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PURIFICATION AND CHARACTERIZATION OF SERINE:GLYOXYLATE AMINOTRANSFERASE FROM KIDNEY BEAN *(PHASEOLUS VULGARIS)*

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SUMMARY

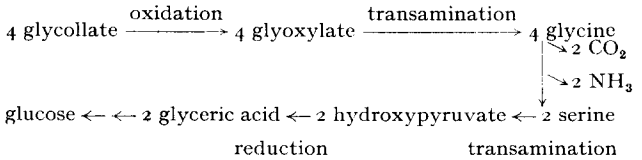
1. Homogenates of kidney bean leaves were shown to catalyse aminotransferase reactions between the following compounds; serine:glyoxylate, alanine:glyoxylate, glutamic acid:glyoxylate and serine:pyruvate. A 100-fold purification of serine:glyoxylate aminotransferase yielded a preparation essentially free of alanine:glyoxylate aminotransferase and glutamate:glyoxylate aminotransferase; however, there was co-purification of serine:pyruvate aminotransferase.

2. For serine:glyoxylate aminotransferase and serine:pyruvate aminotransferase double-reciprocal plots of the velocity of the reaction against one variable substrate at a series of fixed concentrations of the second substrate yielded sets of parallel lines and indicated a ping-pong reaction mechanism. The K_m for glyoxylate and serine in the serine:glyoxylate aminotransferase reaction were $6 \cdot 10^{-4}$ M and $7.1 \cdot 10^{-4}$ M, respectively; and for pyruvate and serine in the serine:pyruvate aminotransferase reaction were $3.8 \cdot 10^{-2}$ M and $3.9 \cdot 10^{-4}$ M, respectively.

3. Serine:glyoxylate aminotransferase and serine:pyruvate aminotransferase activities were inhibited by low concentrations of hydroxylamine but were less sensitive to *N*-ethylmaleimide and *p*-chloromercuribenzoate. Serine:glyoxylate aminotransferase activity was specifically inhibited by NH_4^+, the inhibition was linear competitive with serine and non-linear non-competitive with glyoxylate, the mechanism of NH_4^+ inhibition is discussed. In contrast, serine:pyruvate aminotransferase activity was not inhibited by NH_4^+.

INTRODUCTION

In plants, which generate glycollate photosynthetically, it has been proposed that gluconeogenesis may proceed as follows[1]:



Two aminotransferase reactions are involved in the above scheme: The formation of glycine from glyoxylate and the formation of hydroxypyruvate from serine. Aminotransferase reactions between glyoxylate and either serine, alanine or glutamic acid have been reported in extracts from oat leaves[2] and hydroxypyruvate formation from serine with either pyruvate or glyoxylate as the amino acceptor has been reported in extracts of spinach leaves[3]. However, there is little information concerning the specificity or kinetic properties of the enzymes catalysing these reactions. For instance, Yamazaki and Tolbert[3] described a serine:pyruvate aminotransferase, in peroxisomes isolated from spinach leaves, which was subsequently reported[4] to be 4 times as active functioning as a serine:glyoxylate aminotransferase. Conversely, a serine:glyoxylate aminotransferase isolated from oat leaves was unable to use pyruvate as an amino acceptor[2].

The present communication describes the specificity and kinetic behaviour of a serine:glyoxylate aminotransferase isolated from kidney bean leaves.

EXPERIMENTAL

Materials

Enzymes were isolated from the leaves of 14-day-old, greenhouse grown, kidney bean seedlings (*Phaseolus vulgaris* L. cv. Red Kidney). DEAE-cellulose (DE-52) was obtained from W. and R. Balston Ltd, England; Agarose gel (A 0.5 M) and brushite (Biogel BT $CaHPO_4 \cdot 2\ H_2O$) from Biorad Laboratories, Richmond, Calif.; Sephadex G-200-120 from Sigma Chemical Co., St. Louis, Mo.; ^{14}C-labelled compounds from Amersham-Searle, Arlington Heights, Ill.; and Aquasol liquid scintillation cocktail from New England Nuclear, Boston, Mass. Analytical grade chemicals were obtained from commercial suppliers.

Isolation of serine:glyoxylate aminotransferase

Leaves (50 g) were homogenized manually in 75 mM phosphate buffer, pH 7.5 (200 ml), and the resultant slurry was squeezed through cheesecloth and centrifuged at 10 000 × *g* for 20 min. A 5-ml aliquot of the supernatant was dialyzed for 6 h against 5 mM phosphate buffer, pH 7.5 (2 × 1 l), and is designated dialyzed homogenate.

The remaining supernatant was added to DEAE-cellulose (35 ml of packed gel) which had been equilibrated overnight with 75 mM potassium phosphate buffer (pH 7.5) and the resultant slurry was placed on a magnetic stirrer and allowed to equilibrate for 30 min. The DEAE-cellulose was sedimented by centrifugation at 10 000 × *g* for 15 min and discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant to give 60% saturation, the precipitated protein was collected by centrifugation, and dissolved in 5 mM potassium phosphate buffer, pH 7.5 (7 ml). A 1-ml aliquot of this protein solution was dialyzed for 6 h against the same buffer (2 × 1 l) and is designated DEAE-cellulose fraction.

A 5-ml aliquot of undialyzed DEAE-cellulose fraction was applied to an agarose gel column (45 cm × 2.5 cm) which had been equilibrated with 5 mM potassium phosphate buffer (pH 7.5). The elution rate was 20 ml/h and 5-ml fractions were collected. Maximum serine:glyoxylate aminotransferase activity was eluted between 90 and 103 ml. The void volume of the column was approximately 60 ml.

The fractions with maximum activity were pooled and are designated agarose gel fraction.

A 10-ml aliquot of the agarose gel fraction was applied to a brushite column (10 cm × 1.6 cm), which had been equilibrated with 10 mM potassium phosphate buffer, and nonabsorbed protein was washed from the column with 20 ml of the same buffer. Serine:glyoxylate aminotransferase was eluted with 40 ml of 50 mM potassium phosphate buffer (pH 7.5).

A 3-ml fraction of the brushite eluate was applied to a Sephadex G-200 column (43 cm × 2.5 cm), which had been equilibrated with 10 mM potassium phosphate buffer (pH 7.5). Maximum serine:glyoxylate aminotransferase activity was eluted between 100 and 110 ml. The void volume of the column was approximately 65 ml.

All operations were performed at 0–4 °C and all buffers contained 0.2 mM dithiothreitol. Protein was determined by the method of Lowry *et al.*[5].

Assay of serine:glyoxylate aminotransferase activity

Reaction mixtures (1 ml) contained the following components in μmoles: Hepes buffer, pH 8.0 (60), [$^{14}C_3$]serine (1, spec. act. 0.25 Ci/mole), glyoxylate (1) or pyruvate (10) and protein. Reaction mixtures were incubated for 30 min at 30 °C, the reaction was terminated by the addition of 0.2 ml of 1.5 M trichloroacetic acid and precipitated protein was sedimented by centrifugation and discarded. The supernatant was applied to a Dowex 50 (H^+ form) column (7 cm × 0.9 cm) and the radioactive product (hydroxypyruvate) was washed from the column with 20 ml of distilled water. A 5-ml aliquot of the effluent was added to 10 ml of Aquasol and counted using a liquid scintillation counter. Hydroxypyruvate was identified by preparation of the dinitrophenylhydrazone and paper chromatographic comparison with the authentic hydroxypyruvate dinitrophenylhydrazone using butanol–ammonia as a solvent[7].

Assay of alanine:glyoxylate and glutamate:glyoxylate aminotransferase activities

Reaction mixtures (1 ml) contained the following components in μmoles: Hepes buffer, pH 8.0 (60), L-alanine (1) or DL-glutamic acid (2), [$^{14}C_2$]glyoxylate (1, spec. act. 0.25 Ci/mole) and protein. Reaction mixtures treated as above. The radioactive product (glycine) was eluted from the column with 15 ml of 3 M NH_4OH, and was identified by paper chromatographic comparison with authentic glycine using butanol–acetic acid–water and water-saturated phenol as solvent systems[1].

The use of radioactive method for the assay of glyoxylate:aminotransferase activity is preferred over other methods[8] because it is specific, sensitive and can be used to assay exchange reactions.

RESULTS AND DISCUSSION

Isolation and general properties of serine:glyoxylate aminotransferase

In preliminary studies it was shown that greater than 90% of the total plant serine:glyoxylate aminotransferase activity was present in the leaves. As the leaves expanded there was a concomitant increase in enzymatic activity. For instance, extracts of primary leaves from 7-day-old plants (lamina length 22 mm) had a spec. act. of 30 μmoles/g fresh weight per h and a total activity per primary leaf pair of 3 μmoles/h, while extracts of 21-day-old leaves (lamina length 62 mm) had a specific

activity of 241 μmoles/g fresh weight per h and a total activity of 183 μmoles/h.

Previous studies show that enzymes which metabolize glyoxylate are often localized in glyoxysomes[4]. To determine the cellular localization of the enzyme bean leaves were homogenized in 0.3 M mannitol containing 10 mM potassium phosphate buffer (pH 7.2), 1 mM EDTA and 0.2 mM dithiothreitol and the homogenate subjected to differential centrifugation according to the method of Tolbert and coworkers[9]. Greater than 80% of the serine:glyoxylate aminotransferase activity was present in the supernatant fraction and less than 20% in the pellet (mitochondria, glyoxysomes); therefore in subsequent experiments no attempt was made to isolate glyoxysomes. However, it should be noted that kidney beans are not a good source of intact glyoxysomes when compared to other plants[9].

Glyoxylate:aminotransferase activity was linear with time and enzyme concentration, when assayed under conditions at which less than 20% of the least concentrated substrate was reacted. The pH optimum for serine:glyoxylate aminotransferase was 8.2 in either Hepes or Bicine buffers. However, Hepes was used in all experiments, because of an interaction between serine and Bicine, which resulted in the production of an unidentified anionic compound in the absence of added keto acid.

Some of the aminotransferase activities present in bean leaf homogenates are summarized in Table I. Serine, alanine and glutamic acid acted as amino donors to glyoxylate and serine was an amino donor to pyruvate. In addition exchange reactions occur between serine:hydroxypyruvate and glyoxylate:glycine.

The purification procedure resulted in a 100-fold increase in specific activity for serine:glyoxylate aminotransferase (Table II). This preparation was slightly contaminated with alanine:glyoxylate aminotransferase but contained no detectable glutamic acid:glyoxylate aminotransferase activity. During purification there was a change in ratio of the following aminotransferase reactions: serine:glyoxylate, alanive: glyoxylate and glutamic acid: glyoxylate, which indicates that each is catalyzed by a separate enzyme. Brock and coworkers[2], working with oat leaves, showed that glyoxylate aminotransferase reactions are catalyzed by proteins separate from other aminotransferases, but conflicting results were obtained regarding the specificity of glyoxylate aminotransferases. The relatively constant ratio of serine:glyoxylate and serine:pyruvate aminotransferase activities indicates that either these reactions are catalyzed by the same protein or by two proteins which are of similar size and ionic

TABLE I

SPECIFIC ACTIVITIES OF SOME AMINOTRANSFERASE REACTIONS CATALYZED BY DIALYZED HOMOGENATES OF KIDNEY BEAN LEAVES

Substrates		*Specific activity (μmoles/mg per h)*
Amino donor	*Amino acceptor*	
Serine	Glyoxylate	7.69
Serine	Pyruvate	0.49
Serine	Hydroxypyruvate	4.52
Serine	None	0.02
Alanine	Glyoxylate	7.03
Glutamic acid	Glyoxylate	4.89
Glycine	Glyoxylate	0.88
None	Glyoxylate	0.04

TABLE II

PURIFICATION OF SERINE:GLYOXYLATE AMINOTRANSFERASE FROM KIDNEY BEAN

Purification step	*Total protein (mg)*	*Specific activity (μmoles/mg per h)*	*Relative purification*	*% activity relative to SGA**		
				SPA	*AGA*	*GGA*
Dialyzed homogenate	742.0	4.0	1	6.0	95.0	68.5
DEAE-cellulose	353.0	7.8	2	7.1	94.6	61.4
Agarose Gel A-0.5 M	26.2	32.1	8	7.0	38.7	21.2
Brushite	7.8	76.8	19	7.0	9.8	0.8
Sephadex G-200	0.2	418.0	105	6.0	3.7	0

* SGA, serine:glyoxylate; SPA, serine:pyruvate; AGA, alanine:glyoxylate; GGA, glutamate:glyoxylate.

properties. The former conclusion is an agreement with the unsubstantiated report of Tolbert[4] that pyruvate may act as an alternate aminoacceptor from serine, being one quarter as active as glyoxylate. The serine:glyoxylate aminotransferase from oat leaves was reported[2] to be inactive with pyruvate as an amino acceptor. However, the very unfavorable kinetics of the latter reaction (see Kinetics section) may have resulted in this activity being overlooked. In conclusion, no evidence was obtained in the present study to indicate that serine:glyoxylate and serine:pyruvate aminotransferase activities are catalyzed by separate proteins.

KINETICS

A double-reciprocal plot of the initial velocity against glyoxylate concentration at a series of fixed concentrations of serine yielded a set of parallel lines, one for each concentration of serine (Fig. 1, see also Figs 2, 3 and 4). This is consistent with the

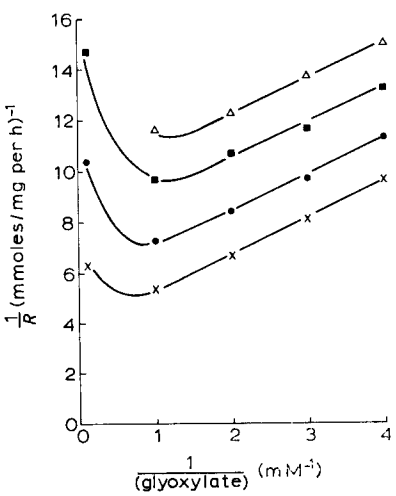


Fig. 1. Lineweaver–Burk double-reciprocal plots of initial velocity of serine:glyoxylate aminotransferase activity against glyoxylate concentration at a series of serine concentrations. Reaction conditions in text. Serine concentrations: ×—×, $1 \cdot 10^{-3}$ M; ●—●, $5 \cdot 10^{-4}$ M; ■—■, $3.3 \cdot 10^{-4}$ M; △—△, $2.5 \cdot 10^{-4}$ M.

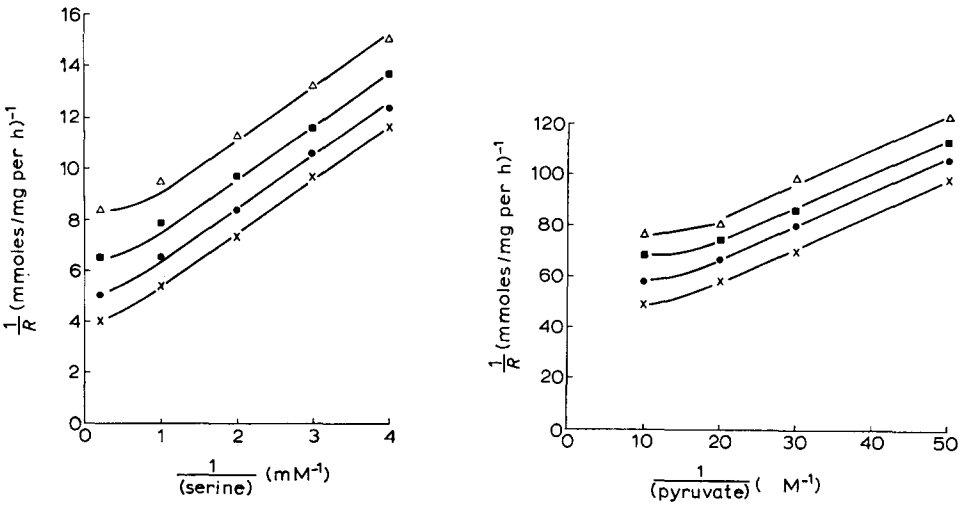


Fig. 2. Lineweaver–Burk double reciprocal plots of initial velocity of serine:glyoxylate aminotransferase activity against serine concentration at a series of fixed glyoxylate concentrations. Reaction conditions in text. Glyoxylate concentrations: ×—×, $1 \cdot 10^{-3}$ M; ●—●, $5 \cdot 10^{-4}$ M; ■—■, $3.3 \cdot 10^{-4}$ M; △—△, $2.5 \cdot 10^{-4}$ M.

Fig. 3. Lineweaver–Burk double-reciprocal plots of initial velocity of serine:pyruvate aminotransferase activity against pyruvate concentration at a series of fixed serine concentrations. Reaction conditions in text. Serine concentrations: ×—×, $1 \cdot 10^{-3}$ M; ●—●, $5 \cdot 10^{-4}$ M; ■—■, $3.3 \cdot 10^{-4}$ M; △—△, $2.5 \cdot 10^{-4}$ M.

ping-pong reaction mechanism typical of aminotransferase reactions[10,11] and is in agreement with previous studies on serine:glyoxylate aminotransferase from oats[2]. At concentrations of glyoxylate higher than 1 mM double-reciprocal plots are not linear, due to an inhibition of aminotransferase activity at high glyoxylate concentrations (Fig. 1). The enzyme appears to have allosteric properties, such that glyoxylate binding changes the conformation of the enzyme and decreases the affinity of

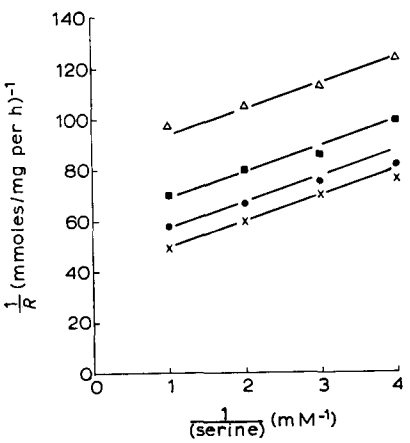


Fig. 4. Lineweaver–Burk double-reciprocal plots of initial velocity of serine:pyruvate aminotransferase activity against serine concentration at a series of fixed pyruvate concentrations. Reaction conditions in text. Pyruvate concentrations: ×—×, $1 \cdot 10^{-1}$ M; ●—●, $5 \cdot 10^{-2}$ M; ■—■, $3.3 \cdot 10^{-2}$ M; △—△, $2 \cdot 10^{-2}$ M.

the enzyme for serine. The allosteric properties of other enzymes with ping-pong reaction mechanisms have recently been examined by Sumi and Ui[12].

The K_m and V values were calculated by the method of Velick and Vavra[10]. For instance, in Fig. 1 lines were extrapolated to the abscissa to yield a set of apparent K_m values for glyoxylate, when these were plotted against the reciprocal of the serine concentration a straight line was obtained which could be extrapolated to infinite serine concentration to yield a K_m for glyoxylate. The intercepts on the ordinate (apparent V) when plotted against the reciprocal of the serine concentration gave a straight line which could be extrapolated to infinite serine concentration to yield the V of the reaction. The data in Figs 2, 3, and 4 were treated in the same way. An average of three experiments yielded a K_m for glyoxylate of $6 \cdot 10^{-4}$ M and for serine of $7.1 \cdot 10^{-4}$ M and a V of 435 μmoles/mg per h. Similarly, from the data in Fig. 3 and Fig. 4, K_m values of $3.8 \cdot 10^{-2}$ M for pyruvate and $3.9 \cdot 10^{-4}$ M for serine, and a V of 37 μmoles/mg per h were calculated. As indicated by the K_m values the enzyme has a much greater affinity for glyoxylate as the amino acceptor, which may explain why previous investigators[2] reported that pyruvate was not a substrate of the enzyme. Experiments are in progress to examine the kinetics of the two reverse reactions to determine whether the low affinity of the enzyme for pyruvate indicates that this reaction normally proceeds in the direction of pyruvate and serine synthesis. Serine synthesis from alanine and hydroxypyruvate has been demonstrated in plants[13,14]. In animals, it was shown that serine:pyruvate aminotransferase is 2–3 times more active in the direction of serine synthesis. However, it was still concluded[15,16] that the function of the enzyme was the initiation of gluconeogenesis from serine. In conclusion, kinetic evidence obtained in the present study indicates that *in vivo* glyoxylate may be more important than pyruvate in the conversion of serine to hydroxypyruvate.

INHIBITORS

Serine:glyoxylate and serine:pyruvate aminotransferase activities were inhibited by hydroxylamine at low concentrations, but were only inhibited by sulfhydryl reagents such as *p*-chloromercuribenzoate and *N*-ethylmaleimide at relatively high concentrations (Table III). Similar results were obtained with a serine:glyoxylate aminotransferase from oats[2] and suggested the presence of pyridoxal phosphate and the probable absence of any cysteine groups at the active site of the enzyme.

Serine:glyoxylate aminotransferase was inhibited by low concentrations of ammonium salts. The inhibition was apparently specifically due to NH_4^+, since equimolar quantities of different ammonium salts were equally effective as inhibitors. By contrast, serine:pyruvate aminotransferase activity was only inhibited by high concentrations of NH_4^+, at which concentrations nonspecific ion effects were observed.

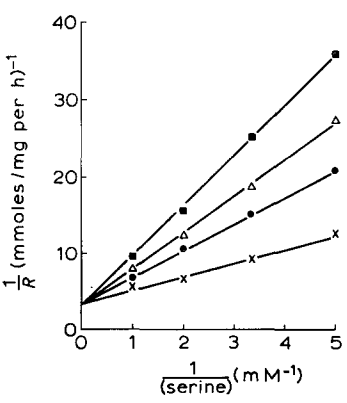
A kinetic investigation of the NH_4^+ inhibition revealed that NH_4^+ is a linear competitive inhibitor of serine (Fig. 5) and a non-linear noncompetitive inhibitor of glyoxylate (Fig. 6). Several experiments were conducted to determine whether the non-linearity in the latter instance was due to an effect of ammonium upon the enzyme or reaction of ammonium with one of the substrates or products. Firstly, when an ammonium salt was incubated with [^{14}C]glyoxylate there was no evidence for the formation of glycine or any other cationic material (*i.e.* material which is

TABLE III

EFFECT OF VARIOUS INHIBITORS ON SERINE:GLYOXYLATE AND SERINE:PYRUVATE AMINOTRANSFERASE ACTIVITIES

Inhibitor	*Concentration (M)*	*Inhibition (%)*	
		Serine:glyoxylate	*Serine:pyruvate*
Hydroxylamine	$1 \cdot 10^{-5}$	98	94
p-Chloromercuribenzoate	$1 \cdot 10^{-3}$	37	51
N-Ethylmaleimide	$1 \cdot 10^{-3}$	41	29
NH_4Cl	$1 \cdot 10^{-2}$	100	57
	$1 \cdot 10^{-3}$	92	7
	$1 \cdot 10^{-4}$	42	0
Ammonium acetate	$1 \cdot 10^{-2}$	100	42
	$1 \cdot 10^{-3}$	92	6
	$1 \cdot 10^{-4}$	42	0
$(NH_4)_2SO_4$	$5 \cdot 10^{-3}$	100	15
	$5 \cdot 10^{-4}$	91	6
	$5 \cdot 10^{-5}$	45	0
Na_2SO_4	$1 \cdot 10^{-2}$	5	21
K_2SO_4	$1 \cdot 10^{-2}$	3	14
$MgSO_4$	$1 \cdot 10^{-2}$	23	11

bound by Dowex 50, H^+ form). Secondly, if ammonia is reacting with hydroxypyruvate (the product usually assayed) to form an amino compound an apparent inhibition of the reaction would be observed. However, if serine and [^{14}C]glyoxylate are incubated and [^{14}C]glycine formation used as an assay, reaction of ammonium with hydroxypyruvate might be expected to increase the rate of [^{14}C]glycine formation because of the continuous removal of hydroxypyruvate. When the latter



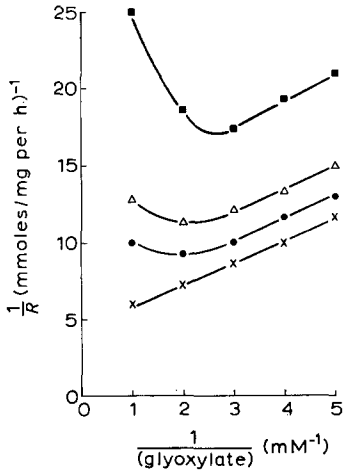


Fig. 5. Lineweaver–Burk double-reciprocal plots of initial velocity of serine:glyoxylate aminotransferase activity against serine concentration at a series of fixed NH_4Cl concentrations. Reaction conditions in text. Glyoxylate concentration $1 \cdot 10^{-3}$ M; NH_4Cl concentration: ×—×, none; ●—●, $5 \cdot 10^{-4}$ M; △—△, $1 \cdot 10^{-3}$ M; ■—■, $2 \cdot 10^{-3}$ M.

Fig. 6. Lineweaver–Burk double-reciprocal plots of initial velocity of serine:glyoxylate aminotransferase activity against glyoxylate concentration at a series of fixed NH_4Cl concentrations. Reaction conditions in text. Serine concentration $1 \cdot 10^{-3}$ M; NH_4Cl concentrations: ×—×, none; ●—●, $1 \cdot 10^{-3}$ M; △—△, $2 \cdot 10^{-3}$ M; ■—■, $5 \cdot 10^{-3}$ M.

experiment was performed, glycine formation was inhibited to the same extent as hydroxypyruvate formation. It is concluded that NH_4^+ inhibition of serine:glyoxylate aminotransferase activity is due to a direct effect upon the enzyme.

It is proposed that ammonium binding to the enzyme causes an increase in glyoxylate binding, which reduces the affinity of the enzyme for serine (as shown previously glyoxylate is inhibitory at high concentrations), and therefore inhibits the overall reaction. This conclusion is supported, firstly, by the observation that ammonium is most effective as an inhibitor at high glyoxylate concentrations, and secondly, by experiments in which various combinations of inhibitor and substrates were preincubated with the enzyme prior to the addition of the other components of the reaction mixture. Preincubation of the enzyme with glyoxylate and NH_4Cl (10^{-4} M) or NH_4Cl alone, gave maximum inhibition, 89 and 85%, respectively. Significantly less inhibition was observed when the enzyme was preincubated with serine and NH_4Cl (62% inhibition) or serine alone (55% inhibition).

The absence of NH_4^+ inhibition of serine:pyruvate aminotransferase activity may indicate that this activity is catalyzed by an enzyme other than the one which catalyzes the serine:glyoxylate aminotransferase reaction. However, if the above interpretation of the mechanism of NH_4^+ inhibition is correct, the relatively low affinity of the enzyme for pyruvate and the apparent absence of any effect of pyruvate on serine binding might lead to the prediction that ammonium would not be inhibitory.

In conclusion, the physiological significance of NH_4^+ inhibition of serine:glyoxylate aminotransferase cannot be evaluated at this time, because the effect of NH_4^+ on other aminotransferases is not known.

NOTE ADDED IN PROOF (Received July 27th, 1973)

After this manuscript was submitted, I learned of a paper by Rehfeld and Tolbert[17]; these authors studied a peroxisomal serine:glyoxylate aminotransferase isolated from spinach leaves which utilized pyruvate as an alternate amino acceptor.

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