STUDIES ON THE ENZYMATIC DEGRADATION OF COCARBOXYLASE

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(Received August 27, 1957)

It can hardly be said that sufficient knowledge has been accumulated in the literature concerning the enzymatic degradation of cocarboxylase. Lohmann and Schuster (1) studied the nature of the phosphoric acid of cocarboxylase (thiamine diphosphate, TDP), and found that 50 per cent of the total phosphoric acid was hydrolysed in 1*N* hydrochloric acid at 100° within 15 minutes, whereas the remaining 50 per cent was much more resistant to the hydrolysis for liberating inorganic phosphate (iP) by heating for 90 minutes. Using the dialysate of kidney extract, they examined the action of so-called alkaline phosphatase on TDP, and stated that the easily hydrolysable phosphoric acid was liberated very quickly, whereas the hardly hydrolysable one much slower. Partially purified acid phosphatase from prostate was also used. By this enzyme, however, both kinds of the phosphoric acid were liberated at the same rate.

It has been known for many years that alkaline-washed dry bakers' yeast (etiozymase) produces carbon dioxide from pyruvate in the presence of TDP and the action is greatly stimulated by thiamine (2). As to this phenomenon Westenbrink *et al.* (3) presumed that the phosphoric acid molecules were liberated one by one by the action of a single enzyme of bakers' yeast, and thiamine inhibits the activity of this enzyme.

The author (4) studied more directly the preventive effect of thiamine on the inactivation of TDP by etiozymase. Recently, using the partially purified apocarboxylase preparations, Aoshima (5) showed the inhibitory action of thiamine on the liberation of iP from TDP.

As to whether a specific thiamine-pyrophosphatase is present or not, Naidoo and Pratt (6) presumed the presence of a specific enzyme differing from other phosphatases in the brains of the rat, *etc.*, mainly from the results of histochemical studies.

On the other hand, Kornberg and Pricer (7) described that there was a strong TDP-decomposing activity (TDPase activity) in the crude extract of potatoes, and most of this activity disappeared during the course of the purification of nucleotide pyrophosphatase. They pointed out, however, even the most purified enzyme preparation had some TDP-decomposing activity, though far weaker than the activity for DPN.

Plaut (8) purified an enzyme specific for inosine diphosphate from the acetone

powder of bovine liver mitochondria. It is not known whether this enzyme can act upon TDP or not.

Miura, Fujiwara, *et al.* (9) made a comparative study for TDP-decomposing activity using various tissue homogenates of rats.

The author¹ has studied the nature of TDPase activities of the homogenates of rat brain, dog kidney, and bovine kidney, and also examined the enzymological properties of the preparations obtained from extracts of dog kidney, bovine kidney, and dry yeast. In this paper, the results of the studies are reported.

Experimental

1. Choice of Methods for Estimating the Degradation of TDP.

For estimating the amount of TDP decomposed, three methods can be used. *i.e.*, (a) manometric determination of the decrease in TDP using etiozymase or apocarboxylase, (b) colorimetric determination of inorganic phosphate liberated, and (c) fluorometric determination of thiamine liberated. With each of these methods, a side-view of the decomposition of TDP can be obtained. However, quantitative aspects of all the possible reaction products cannot be clarified with a single method alone. In the present studies iP determinations were mainly used, and when necessary, the manometric estimation of TDP using apocarboxylase was also used. In addition, the detection of TDP, thiamine monophosphate (TMP), and thiamine was applied using paper electrophoresis.

For determining iP, Gomori's original method (10) was adopted. By this method, iP liberation from TDP in the course of assay procedure was not observed.

2. Influence of Thiamine for the Determination of iP.

Thiamine reacts with phosphomolybdic acid to form an insoluble complex. Thus, when thiamine exists in the reaction mixtures together with iP, the colored solutions become turbid. The influence of this turbidity upon the determination was not so large provided that the colored solution was diluted, and, in addition, that the concentrations of thiamine and iP in the samples were restricted in a settled range. In the early stages of this study, iP was determined under this condition. Later, however, Uehara's method (11), in which the turbidity was counteracted by carrying out the photometric measurements in the half-saturated ammonium chloride solution, was applied to Gomori's method, and good results were obtained. The outlines of the latter method are as follows.

To a 25 ml-graduated test tube, less than 10 ml of the sample (iP not exceeding 100 γ , thiamine not exceeding 200 γ), 2.5 ml of Gomori's acid-molybdate reagent, and 10 ml of saturated ammonium chloride solution are added in this order, and mixed well. Then, 1 ml of 1 per cent monomethyl *p*-aminophenol sulfate in 3 per cent NaHSO₃ is added and made up to 25 ml with distilled water. After mixing well the solution, the test tube is kept at 25° for 45 minutes in a water bath. Photometric reading is made in 45 to 90 minutes (*d* = 25 mm, red filter). A blank test is to run parallel using water in place of sample solutions. The turbidity due to thiamine always disappeared during the incubation period. Under these conditions, 200 γ of thiamine did not show any influence on the determination of iP, and the added ammonium chloride gave scarcely any decrease in the extinction.

¹ Proceedings have been reported at several meetings (22, 23)

3. An Example for the Simultaneous Determination of the *iP* Liberated and the Decrease in TDP Activity.

The TDP-decomposing enzyme used in this experiment was prepared from bovine kidney autolysate by acetone fractionation, trypsin treatment, and ammonium sulfate fractionation. Cocarboxylase activity was determined manometrically using apocarboxylase prepared from yeast according to the method of Yamamura *et al.* (12). As is illustrated in Fig. 1, the decrease of TDP and the liberation of *iP* coincided well.

4. Conditions for Estimating the Activity of TDP-decomposing Enzyme.

The activity of phosphatases is often greatly influenced by the composition of buffer solutions used (13). In the present study, Michaelis' veronal-acetate-HCl buffer (pH 4–9) was used because of its broad buffering range and relative constancy of the composition over a wide range of pH. The basic composition of reaction mixture, expressed in ml, was as follows. If needed, each component and the total fluid volume were multiplied.

Buffer solution, 1.0; 1 per cent magnesium sulfate, 0.2; *M*/250. substrate, 0.4; enzyme solution plus water, 0.4; total volume, 2.0.

The activity of the enzyme preparation was assayed under the conditions where the substrate decomposition was kept less than 30 per cent, and the amount of the enzyme was varied. Incubation was carried out at 38° for 10–15 minutes. When the fluid containing insoluble particulate materials, *e.g.*, homogenate, were used as enzyme, the incubation was carried out with continuous shaking. The reaction was stopped by adding equal volumes of 10 per cent trichloroacetic acid, followed by the filtration of the precipitate formed. Liberated *iP* was determined with 2 ml of the filtrate. Control experiments ran parallel with all the samples, and the blank values (reaction time 0 minute) was subtracted from each main value to correct the small amount of *iP* contained in the reagents and enzyme preparations.

5. Miscellaneous.

The protein concentration in the enzyme solutions was determined with micro-Kjeldahl method as modified by Parnas. The protein concentration in the fractions obtained by column chromatography and zone electrophoresis was estimated with Folin's phenol reagent (14) as modified by Lowry *et al.* (15). For the estimation of inorganic pyrophosphate (*iPP*), Kornberg's method (16) was applied.

TDP was kindly supplied from Dr. Matsukawa of the Takeda Research

FIG. 1 Degradation of TDP and Liberation of Inorganic Phosphate.

Laboratory. The purity of the samples was estimated to be 90 per cent by both electrophoretic and enzymatic analysis. They contained small amount of TMP and iP but no thiamine. TMP also supplied from Dr. Matsukawa, contained neither TDP nor thiamine as tested by electrophoresis.

RESULTS AND DISCUSSION

TDP-Decomposing Activity of Rat Brain Homogenate

1. pH-Activity Curve

Figs. 2—4 illustrate the pH-activity curve obtained from an experiment using 10 per cent rat brain homogenate (in 1 per cent KCl).

When TDP was used as substrate, the homogenate showed an accessory activity peak at pH 6 to 7 which may correspond to TDPase found by Naidoo and Pratt in histochemical studies. Under the author's experimental conditions, however, a much higher decomposing activity was observed in the more alkaline region (adjacent to pH 9). But the relative activity at pH 6 to 7 varied

FIG. 2 *Degradation of TDP by Rat Brain Homogenate.*

1. Total iP liberated.
2. iP liberated from endogenous substrate.
3. iP liberated from TDP by spontaneous decomposition plus iP contained in TDP sample.
4. iP liberated from TDP by enzyme reaction.

FIG. 3 *Degradation of GP by Rat Brain Homogenate.*

from rat to rat and, in some rare cases, it was even higher than that of pH 9.2 in agreement with the findings of Naidoo and Pratt in chicken brains. The decomposing activity for β -glycerophosphate (GP) showed typical peaks of acid and alkaline phosphatases (Fig. 3). On the contrary, pH-activity curve for TMP was similar to that of TDP (Fig. 4).

2. Influence of Osmotic Factor

It is well known that the mitochondrial adenosine triphosphate-decomposing activity (ATPase activity)

depends upon the osmotic factor, exhibiting the lowest activity in isotonic media (17). TDPase activity at pH 6.9 was examined in this respect, and it was found that, though less significant than in the case of ATPase, it clearly depends upon the osmotic pressure and exhibits the lowest activity in isotonic media. From this result, at least some part of TDPase activity is assumed to be bound with cellular structures.

3. Influence of Fluoride

Influence of fluoride was examined with 10 per cent rat brain homogenate (Table I). At the concentration of $M/25$, inhibition by fluoride was

FIG. 4. Degradation of TMP by Rat Brain Homogenate.

TABLE I

Effect of Fluoride on the Activity of TDPase and GPase of Rat Brain Homogenate.

	pH	iP liberated in γ/hr	
		Without NaF	With NaF ^a
TDPase	9.2	7.10	4.65
	6.9	4.85	4.10
GPase	9.2	6.35	1.85

^a Final concentration, $M/25$.

insignificant against the decomposition of TDP compared with against that of GP, especially at pH 6.9. Decomposition of GP was strongly inhibited by fluoride at pH 9.2. These findings coincide well with the histochemical evidence of Naidoo and Pratt (6).

Fractionation of TDPase in Rat Brains by Differential Centrifuging, and Trials for Extracting the Particle-bound Activity

1. Fractionation by Differential Centrifuging

Since at least some part of the TDPase activity was assumed to be bound with structural proteins, the possibility of physiological presence of a soluble enzyme and of extracting the bound enzyme into solution was tested. For the first step, the differential centrifuging was carried out. As the cell composition of the brain is different from that of the liver, *etc.*, and is hardly fractionated in an ordinary way, the activity was determined with fractions obtained by various centrifugal forces. The results are illustrated in Table II. It is apparent that a considerable part of the enzyme activity decomposing TDP at pH 6.9 is bound to cell structure. However, the possibility of physiological existence of some part of the activity in the soluble fraction is not to be excluded.

TABLE II
TDPase Activity of Rat Brain Homogenate Fractions Obtained by
Centrifuging (pH 6.9)

Centrifuging		Fraction	Nitrogen content	iP liberated	Microscopic findings
<i>rpm</i>	<i>min</i>		<i>mg N/ml fraction</i>	<i>γ/hr</i>	
2,500	10	Homogenate	1.48	4.90	Nucleus, large and small granules
		Supernatant	0.40	1.75	Ssmall granules
		Washed precipitate	0.76	2.56	Nucleus, large granules and some small granules
24,000	20	Homogenate		7.03	
		Supernatant		4.13	
		Precipitate		7.00	
150,000	60	Supernatant		5.40	
		Precipitate		7.80	

2. Trials for Extracting the Bound Enzyme

Trials for extracting the enzyme activity bound to insoluble part, and thus for increasing the yield of soluble enzyme were carried out. Freezing and thawing as well as extraction with surface-active substances failed to show good results. When brains were cut into small pieces and autolyzed in water aseptically, the activity of the soluble part increased considerably. On the contrary, when brains were first homogenized and then autolyzed, the effect of autolysis was not observed.

At any rate, as the activity of rat brain enzyme was not so high, it was difficult to examine the nature of the enzyme more thoroughly.

TDP-Decomposing Activity
in Dog Kidneys

1. pH-Activity Curve of the Dog
Kidney Homogenates

With dog kidney homogenate, relatively strong activity was observed at pH 8 to 9, and differing from the case of rat brains, the secondary low peak was observed

FIG. 5 Degradation of TDP by Dog Kidney Homogenate.
1. Total iP liberated.
2. iP liberated from endogenous substrate.
3. iP liberated from TDP by enzyme reaction.

adjacent to pH 4.9, as illustrated in Fig. 5.

2. pH-Activity Curve of the Soluble Enzyme Preparation from the Supernatant of Dog Kidney Homogenate

A fresh dog kidney weighing 52 g was cut into small pieces, 120 ml of water was added, and organ was homogenized for one minute in a Waring blender. The homogenate was centrifuged at 2,500 rpm for 15 minutes. The pH of the supernatant (110 ml) was adjusted to 5.2 with diluted acetic acid and the solution was centrifuged at 2,000 rpm for 15 minutes. The resulting supernatant (80 ml) was clear and colored red brown. The proteins of the supernatant were precipitated by adding acetone (final concentration, 60 v/v per cent) at 0° and centrifuged. The supernatant was decanted off, and the precipitate was dissolved in water and reprecipitated with saturated ammonium sulfate, followed by centrifugation. The precipitate was dissolved in small amount of one per cent KCl solution, and dialysed against water for 24 hours. A small amount of insoluble protein formed was centrifuged off, and the supernatant was used for the experiment. From Fig. 6, it is seen that the enzyme activity was not so high, and the maximum degradation of TDP occurred at an acidic region.

3. pH-Activity Curve of Dog Kidney Autolysate

Dog kidney autolysate was tentatively prepared according to the initial step

FIG. 6 Degradation of TDP by the Soluble Enzyme Preparation from the Supernatant of Dog Kidney Homogenate.

FIG. 7 Degradation of TDP by Dog Kidney Autolytic Extract.

of the method of Abul-Fadl *et al.* (18) who used the procedure for purifying alkaline phosphatase. As this extract showed a relatively high activity at pH 9.2, its pH-activity relationship was examined. The method for the preparation was largely the same as that of bovine kidney extract described below. The pH-activity curve is illustrated in Fig. 7. In this case, the TDP-decomposing activity was stronger at an alkaline region, suggesting that the activity for decomposing TDP is not confined to a single enzyme. Under these conditions non-enzymatic iP liberation from TDP at pH 9.2 was not observed. For examining the nature of these kinds of enzyme thoroughly, bovine kidney was used.

Studies on the TDP-decomposing Enzyme of Bovine Kidney

1. pH-Activity Curve of Bovine Kidney Homogenate

FIG. 8 Degradation of TDP, GP and iPP by Bovine Kidney Homogenate.

As is illustrated in Fig. 8, extremely high iPPase activities were observed in bovine kidney homogenates. Relatively strong TDP decomposition was found at pH 9 and 6, whereas relatively strong GP decomposition at pH 9 and 5. In this experiment, bovine kidneys were obtained from a butchery as freshly as possible, but 2 to 3 hours elapsed after butchering.

2. Preparation of the Crude Enzyme

Largely based upon the method of Abul-Fadl *et al.* (18), crude enzyme was prepared as follows. 6,000 g of bovine kidneys were decapsulated, and the adipose tissue and pelvis were removed. All the cortex and the soft part of the medulla were collected, and minced well. 4050 g of the mince was obtained. 2535 ml of water, 1015 ml of acetone, 200 ml each of toluene and ethyl acetate were added to the mince and mixed well. The whole was allowed to stand at room temperature (20°) for 3 days

with occasional stirring, followed by pressing through a cheese cloth, whereby a turbid fluid was obtained. By filtering through Toyo Filter paper No. 2, a faint yellow-colored clear extract was obtained (3750 ml). 2500 ml of ice-cold pure acetone (2/3 volumes of the extract) was added slowly at 0°, and the resulting precipitate was centrifuged down at 2500 rpm for 30 minutes. The precipitate was transferred to a Buchner funnel with a small volume of cold pure acetone. The cake of the precipitate was washed twice with the same solvent and dried in a vacuum desiccator. Almost white protein powder (acetone-dried

crude enzyme), was obtained, which showed relatively high TDPase and GPase activities at pH 9.2.

3. Denaturation, Inhibition and Activation

Using the crude enzyme, the following experiments were performed to elucidate the outlines of the difference between TDPase and GPase.

Heat Treatment — The degree of the denaturation at pH 9.2 was examined by preincubation of the above enzyme at 50° and 55°. The activities for both TDP and GP were determined at pH 9.2. TDPase activity was slightly more labile to heat treatment, but the decisive difference to permit the separation of the two enzymes was not observed. When preincubated at pH 5.0, both TDPase and GPase were rapidly inactivated.

Inhibition by Metal Ions — The activity of both TDPase and GPase was inhibited by metal ions. The activity was determined 10 minutes after adding an inhibitor. The inhibition of TDPase activity by Cu^{++} or Zn^{++} was slightly more pronounced than that of GPase, but without decisive difference (Table III).

TABLE III
Inhibition of TDPase and GPase of Bovine Kidney by Cu^{++} and Zn^{++} .

Metal salt	Final concentration	Inhibition	
		TDPase	GPase
	<i>M</i>	<i>per cent</i>	<i>per cent</i>
CuSO_4	0	0	0
	0.0005	35.4	1.7
	0.001	38.5	5.4
	0.002	63.7	25.5
ZnSO_4	0	0	0
	0.0005	43.5	30.4
	0.001	58.5	37.2
	0.002	62.7	43.7

Activating Effect of Magnesium Ion and Inhibiting Effect of Fluoride

The final concentration of Mg^{++} used was 0.1 per cent as MgSO_4 , and that of fluoride M/50. This enzyme reaction was found to be apparently activated by

TABLE IV
Influence of Magnesium Salt and Fluoride on the TDPase and GPase of Bovine Kidney

Addition	Activity	
	TDPase	GPase
	<i>per cent</i>	<i>per cent</i>
Complete system	100	100
Without Mg^{++}	77.7	84.1
With F^-	84.3	87.5
With $\text{Mg}^{++} + \text{F}^-$	70.3	80.1

Mg⁺⁺. However, the inhibitory effect of fluoride was not so great, and it was influenced by the addition of Mg⁺⁺ (Table IV).

4. Purification of the Enzyme

Ammonium Sulfate Fractionation — Acetone-dried crude powder was dissolved in water, the insoluble protein was centrifuged off, and the clear supernatant was fractionated with ammonium sulfate. The results are illustrated in Table V. The enzyme activity was relatively polydisperse, being distributed in many fractions. Protein concentration and total activity in a fraction were highest at 0.6 to 0.8 saturation, and the activity ratio (TDPase/GPase) was also greatest at that fraction. However, as a whole, the variation of the ratio was not so remarkable.

TABLE V
Effect of Ammonium Sulfate Fractionation on Acetone-Dried Crude Enzymes of Bovine Kidney

Crude enzyme powder, 12 g → dissolved in 500 ml of dist. water
→ centrifuged at 3,000 rpm, 20 min → supernatant, 470 ml
→ fractionation → dialysis

Fraction	Saturation	Volume	Total protein	Protein	Specific activity (γ iP/mg N/hr)		
					TDPase	GPase	TDPase/GPase
		ml	mg	per cent			
Homogenate		—	—	1.12	80.0	70.8	1.13
Crude extract		470	5781	1.23	204.8	214.0	0.95
I	0 —0.5	62.5	969	1.55	192.8	184.8	1.04
II	0.5 —0.6	17.5	273	1.60	169.2	165.2	1.02
III	0.6 —0.7	62.5	1275	2.04	175.2	136.0	1.28
IV	0.7 —0.8	50.0	456	0.91	391.2	304.0	1.29
V	0.8 —0.95	15.0	39	0.26	1092	1180	0.92
VI	0.95—1.0	7.0	5	0.08	1052	2048	0.50

Trypsin Treatment — Fractions I, II, and III in ammonium sulfate fractionation (Table V) were combined (total volume, 124 ml; total protein, 2200 mg; ca. 1.8 per cent), and 125 mg of crystalline trypsin supplied from the Mochida Chemical and Co. were added to the combined solution. pH was adjusted to 8 by adding sodium bicarbonate. After 3 hour incubation at 37°, kaolin was added to adsorb trypsin and the mixture was centrifuged. This adsorption was repeated twice. The supernatant was refractionated with ammonium sulfate. The results of the refractionation are given in Table VI. An increase in the specific activity of the enzymes was noticed, but the activity ratio TDPase/GPase, showed a considerable variation, and the separation of the two activities did not result.

Zone Electrophoresis — The following results were obtained from zone electrophoresis of the crude enzyme using a starch column. In Fig. 9, electrophoresis was carried out under conditions similar to that of the separation of serum proteins (veronal buffer, pH 8.6. $\mu=0.1$). The distribution of proteins was relatively complicated. The decomposing activities for both TDP and GP had an electrophoretic mobility similar to that of β -globulin, both activities show-

FIG. 9 *Effect of Zone Electrophoresis on the Enzyme Activity of Bovine Kidney.*
Condition for zone electrophoresis: starch column, 1.5 × 4.5 × 30 cm; 240 V;
10 mA; 9.5 hr; veronal buffer; pH 8.6; $\mu = 0.1$

TABLE VI

Effect of Trypsin Treatment and Refractionation with Ammonium Sulfate on the Enzyme Activity of Bovine Kidney

Fr. I, II, and III of Table V (total volume 124 ml, protein 2200 mg, ca. 1.8%) + crystalline trypsin 124 mg → pH 8.0, 37°, 3 hr → removal of trypsin by kaolin adsorption (twice) → fractionation → dialysis

Fraction	Saturation	Volume	Total protein		Specific activity (γ iP/mg N/hr)		TDPase/ GPase
				Protein	TDPase	GPase	
		ml	mg	per cent			
I	0 —0.5	15	180	1.2	370	628	0.59
II	0.5—0.6	8	74	0.93	689	864	0.80
III	0.6—0.7	7	35	0.50	1700	1500	1.13
IV	0.7—0.8	5	38	0.75	1524	1148	1.33
V	0.8—0.9	8.5	12	0.24	2188	3480	0.64
VI	0.9—1.0	4	4.6	0.14	772	1692	0.46

FIG. 10 *Effect of Zone Electrophoresis on the Enzyme Activity of Bovine Kidney.*
Conditions for zone electrophoresis: starch column, 1.5 × 3 × 30 cm; 160 V;
10 mA; 9 hr; veronal buffer; pH 7.5, $M/20$

ing a single peak, which was however considerably broad. Fig. 10 shows an electrophoretic diagram at pH 7.6 (veronal buffer, $M/20$). In this case, the velocity of migration decreased, and both TDPase and GPase migrated to the negative pole. Fig. 11 shows an electrophoretic diagram at pH 6.6 (citrate buffer, $M/20$). The sample was started from the middle of the starch column. The peak of the activities for both TDP and GP did not migrate to either side, but dispersed broadly. In any case the separation of TDPase and GPase was not effected.

FIG. 11 Effect of Zone Electrophoresis on the Enzyme Activity of Bovine Kidney.

Conditions for zone electrophoresis: starch column, $1.5 \times 3 \times 30$ cm; 160 V; 15 mA; 14 hr; citrate buffer, pH 6.6, $M/20$

The active part in the crude enzyme obtained by electrophoresis under the same conditions as in Fig. 9, *i.e.*, a section of 5 cm from the starting line for the positive pole was cut out, and the proteins extracted from this section were refractionated with ammonium sulfate. The results of the refractionation is

FIG. 12 Zone Electrophoresis of the Ammonium Sulfate Fractions Obtained from Zone Electrophoresis

Conditions for zone electrophoresis: starch column, $1.5 \times 3 \times 30$ cm; 300–240 V; 10 mA; 30 hr; veronal buffer; pH 8.6, $\mu = 0.1$

shown in Table VII. The activity ratios, TDPase/GPase, were relatively constant, but fractions with high specific activities were easily obtained. The specific activity was especially high at more than 0.6 saturation. However, a considerable activity was also observed with the fraction of less than 0.5 saturation. Fractions II to IV in Table VII were combined and the second electrophoresis was carried out. The result is illustrated in Fig. 12. From this figure, it is noticeable that the enzyme was partially purified, but the separation of the decomposing activities for TDP and GP was not observed.

TABLE VII
*Ammonium Sulfate Fractionation of an Active Extract Obtained
by Zone Electrophoresis.*

Fraction	Saturation	Specific activity (γ iP/mg N/hr)		
		GPase	TDPase	TDPase/GPase
Crude extract		1050	1353	1.29
I	0—0.5	543	702	1.29
II	0.5—0.6	1098	1548	1.41
III	0.6—0.7	2463	3045	1.24
IV	0.7—0.8	2517	3054	1.21
V	0.8—0.9	2517	2886	1.15
VI	0.9—1.0	2355	3000	1.27

Ion Exchange Chromatography—Using Amberlite XE-64, the crude enzyme solution was chromatographed. The resin was bufferized to a slightly acidic pH with citrate buffer. The adsorbed proteins were eluted with sodium borate (pH 9.2). When the resin was bufferized to pH 5.4, the enzyme activity was not adsorbed (Fig. 13 *a*). After bufferizing the resin to pH 5.15, the enzyme activity was completely adsorbed (Fig. 13 *b*). In the case of bufferizing the resin to the intermediate pH of 5.3, the enzyme activity appeared in both the non-adsorbed and adsorbed fractions (Fig. 13 *c*). However, in any case, the separation of TDPase and GPase was not effected.

Discussion on the Purification of the Enzyme Protein—As to whether the TDPase and the GPase are the same enzyme protein or not, several efforts were performed besides these experiments. When the enzyme solution was chromatographed with a special technique, results suggesting a partial separation of TDPase and GPase were obtained. With this technique, however, the denaturation of the enzyme protein was so pronounced that decisive data could not be obtained. On the other hand, enzyme preparations showing the higher TDPase/GPase ratio of 2 to 2.5 were obtained with repeated fractionations with acetone. However, in spite of many efforts, much higher ratio could not be obtained. At any rate, it was presumed that the TDPase and the GPase would be of a very similar nature.

5. pH-Activity Curve and Substrate Specificity of the Partially Purified Enzyme

pH-Activity Curve—Fig. 14 shows the pH-activity curve of the enzyme preparation treated with trypsin. Strong GPase, TDPase, and TMPase activities were observed at pH 9. However, it was remarkable that iPPase active in

FIG. 13 Effect of Ion Exchange Chromatography on the Crude Enzyme of Bovine Kidney.

Column: Amberlite XE-64, approx. 25 mg crude enzyme chromatographed.
— Protein, — GPase, TDPase

alkaline region was scarcely detected. Preparations purified further by acetone fractionation showed no iPPase activity at pH 9.

Substrate Specificity— Although the separation of activities for TDP and GP was not accomplished, the substrate specificity was once examined with the

FIG. 14 pH-Activity Curve of Partially Purified Bovine Kidney Phosphatase for Various Substrates.

liberated iP in γ per 2 ml of the reaction mixture during 15 minutes at pH 9.2 was 63.7, 55.0, 74.1, and 62.1 for ATP, adenosine-5'-phosphate (A5'P), TDP, and GP, respectively, in equimolar concentration. Thus ATP and A5'P were also decomposed by this enzyme preparation.

6. Experiments on the Mechanism of the TDP-Decomposition Reaction

Production of iP Rather than iPP by the Decomposition of TDP

As iPPase activity capable of acting at pH 9.2 was scarcely contained in this enzyme preparation, iPP must accumulate if iPP is produced by splitting TDP. However, iPP accumulation was not detected, when determined by Kornberg's method (6), but iP was found to be liberated from TDP. Thus the reaction

FIG. 15 Analysis of Reaction Products by Paper Electrophoresis

Enzyme : partially purified kidney phosphatase
Electrophoresis : Toyo Filter Paper No. 50,
2 × 25 cm, 1.35 mA/cm, 1300 → 500 V, 3 hr,
M/60 acetate buffer, pH 5.4
Detection : Thiochrome Reaction
iP determination : Gomori's method
The figures on the right, γ P per 2 ml reaction mixture.

enzyme prepared as pure as possible. As shown previously, TMP was decomposed by this enzyme. The libe-

FIG. 16 Lineweaver-Burk Plot for the Action of Bovine Kidney Enzyme on TDP and GP.

are excluded to be the actual process.

Analysis of the Reaction Products by Paper Electrophoresis — In Fig. 15, the production of iP and the rise and fall of TDP, TMP, and thiamine are shown following the time course of the reaction. In the Figure, the intensity of the thiochrome reaction is expressed by the density of oblique lines.

As stated above, TDP samples used contained a trace of TMP as a contaminant. As relatively high TMPase activity was present in the enzyme preparation, the spot of the contaminated TMP will disappear in a short time if TMP was not produced from TDP. In this experiment, however, the spot of TMP did not disappear but showed a tendency to increase in intensity as the reaction proceeded. This is to be interpreted that TMP is produced from TDP. Thus it is assumed that the degradation reactions expressed as

must be the actual case.

7. Kinetic Analysis

Substrate Concentration-Reaction Velocity Curve — As illustrated in Fig. 16, the velocity of the decomposing reaction reached a maximum at the substrate concentration of about $10^{-3} M$. It is very interesting that the reaction velocity decreased at higher substrate concentrations. Such inhibition by high substrate concentration was also noticed with TMP, but not in the case with GP.

According to the method of Lineweaver and Burk (19), reciprocals of the reaction velocity and the substrate concentration were plotted. The linear part of the plots (excluding the inhibited part) were extrapolated and the apparent Michaelis constants were calculated. Thus the values of 2×10^{-3} and $5 \times 10^{-3} M$ were obtained for TDPase and GPase, respectively.

Analysis of the Enzymes with the Competition between Substrates — As reported by Blaschko *et al.* (20), if two kinds of substrate (*A* and *B*) are either decomposed by appropriate respective enzymes or exert no influence on each other², there would be the following relation.

$$v_{A+B}^{\max} = v_A^{\max} + v_B^{\max}$$

where v_A represents the reaction velocity for substrate *A*, v_B that for substrate *B*, v_{A+B} that in the presence of *A* and *B*, and $v^{\max}$ the maximum velocity.

On the contrary, if two kinds of substrate are decomposed by a single enzyme, there would be the following relation.

$$v_{A+B} < v_A + v_B$$

The higher the concentration of substrate, the greater the difference. Finally, v_{A+B} approaches to the maximum reaction velocity of the substrate whose Michaelis constant is smaller than the other. In another words, the velocity for the mixed substrates approaches to that of the substrate which combines with the enzyme more tightly.

Applying this theoretical ground, experiments were performed to analyze the enzyme preparation. Fig. 17 illustrates the results concerning TMP and

² In many cases, this supposition is satisfied only approximately.

GP. As the substrate concentration increased, $v_{\text{TMP+GP}}$ approached to v_{TMP} . Fig. 18 illustrates the results concerning TDP and GP. Also in this case, as the substrate concentration increased, $v_{\text{TDP+GP}}$ approached to v_{TDP} . Fig. 19 shows the results concerning TDP and TMP. $v_{\text{TMP+TDP}}$ reached a maximum at a concentration of about M/1000 each and, when the concentration was increased further, $v_{\text{TDP+TMP}}$ become smaller than either v_{TDP} or v_{TMP} .

From these results, next assumption will be drawn provided that the substrate inhibition is tentatively not brought into consideration. That is, in a reaction system expressed as

the enzymes E_1 , E_2 , and E_3 are equal. It means that a single enzyme (so-called kidney alkaline phosphatase) acted upon TDP and liberated the phosphoric acid one by one.

However, decisive conclusion should be withheld until the nature of the inhibition caused by increasing the substrate concentration is clarified.

No inhibition being observed with GP, it is apparent that the inhibition mentioned above is not an inhibition caused by iP produced.

Inability of Thiamine to Inhibit the Reaction—No inhibition was observed by the addition of thiamine up to about the equal concentration to the substrates (Table VIII).

FIG. 17 Competition of Bovine Kidney Enzyme between TMP and GP

FIG. 18 Competition of Bovine Kidney Enzyme between TDP and GP

FIG. 19 Competition of Bovine Kidney Enzyme between TMP and TDP

TABLE VIII
Invalidity of Thiamine to Inhibit Kidney Enzyme

Substrate (M/1700)	iP liberated ($\gamma/15$ min)		
	Without thiamine	With thiamine (final M/5,000)	With thiamine (final M/1,000)
TDP	22.9	22.3	22.4
TMP	13.8	14.3	13.8
GP	13.1	13.1	12.7

TDP-Decomposing Enzyme in Yeast Autolysate

1. *Preparation of the Extract*

Packed dry bakers' yeast³ supplied from the Oriental Yeast and Co.⁴, was suspended in M/2 phosphate buffer (pH 7.2), autolyzed at 38° for 2.5 hours, and centrifuged. To the supernatant, solid ammonium sulfate was added to saturation, and the resulting precipitate was separated by centrifuging at 10,000 rpm for 60 minutes. The precipitate was dissolved in a small volume of water, dialyzed against running tap water for 24 hours, and was used for experiments.

2. *pH-Activity Curve*

Conditions employed for the determination of the activities were almost the same as described above except that citrate buffer was used. Fig. 20 illustrates

FIG. 20 *pH-Activity Curve of TDP-Decomposing Enzyme of Yeast.*

FIG. 21 *The Effect of Thiamine on the Substrate Concentration-Reaction Velocity Curve of TDP-Decomposing Enzyme of Yeast (pH 5.2)*

³ Air dried yeast, made from a heat-stable strain. ⁴ Chūō-ku, Tokyo

the pH-activity curve for TDP-decomposing activity showing the strong decomposition in acidic regions.

3. Kinetic Analysis

The substrate concentration-reaction velocity curve at pH 5.2 showed that thiamine apparently inhibited the decomposition of TDP by this enzyme. Substrate inhibition was, under the conditions tested, not observed. It is worthy of note that the yeast enzyme is different from the bovine enzyme in these respects. Fig. 21 shows the Lineweaver-Burk plot, from which thiamine is assumed to inhibit the decomposition of TDP competitively. Further detailed studies are necessary for clarifying the decomposition of TDP by yeast phosphatase.

Inability of Inosine Diphosphatase to Degrade TDP

The acetone powder of bovine liver mitochondria was prepared following the method of Plaut (7), and the cold water extract of the acetone powder was used. The reaction mixture contained 1.0 ml of *M*/10 tris buffer, 0.4 ml of *M*/250 substrate, 0.4 ml of 1 per cent MgSO_4 and 0.2 ml of the enzyme. After 20 minute incubation at 37°, the liberated inorganic phosphate was estimated. When inosine diphosphate (IDP) was used as substrate, the liberated iP was 15 γ , whereas TDP and TMP failed to liberate iP. In this case, non-enzymatic decomposition of IDP during the colorimetric estimation was not observed. Thus this enzyme is shown to decompose IDP, but not TDP and TMP.

SUMMARY

Thiamine diphosphate-decomposing activities in the homogenates of rat brain, dog kidney' and bovine kidney were studied. Those in the autolysate of dog kidney, bovine kidney and yeast were fractionated, some of which were partially purified, and the enzymological properties were also studied with the following results.

1. When the liberation of inorganic phosphate was measured for determining thiamine diphosphate-decomposing activities, a turbidity caused by the reaction between thiamine and phosphomolybdate appeared. However, under suitable conditions, and in a settled concentration range, the extinction could be used as an indicator of degradation. Furthermore, the method of using ammonium chloride to extinguish the turbidity was retested and proved to be excellent and useful. It was applied to the present experiments.

2. β -Glycerophosphate-dephosphorylating activity in rat brain homogenates showed, under conditions used, two maxima at pH 9 and 5. On the contrary, thiamine diphosphate-decomposing activity showed the highest value at approximately pH 9, and, in addition, an accessory peak between pH 6 and 7. The degree of the inhibition for thiamine diphosphate-decomposing activity by fluoride was much less than that for β -glycerophosphate-dephosphorylating activity, especially at pH 6.9. The activity for thiamine diphosphate was influenced to some extent by the change in osmotic pressure. The enzyme was presumed to exist in both soluble and insoluble fractions.

3. Thiamine diphosphate-degrading activity in dog kidney homogenates showed a maximum between pH 8 and 9, but the activity was also observed below pH 6. The accessory activity peak was observed adjacent to pH 4.9.

An enzyme preparation was obtained from the supernatant of the kidney homogenate by fractionating at various pH's. It decomposed thiamine diphosphate at acidic pH range, but its activity was not so high. Another enzyme preparation was obtained from the extract prepared by autolyzing kidneys in the mixed solvent containing water, acetone, toluene and ethyl acetate. This preparation degraded thiamine diphosphate more markedly at pH 9.2.

4. Bovine kidney homogenates showed strong decomposing activities for inorganic pyrophosphate over a wide pH range. Besides these, they showed relatively high decomposing activities for thiamine diphosphate at pH 9 and 6, and also for β -glycerophosphate at pH 9 and 5 (so-called alkaline and acid phosphatases).

5. Using the autolysate of bovine kidneys as the starting material, attempts for purification of the enzyme were made. In the partially purified enzyme preparations, no inorganic pyrophosphatase activity at pH 9.2 was found. However, decomposing activities for both thiamine diphosphate and β -glycerophosphate were found, and it was difficult to separate them. These two activities showed the same behaviour when tested with ordinary zone electrophoresis and column chromatography. With ammonium sulfate fractionation, trypsin treatment, and acetone fractionation, especially with repeated acetone fractionations, the activity ratio (TDPase/GPase) somewhat shifted to the larger. But it was not clarified whether they could be separated or not. It was presumed that the thiamine diphosphate-decomposing enzyme and the β -glycerophosphate-decomposing enzyme would be of a very similar nature.

6. Thiamine diphosphate-decomposing enzyme preparations obtained from bovine kidneys could also decompose ATP, adenosine-5'-phosphate, thiamine monophosphate, and β -glycerophosphate.

Presumptive mechanism for degradation is as follows.

The activities for both thiamine diphosphate and thiamine monophosphate was inhibited by the increase in substrate concentration, but that for β -glycerophosphate was not. Thiamine showed no inhibitory action for these enzyme reactions. If the inhibition due to increase in substrate concentration is tentatively not brought into consideration, it is possible to assume from the kinetic analysis that the reactions mentioned above are catalyzed by a single enzyme.

7. Thiamine diphosphate-decomposing activity in the enzyme preparation obtained from yeast extracts showed strong action at acidic pH. With this enzyme, substrate inhibition was not observed. It was reinvestigated and confirmed that thiamine inhibits the decomposition of thiamine diphosphate by yeast enzyme. Furthermore, results were obtained indicating this inhibition to be of a competitive nature.

8. Thiamine diphosphate was not degraded by inosine diphosphatase.

ACKNOWLEDGEMENT

The author is deeply indebted to Prof. Norio Shimazono for his kind advice and encouragement, and also to Dr. Taizo Matsukawa for the kind gift of synthetic cocarboxylase.

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