ENZYMATIC BREAKDOWN OF THREONINE BY THREONINE ALDOLASE*

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Braunstein and Vilenkina (1) reported the presence of an enzyme (or enzyme system) in various animal tissues which forms glycine from serine, threonine, and certain other α-amino-β-hydroxy acids. They named this enzyme glycinogenase. Vilenkina (2) showed that the enzyme system yielded as much glycine from DL-threonine as from DL-serine, that it operated selectively on the L-amino acid isomers; and that allothreonine was split more readily than was threonine. The products of the breakdown of threonine were glycine and acetaldehyde (3). The enzyme preparations used by Braunstein and Vilenkina were slices, homogenates, and acetone powder extracts of various tissues of various species. The present available information on this enzyme is limited to preparations not purified further than the extract of acetone powder of tissues.

The present investigation was undertaken to isolate a more purified enzyme preparation and to study its general chemical characteristics, substrate specificity, and kinetics. The cleavage of threonine into glycine and acetaldehyde is similar to the reverse of an aldol condensation and threonine is the chief substrate commonly present in nature, serine not being attacked. Consequently, "threonine aldolase" appears to be a more appropriate name, and the enzyme is so designated in this paper.

Experimental Methods

Measurement of Threonine Aldolase Activity.—Threonine aldolase activity was estimated by measuring the rate of acetaldehyde formation. Incubations were carried out in tightly stoppered culture tubes, or in stoppered 10 ml. flasks, for 30 minutes at 37.3°C. The enzyme assay system contained, unless otherwise stated, 2 ml. of 0.15 M phosphate buffer, pH 7.6–7.7; 0.25 ml. of 0.25 M DL-allothreonine (or other amino acid) and 1 ml. of enzyme solution. When other components had to be added, the incubation volume was maintained at a total of 3.25 ml. by reducing the amount of phosphate and adding water when necessary. After incubation, the tubes were immediately

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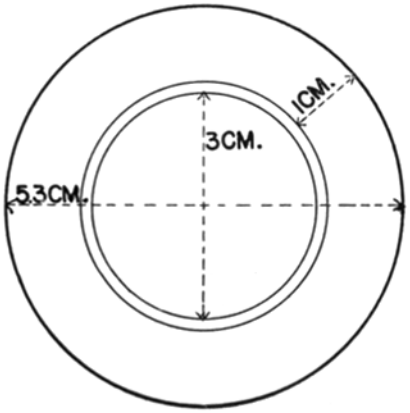

FIG. 1 *a*

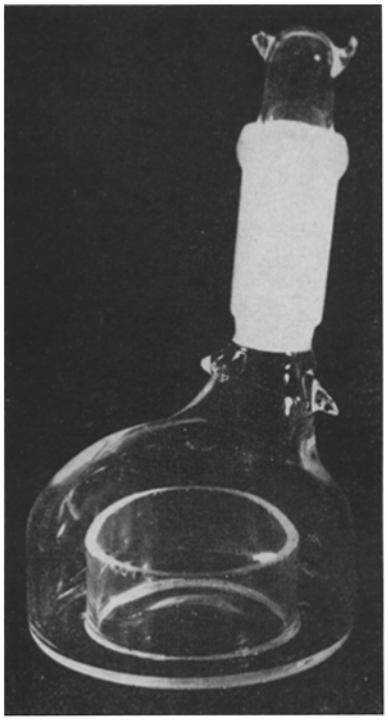

FIG. 1 *b*

FIG. 1. Glass diffusion cell. (*a*) Diagram of cross-section of diffusion compartment. (*b*) Photograph of diffusion cell (actual size).

cooled in an ice-salt mixture to stop the reaction, and the acetaldehyde determined. Appropriate blanks were run parallel with the experimental flasks in most experiments.

Determination of Acetaldehyde.—This was determined by the method of Burbridge *et al.* (4) with some modification. A set of glass diffusion cells[1] (shown in Fig. 1) was used. 3 ml. of buffered semicarbazide hydrochloride solution were pipetted into the center chamber and 0.25 ml. each of 2/3 N H_2SO_4 and 10 per cent sodium tungstate were introduced into the outer chamber. 1 ml. of the cold incubation mixture was transferred into the outer chamber, and the greased stopper was immediately inserted and tightened with a rubber band. The contents of the outer chamber were mixed by gently rotating the cell. The cells were then placed in a water bath at a temperature of around 35°C., and diffusion was allowed to proceed for 90 minutes. At the end of this time 2 ml. of the contents of the inner chamber were pipetted into a 10 ml. volumetric flask and brought to volume with distilled water. The optical densities of these solutions were read within 90 minutes on a Beckman DU spectrophotometer at the wave length of 224 mμ in 1 cm. cells. The reference blank for these readings was 2 ml. of the semicarbazide solution diluted to volume in a 10 ml. volumetric flask. From the optical densities obtained the corresponding micromoles of acetaldehyde were read off from a standard curve. The final value of acetaldehyde formed in 3.25 ml. of the incubation mixture was obtained by multiplying by the factor 4.88, in order to correct for dilution.

Determination of Nitrogen.—At each stage in the purification of threonine aldolase the nitrogen content was determined by the micro-Kjeldahl method.

Preparation of Solutions.—(*a*) *Enzyme solution*: A water solution containing 2.0 mg. of lyophilized dry enzyme powder was used for incubation, unless otherwise indicated. (*b*) *Substrate solution*: Amino acid solutions were made up in desired concentrations with 0.15 M phosphate buffer at pH 7.6–7.7, the final pH of the solution being about 7. The DL-allothreonine was purchased from the National Biochemical Corporation, Cleveland, Ohio; the L-threonine, from the Schwarz Laboratory, Inc., Mount Vernon, New York; the DL-threonine, from the H. M. Chemical Co., Ltd., Santa Monica, California; and both the D- and L-allothreonine were gifts from Dr. J. P. Greenstein[2] of the National Cancer Institute. The D-allothreonine was purified by recrystallization from a water-alcohol mixture, the L-compound was used without further purification. (*c*) *Phosphate buffer*: 0.15 M phosphate buffer was made up by dissolving 19.45 gm. of Na_2HPO_4 and 1.79 gm. of $NaH_2PO_4 \cdot H_2O$ with water to 1 liter; the pH of the solution was adjusted to 7.6–7.7 with the aid of a pH meter. (*d*) *Tris[3]-maleate buffer*: It was made up by acidifying 0.2 M tris buffer with 0.2 M maleic acid to the desired pH. (*e*) *Alkaline saline*: 0.154 M KCl and 8 ml. of 0.04 M $KHCO_3$ were dissolved in 1 liter of solution.

Measurement of pH.—All pH values were determined with a Beckman pH meter, model G, the temperature being 25°C., unless otherwise indicated.

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[1] Developed in cooperation with Dr. E. M. Gal.

[2] We are greatly indebted to Dr. Greenstein for his generosity in furnishing these compounds.

[3] Tris(hydroxymethyl)aminomethane.

EXPERIMENTAL RESULTS AND DISCUSSION

Purification of Enzyme

The procedure finally adopted to prepare a partially purified threonine aldolase preparation involves the following steps: (1) preparation of an acetone powder; (2) extraction of acetone powder; (3) heat precipitation of non-threonine aldolase proteins; (4) adsorption on and elution from alumina Cγ; (5) fractionation with ammonium sulfate; (6) lyophilization to obtain a dry powder. All steps (except the heating process) were performed in a cold room at a temperature of 1–3°C.

Fairly good threonine aldolase activity was found in rat, guinea pig, and sheep livers; very low activity in pig and horse liver.

Preparation of Acetone Powder.—Rat liver was used in the experiments reported in this paper. The animals were sacrificed by a blow on the head, the liver removed immediately, and cooled in a beaker surrounded by ice water. The livers were washed briefly with ice-cooled water and the superficial liquid was removed by blotting with filter paper. They were then homogenized in a Waring blendor with an equal volume (*w*:*v*) of alkaline saline, and the homogenate centrifuged briefly (1 to 2 minutes) at low speed in a refrigerated centrifuge to separate from the gross particulate matter. The upper layer of the homogenate was decanted and mixed, with stirring, with 3 volumes of precooled acetone. Then the mixture was allowed to stand for 1 to 2 hours. The lower layer, formed by settling, was centrifuged in 250 ml. centrifuge bottles at 900 *g* in the refrigerated centrifuge for 15 minutes and the supernatant was discarded. The precipitate was washed three or four times with acetone, depending on the thickness of the precipitate in the bottle. After the bottles were wiped dry with a towel and covered with a sheet of cheese-cloth at the mouth and tied with a rubber band, they were dried in a vacuum desiccator over concentrated H_2SO_4. The material took about 1 to 2 days to dry and the residue was pulverized to powder. The dry powder was slightly pink. About 120 to 130 gm. of acetone powder was obtained from 500 gm. of liver.

Extraction of Acetone Powder.—100 gm. of dry acetone powder was extracted with 1 liter of distilled water. The mixture was stirred for 1 hour and centrifuged at 1300 *g* for 30 minutes. A deep red clear extract (S_1) was obtained.

Heat Precipitation of Non-Threonine Aldolase Protein.—The extract (S_1) was heated in 50 ml. portions in a water bath controlled by a thermostat at a temperature of 56°C. for 8 minutes, and cooled in an ice water bath immediately. The precipitate was centrifuged off and discarded. The supernatant (S_2) was reserved for the next step.

Treatment with Alumina Cγ.—The supernatant fluid (S_2) was dialyzed against 0.01 M maleate buffer at pH 7.0 for 30 hours. The dialyzed extract was transferred into a beaker and 29 to 30 mg. of alumina Cγ (5) (alumina Cγ gel contained 28 to 29 mg. dry weight per ml.) were added per milliter of S_2. The mixture was stirred for 10 minutes and centrifuged. The supernatant fluid contained no threonine aldolase activity and was discarded. The precipitate (R_3) was transferred to a beaker and eluted with 0.1 M phosphate buffer, pH 7.0, three times; the volume of phosphate buffer used

each time was equal to half of the volume of S_2 that was used in the beginning of this step. The supernatant (S_4) from each elution was assayed for threonine aldolase activity. The fractions containing more than 5 threonine aldolase units per mg. N were combined to be used for the next step.

Fractionation with Ammonium Sulfate.—To the combined fractions from the previous step, saturated ammonium sulfate solution, at pH 7.3 (70 gm. of ammonium sulfate per 100 ml. of H_2O, pH adjusted to 7.3 with concentrated ammonia) was added to give 33 per cent saturation. The mixture was allowed to stand overnight in the cold

TABLE I

Purification of Threonine Aldolase of Rat Liver

Fraction		Volume or weight	Nitrogen		T.A.U.*		T.A. U./N	Recovery
			Per ml.	Total	Per ml.	Total		
			mg.	*mg.*	*μM*	*μM*		*per cent*
Acetone powder extract	(S_1)	740 ml.	2.640	1953.6	3.9	2886	1.4	100
Supernatant after heating	(S_2)	625 ml.	1.630	1018.8	4.1	2563	2.5	88
Eluate from alumina Cγ	(S_4)	631 ml.	0.325	205.1	1.7	1073	5.4	37
Precipitate from ammonium sulfate fractionation (60 per cent) (after dialysis)	(R_6)	65 ml.	1.045	67.9	8.9	578.5	8.5	20
Powder from lyophilization		365 mg.‡	0.106	57.9§	0.67	364.8‖	6.3	12

* T.A.U. is defined as micromoles of acetaldehyde formed in 30 minutes.

‡ A correction has been made on potassium maleate buffer which was present in the enzyme solution.

§ Obtained by assuming that protein = N × 6.25.

‖ Calculated by the relation total T.A.U. = total N × T.A.U./N.

room and any precipitate that separated was removed by centrifugation. The supernatant fluid (S_5) was brought to 60 per cent saturation by addition of more saturated ammonium sulfate, pH 7.3, and again allowed to stand overnight. The precipitate (R_6) from this was collected by centrifugation at 2200 *g* for 30 minutes, and redissolved in a small volume of 0.01 M maleate buffer, pH 7.0. Any undissolved material was centrifuged off. The clear, deep yellow supernatant fluid was dialyzed against the same buffer until free from sulfate.

Lyophilization.—The clear dialyzed solution from the previous step was lyophilized and a slightly yellowish powder obtained. In Table I are shown the threonine aldolase activity measurements and the recoveries determined at the different stages of purification.

General Properties of Threonine Aldolase

DL-Allothreonine was used as the substrate unless otherwise indicated. The pH-activity curve of threonine aldolase acting on DL-allothreonine is shown in Fig. 2. This figure shows that the optimum pH is at 7.5–7.7. The buffer em-

ployed was a mixture of 0.2 M tris buffer and 0.2 M maleic acid. The pH values of each mixture before and after incubation were checked with a glass electrode for constancy.

The enzyme was found to be unstable and activity was rapidly lost at a pH below 5.

It was determined that the acetaldehyde formed was proportional to the enzyme concentration up to a value of 4 mg. of lyophilized enzyme protein per 3.25 ml. of incubation mixture at an initial substrate concentration of 19.2×10^{-3} M DL-allothreonine. The temperature for this test was 37°C. and the incubation time 30 minutes.

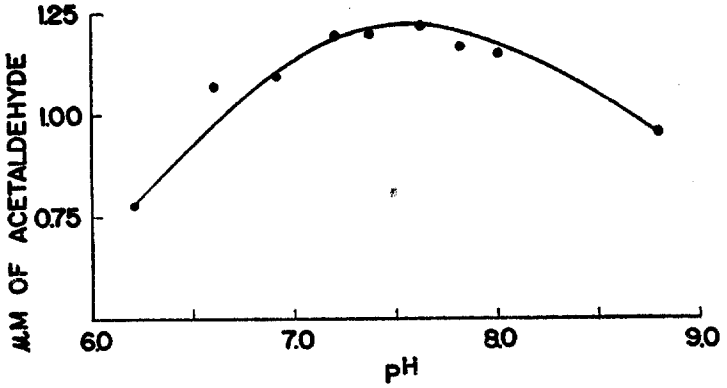

FIG. 2. pH-activity curve of threonine aldolase. See text for experimental conditions.

Similarly the production of acetaldehyde proceeded linearly with time up to a period of 40 minutes under the same conditions as given above. On the basis that D-allothreonine is not attacked by the enzyme it was calculated that the reaction was linear until about 5.1 per cent of the initial L-allothreonine was decomposed.

The effect of substrate concentration on the reaction is typical of the Michaelis-Menten equation, as will be brought out later.

Unsuccessful Attempts to Establish Pyridoxal Phosphate as a Cofactor.—Pyridoxal and a catalytic metal ion (Al, Fe (III) or Cr (II)) (6, 7) have been found to convert threonine and allothreonine rapidly to acetaldehyde and glycine in aqueous solutions between pH 4.0 and 10. Experiments were, therefore, undertaken to discover whether pyridoxal phosphate is or is not a cofactor of threonine aldolase. If pyridoxal phosphate is a coenzyme of threonine aldolase, it appeared that it might be inhibited by desoxypyridoxine.[4]

[4] Inhibition by desoxypyridoxine has been observed in this laboratory with the enzyme system synthesizing serine from glycine and formaldehyde.

An experiment in which varying concentrations of desoxypyridine (from 0.5 to 6 mg. of desoxypyridoxine hydrochloride) were added showed no evidence of inhibition of the enzyme activity.

It was reasoned that if bound pyridoxal phosphate is present as a cofactor, it possibly would be hydrolyzed by incubating with phosphodiesterase and, as a consequence, the threonine aldolase activity would either be reduced or completely lost. Also, the activity might possibly be restored by the addition of pyridoxal phosphate. Two experiments were carried out based on this argument. The result obtained was that the threonine aldolase activity was not inhibited if incubated with purified snake venom phosphodiesterase[5] at pH 9 for only 15 minutes, but it was completely inactivated even in the absence of diesterase if incubated for 25 minutes at this pH.

Umbreit *et al.* (8) successfully demonstrated that pyridoxal phosphate was a cofactor in tryptophan synthesis from indole and serine by employing aged cells of *Neurospora sitophila*. In an experiment with our enzyme a solution which had been standing in the refrigerator for 12 days and another enzyme solution which had been standing at room temperature for 15 hours were both incubated with and without the addition of pyridoxal phosphate. No evidence of activation was observed in either case.

It was found that the enzyme activity was inhibited by hydroxylamine. Table II shows that the activity was completely inhibited by 6.2×10^{-5}M hydroxylamine. Although the reaction product, acetaldehyde, reacts chemically with hydroxylamine, the reduction in acetaldehyde production due to the presence of hydroxylamine far exceeds the acetaldehyde which stoichiometrically is required to react with hydroxylamine. This suggests that a carbonyl group is essential to the enzyme activity. However, it cannot be concluded that the carbonyl group must be from pyridoxal and, also, hydroxylamine may react in some other manner than with a carbonyl group. Therefore, the prediction of Metzler *et al.* (6) that pyridoxal phosphate would turn out to be a cofactor of the enzyme system which splits threonine to glycine and acetaldehyde could not be demonstrated by our experiments with our threonine aldolase preparation.

Balance Study of Decomposition of DL-*Allothreonine by Threonine Aldolase.* —To determine the stoichiometry of the reaction an experiment was performed to compare the acetaldehyde and the glycine production from DL-allothreonine. The incubation was carried out as described previously. From the 3.25 ml. of incubation mixture, 1 ml. was taken to determine acetaldehyde by means of the diffusion method. The remaining solution was immersed immediately into a vigorously boiling water bath for several seconds to precipitate the protein while the top of the tube was firmly corked to avoid loss of

[5] We are greatly indebted to Dr. N. O. Kaplan (The McCollum-Pratt Institute of the Johns Hopkins University) for a sample of purified snake venom phosphodiesterase.

water. The tube was cooled and rotated horizontally so that the water condensed on the wall was collected. A 2 ml. sample used to determine glycine was carefully pipetted into a centrifuge tube and centrifuged. The supernatant fluid was decanted into a small beaker and the precipitate was washed twice with 2 ml. portions of water. The combined supernatants were evapo-

TABLE II

Effect of Hydroxylamine on Threonine Aldolase Activity

Hydroxylamine hydrochloride solution was brought to pH 7.65 with dilute NaOH before using.

Concentration of hydroxylamine	Acetaldehyde formed per incubation	Inhibition
$M \times 10^5$	μM	*per cent*
None	1.35	0
0.62	0.91	32.2
1.24	0.63	53.6
1.86	0.44	67.7
2.48	0.22	79.7
3.10	0.17	87.3
6.20	0	100

TABLE III

Equivalence of Acetaldehyde and Glycine Formation from DL-*Allothreonine*

Acetaldehyde determined by diffusion method; glycine determined by ninhydrin color reaction after it had been separated chromatographically on a dowex-50 column.

Time of incubation	Acetaldehyde formed per incubation	Glycine formed per incubation
min.	μM	μM
60	1.61	1.43
120	2.81	2.92

rated to dryness and the glycine was separated by means of a dowex-50 column as recommended by Stein and Moore (9). The quantity of glycine was determined by the ninhydrin color reaction (10). The result given in Table III shows that equivalent amounts of acetaldehyde and glycine were formed from DL-allothreonine.

The Breakdown of Threonine with Threonine Aldolase Is Irreversible.—An attempt to demonstrate the reversibility of threonine aldolase reaction gave a negative result. The experiment was carried out by incubating glycine-2-C^{14} with acetaldehyde. The protein-free incubation mixture was fractionated on a dowex-50 column (9) and the fractions were counted on a gas flow Gei-

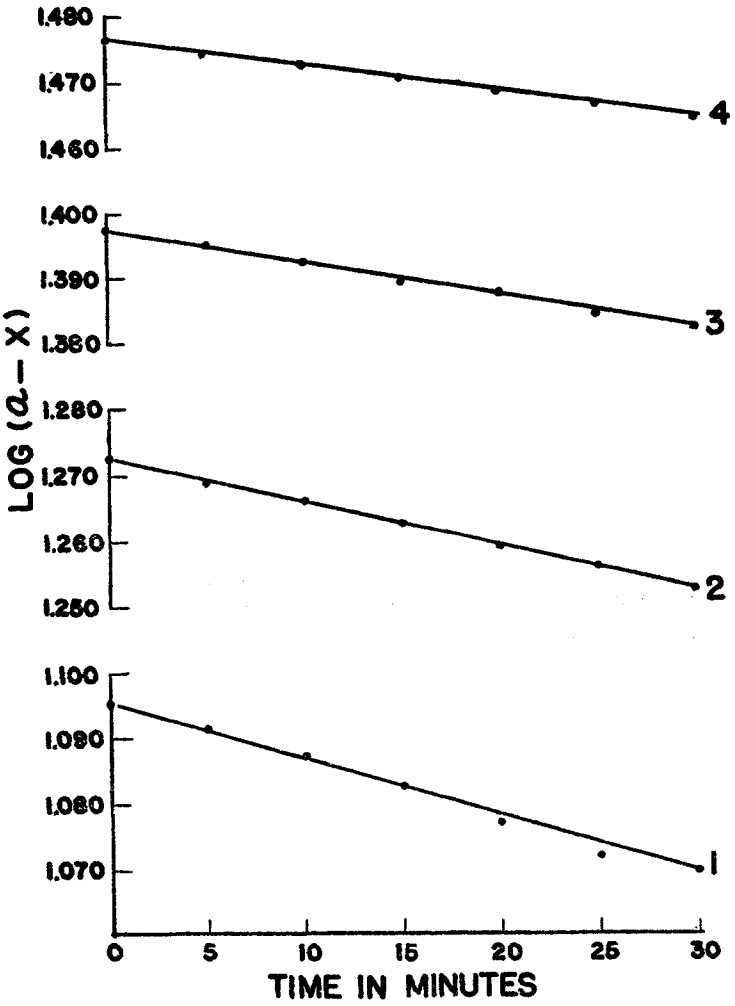

FIG. 3. Logarithmic plots of acetaldehyde formation at different concentrations of DL-allothreonine. Concentrations of DL-allothreonine, curve 1, 3.8×10^{-3} M; curve 2, 5.8×10^{-3} M; curve 3, 7.7×10^{-3} M; curve 4, 9.2×10^{-3} M.

ger-Müller counter for radioactivity. No evidence of radioactive threonine was found.

Substrate Specificity.—The activity of threonine aldolase on the four isomeric forms of threonine has been investigated. It was found that D-threonine and D-allothreonine were not attacked by the enzyme; L-threonine was poorly, while L-allothreonine was most readily attacked.

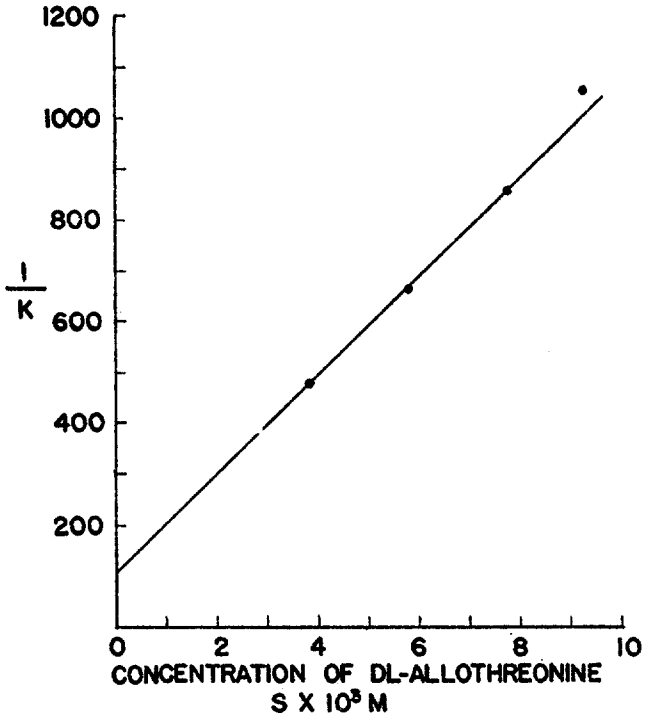

FIG. 4. Determination of K_s for DL-allothreonine from plot of $1/k$ *vs.* substrate concentration. Velocity constants (k) from plots of Fig. 3. Empirical equation of line, $1/k = 105.2 + 96.9 \times 10^3 (S)$. $K_s = 1.1 \times 10^{-3}$ M.

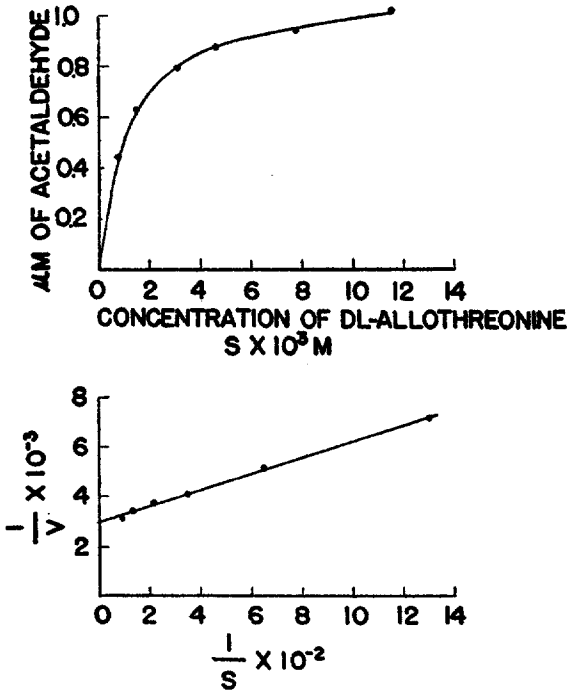

FIG. 5. Activity-concentration curve for DL-allothreonine. For experimental conditions see text.

Serine Is Not Attacked by Threonine Aldolase.—In liver slices, homogenates, and extracts, Braunstein and Vilenkina (1) and Vilenkina (2) demonstrated that glycine was formed from serine as well as from threonine and allothreonine. In an experiment performed by incubating DL-serine (19.2×10^{-3} M with DL-serine-1-C^{14}, 9.8×10^4 counts/100 sec.) with our preparation of threonine aldolase (4 mg.) in 0.15 M phosphate buffer at pH 7.65 for 1 hour, we did not

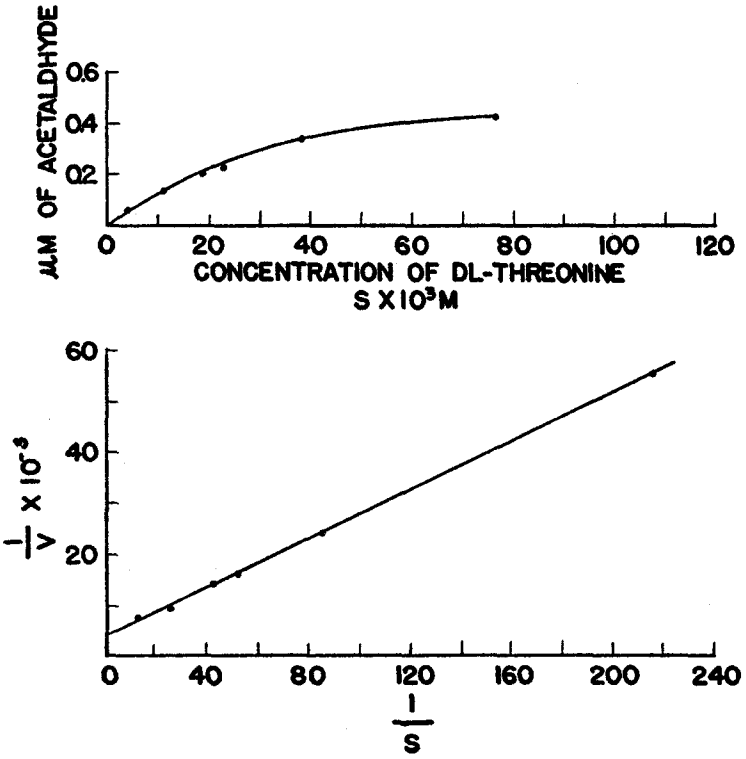

FIG. 6. Activity-concentration curve for DL-threonine. Time of incubation 60 minutes. Other experimental conditions same as for DL-allothreonine.

find any evidence of radioactive glycine when the protein-free incubation mixture was chromatographed on a dowex-50 column (9) and the fractions of effluent were counted for radioactivity. Although we have not tested whether or not boiled liver extract added to our threonine aldolase preparation can activate the serine cleavage, it is safe to say that threonine aldolase is not the same as the enzyme which cleaves serine.

Kinetics of Threonine Aldolase

Order of Reaction and Determination of Ks.—At lower concentrations of substrate, the reaction of the splitting of allothreonine follows a first order re-

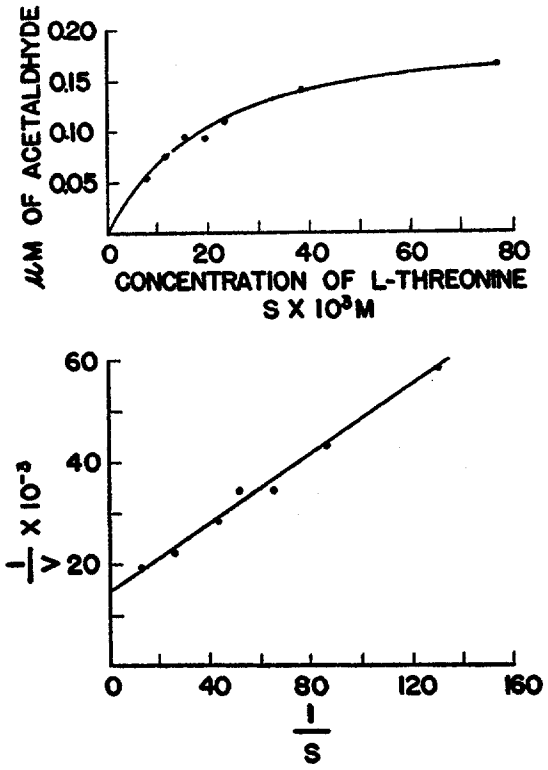

FIG. 7. Activity-concentration curve for L-threonine. Experimental conditions same as for DL-allothreonine.

TABLE IV

Michaelis Constants for Isomers of Threonine

Compounds	*Ks*	*Ks* calculated on basis of L-form only
L-Threonine	25.0×10^{-3} M	
DL-Threonine	56.5×10^{-3} M	28.4×10^{-3} M
DL-Allothreonine	1.0×10^{-3} M*	0.5×10^{-3} M
L-Allothreonine‡	1.0×10^{-3} M	

* The *Ks* value derived from plotting the reciprocals of the velocity constants obtained from Fig. 3 against the concentration of DL-allothreonine was 1.1×10^{-3}.

‡ Early preparation of threonine aldolase which was made up through heating step, then dialyzed and lyophilized.

action course. Typical results are plotted in Fig. 3. The velocity constant for each substrate concentration was calculated from the slope of the line. These constants were utilized to determine the *Ks* of the enzyme-substrate intermediate by plotting $1/k$ against the substrate concentration (11) (Fig. 4).

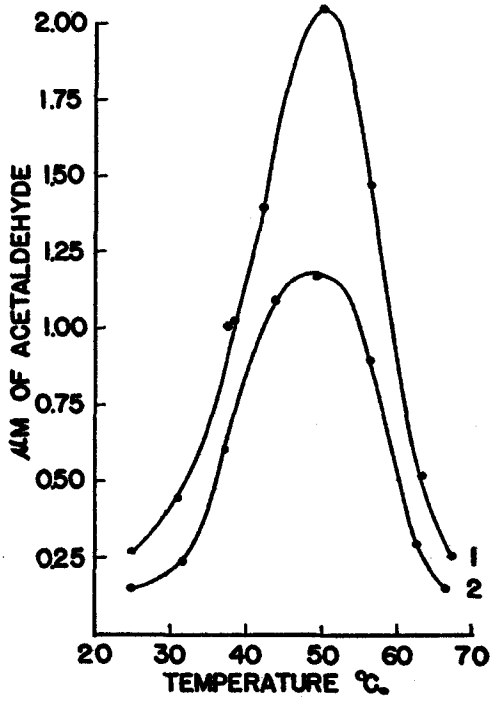

FIG. 8. Temperature-activity curve of threonine aldolase on DL-allothreonine.

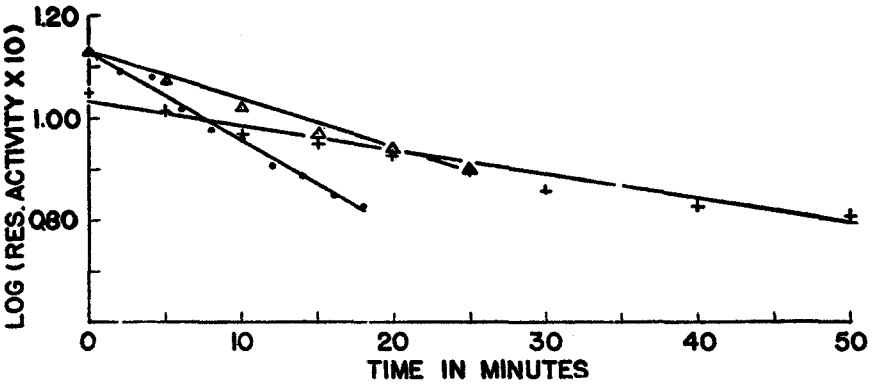

FIG. 9. Logarithmic plots of the rates of thermal inactivation of threonine aldolase. Temperatures, +41.2°C.; △44.6°C.; ●46.6°C.

Michaelis constants were also determined by measuring the initial reaction rates as a function of the substrate concentration and plotting the results according to the method of Lineweaver and Burk (12). The curves for DL-allothreonine, DL-threonine, and L-threonine are given in Figs. 5 to 7. The data

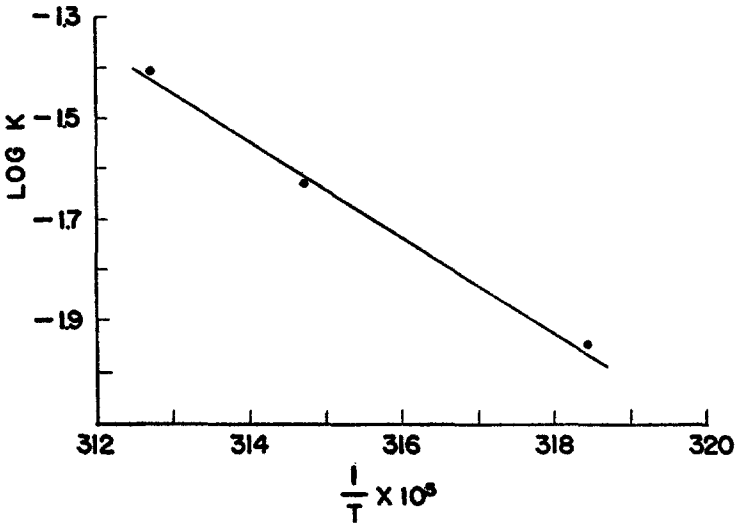

FIG. 10. van't Hoff-Arrhenius plot of thermal inactivation of threonine aldolase.

TABLE V

Thermal Stability of Threonine Aldolase

Enzyme heated at pH 7.0 in 0.1 M phosphate buffer. Enzyme activity assayed at pH 7.65 and temperature of 37°C.

Temperature of heating	Velocity constant of thermal inactivation	Half-life $t_{1/2}$	Energy of thermal inactivation
°C.	$k \times 100$	min.	
41.2	1.13	61.0	
44.6	2.37	29.1	43,000 cal. per mole
46.6	3.90	17.7	

for the Michaelis constants obtained from the different experiments are summarized in Table IV.

Effect of Temperature

Temperature-Activity Curve of Threonine Aldolase.—Temperature-activity curves in phosphate buffer and tris-maleate buffer are shown in Fig. 8. The optimum temperature of threonine aldolase at pH 7.6–7.7 was found to be 50° in phosphate buffer and 48°C. in tris-maleate buffer for a reaction time of 30 minutes.[6] The inhibiting effect of tris-maleate buffer on the activity of the enzyme is obvious.

[6] In the purification procedure heating for 8 minutes at 56.1°C. is employed to remove a part of the non-threonine aldolase protein with little loss of enzyme activity,

Kinetics of Thermal Inactivation of Threonine Aldolase.—Fig. 9 shows that the rate of thermal inactivation of threonine aldolase at 41.2°, 44.6°, and 46.6° in 0.1 M phosphate buffer at pH 7, as would be expected, conforms to a first order reaction. The values of the velocity constant at each temperature tested and the half-lives of the enzyme are given in Table V. From the van't Hoff-Arrhenius plot of the logarithms of the velocity constants against the reciprocals of the absolute temperatures (Fig. 10) the value of 43,000 cal. per mole was calculated for the energy of thermal inactivation of the enzyme.

SUMMARY

1. The enzyme which splits threonine to acetaldehyde and glycine has been partially purified from rat liver (five- to sixfold purification) and the name threonine aldolase proposed for it.

2. The general properties of threonine aldolase have been studied. The enzyme is unstable to a pH below 5. The pH optimum of the enzyme reaction is at 7.5–7.7. The initial rate of production of acetaldehyde is proportional to the enzyme concentration, and when the enzyme concentration is constant, the production of acetaldehyde is proportional to the time, provided that the substrate is in excess. The enzyme is inhibited by the carbonyl group reagent, hydroxylamine. Attempts to demonstrate that pyridoxal phosphate is a cofactor were unsuccessful.

3. The enzyme splits only L-allothreonine and L-threonine and is inactive against the D-forms of these amino acids.

4. The enzyme reaction on DL-allothreonine follows first order kinetics. From the first order velocity constants and the initial rates of the rates of the reaction at various substrate concentrations the Michaelis constant, *Ks*, for this substrate has been evaluated. Michaelis constants have also been determined for threonine.

5. The optimum temperature for the enzymatic breakdown of DL-allothreonine at pH 7.65 was found to be 50°C. in phosphate buffer and 48°C. in tris-maleate buffer. The rate of thermal inactivation of the enzyme threonine aldolase obeys a first order reaction. The heat of thermal inactivation was calculated by the aid of the van't Hoff-Arrhenius equation to be 43,000 cal. per mole for the temperature range 41.2–46.6°C.

6. Equivalent amounts of acetaldehyde and glycine were formed from DL-allothreonine and the enzymatic breakdown of DL-allothreonine was found to be irreversible.

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whereas about 71.4 per cent and 57.5 per cent destruction, respectively, of the purified threonine aldolase activity occurred when it was heated for 5 minutes in 0.1 M phosphate buffer at pH 7.0 at the temperatures of 56.1 and 52°C. This suggests that there may be a protective factor against heat denaturation of the enzyme in the crude solution.

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