBURG and his associates[9] that the mathematical solution of this question can be achieved by assuming that the enzyme reaction rate depends on the concentration of the enzyme-substrate complex. By determining the enzyme substrate dissociation constants they were able to obtain time-laws for some enzyme actions. But even from these studies no generalized time law could be constructed, owing no doubt to the aproximative nature of the calculations.

The necessity of work on these lines is emphasized by the fact that one often encounters kinetic studies in which the simple non catalytical time laws of monomolecular or bimolecular reactions are shown to hold for an enzyme reaction. COHEN for instance in his paper on transamination states that the transamination can be described by a bimolecular reaction constant. This can only be due to experimental error. Apart

from the fact that not even the reversibility of the system is taken into account, such an attempt is meaningless. This is illustrated by Fig. 3. in which the experimental time curve for the alanine-ketoglutaric acid reaction is indicated by the amount of pyruvic acid formed. Substrate concentration: 0,00685 mol/liter for each substrate, enzyme concentration 0,56 units/ml glutamino-pyruvic aminopherase. PH 7,3, temperature 38°. The time curves of a simple bimolecular reaction and that

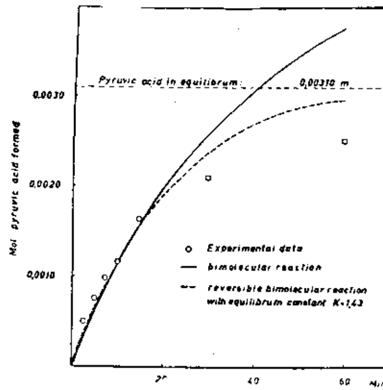

Fig. 3.

of a reversible bimolecular reaction (with equilibrium constant $K = 1{,}43$) are drawn into the same figure so as to meet the facts at one point. (10 min.). It is seen that they do not meet the facts at any other point.

If we assume that the substrates form dissociating complexes with the enzyme, it is possible to obtain an integral equation as the time law of this reversible bimolecular enzyme reaction. This is rather a complicated equation even for the case of equal initial substrate concentration, but it contains only the following quantities: initial concentration of substrates, enzyme concentration, the four enzyme-substrate dissociation constants, the equilibrium constant and the substrate concentration at time *t*. Owing to reasons described above, two of the enzyme-substrate dissociation constants are still missing and we were not able to prove our equation, thus not described here. But it is believed that only on these lines can the kinetic laws of catalyzed reactions be developed.

D e t e r m i n a t i o n   o f   t h e   e n z y m e - s u b s t r a t e

dissociation constants. We suppose that alanine and ketoglutaric acid, which react with one another are bound to different points of the enzyme. In this case the dissociation

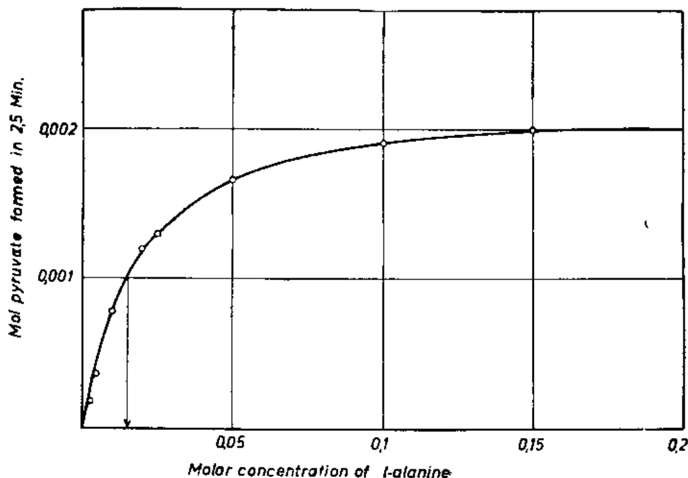

Fig. 4.

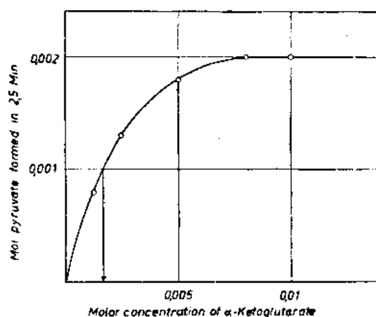

Fig. 5.

of the enzyme-alanine complex is independent of the concentration of the ketoglutaric acid and vice versa. If therefore the concentration of ketoglutaric acid is kept constant and so high that it saturates the enzyme (0,05 M), then the initial velocity is proportional to the concentration of the enzyme-alanine complex. If the initial velocities are now determined for varied alanine concentrations, the curve of Fig. 4. is obtained. The alanine concentration, at which half of the maximum velocity

is observed, is equal to the enzyme-alanine dissociation constant. In a similar way, the enzyme-ketoglutaric acid dissociation constant can be evaluated from Fig. 5. Here the concentration of alanine was kept constant and high (0,1 *M*) and the concentration of α-ketoglutaric acid varied.

We are indebted to Prof. V. BRUCKNER and Mr. C. AUTHERIED for a generous supply of synthetic α-ketoglutaric acid.

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