SUMMARY

1. The action of carboxypeptidase on insulin and acetylinsulin has been investigated. The results of previous workers that the removal of *C*-terminal alanine does not affect the biological activity of the insulin molecule, but that removal of *C*-terminal asparagine does so, have been confirmed.

2. The action of trypsin on insulin has also been investigated. The peptides resulting have been purified, characterized and tested for biological activity. The peptide DHA insulin, which is insulin without the eight amino acid residues of the carboxyl end of the phenylalanyl chain, still retains about 15% of the biological activity of the intact insulin molecule.

I wish to thank Professor F. G. Young, F.R.S., and Dr F. Sanger, F.R.S., for much valuable advice and encouragement; Dr L. F. Smith for the gift of acetylinsulin and assistance in the column procedures; Mr T. Hollingsworth and Mr R. G. Carpenter, of the Department of Human Ecology, Cambridge, for advice on statistical analysis and computation.

REFERENCES

Andersen, W. (1954). *Acta chem. scand.* **8**, 359.
Blackburn, S. & Lowther, A. C. (1950). *Biochem. J.* **46**, xxvii.
Butler, J. A. V., Dodds, E. C., Phillips, D. M. P. & Stephen, J. M. L. (1948). *Biochem. J.* **42**, 116.
Butler, J. A. V., Dodds, E. C., Phillips, D. M. P. & Stephen, J. M. L. (1949). *Biochem. J.* **44**, 224.
Fraenkel-Conrat, J. & Fraenkel-Conrat, H. (1950). *Biochim. biophys. Acta*, **5**, 89.
Gerngross, O., Voss, K. & Herfeld, T. (1933). *Ber. dtsch. chem. Ges.* **66**, 435.
Gey, G. O. & Gey, M. K. (1936). *Amer. J. Cancer*, **27**, 45.
Harris, J. I. & Li, C. J. (1952). *J. Amer. chem. Soc.* **74**, 2945.
Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
Kilby, B. A. & Youatt, G. (1954). *Biochem. J.* **57**, 303.
Lens, J. (1949). *Biochim. biophys. Acta*, **3**, 367.
Levy, A. (1954). *Nature, Lond.*, **174**, 126.
Mirsky, I. A. (1957). *Recent Progr. Hormone Res.* **13**, 429.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Nelson, N. (1944). *J. biol. Chem.* **178**, 375.
Nicol, D. S. H. W. & Smith, L. F. (1956). *Biochem. J.* **64**, 17P.
Polson, A., Mosley, V. M. & Wyckoff, R. W. G. (1947). *Science*, **105**, 603.
Randle, P. J. (1956). *J. Endocrin.* **13**, 1.
Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 541.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sanger, F. & Thompson, E. O. P. (1953). *Biochem. J.* **53**, 366.
Sluyterman, L. A. A. (1955). *Biochim. biophys. Acta*, **17**, 169.
Smith, E. L., Hill, R. L. & Borman, A. (1958). *Biochim. biophys. Acta*, **29**, 207.
Somogyi, M. (1941). *J. biol. Chem.* **141**, 219.
Van Abeele, F. R. & Campbell, E. D. (1951). *Fed. Proc.* **10**, 263.

Biochem. J. (1960) **75**, 401

Glutamic–Aspartic Transaminase of *Dolichos lablab*: Participation by Iron as a Cofactor

By M. V. PATWARDHAN
Division of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, India

(*Received* 14 *August* 1959)

The possibility of a metal participating in enzymic transformations of amino acids that are pyridoxal phosphate-dependent was suggested by the studies of Metzler, Ikawa & Snell (1954). Experimental evidence for metal participation in these types of enzymic reactions was given by Yanofsky (1952), who showed that inhibition of serine dehydrase by metal-binding agents was partially reversed by Mg^{2+} ions. Similar results have been obtained with kynureninase of *Neurospora* (Jakoby & Bonner, 1953), cystathionase of *Escherichia coli* (Wijesundera & Woods, 1953) and tryptophanase (Happold & Struyvenberg, 1954). Eggleston (1958) studied decarboxylases from animal and plant sources and stated that pyridoxal phosphate in some cases acted in conjunction with a metal whereas in others the action was independent of a metal.

Happold & Turner (1957) have shown activation of glutamic acid–aspartic acid transaminase (from heart muscle) by Mg^{2+} ions, but they thought that the effect was due mainly to the acceleration of non-enzymic decarboxylation of oxaloacetic acid, one of the reaction products, thus giving an apparent increase in activity. A preliminary communication from this Institute (Patwardhan, 1958) has

reported the possibility of participation of a metal in transamination catalysed by the enzyme isolated from a plant source, *Dolichos lablab*. The present paper gives more data in support of the idea of iron involvement in the reaction. Purification of the enzyme and some of its properties are also reported.

EXPERIMENTAL

Enzyme. The source of the enzyme was fresh, green beans of *Dolichos lablab*, a seasonal dicotyledonous legume. In a general study of the composition of this material because of its hypoglycaemic effect (Srinivasan, 1957), strong transaminase activity was noticed and this formed the starting-point of the present investigation. The cotyledons were collected from fresh beans by removing the epidermis. An acetone-dried powder of the cotyledons was prepared by homogenizing them, with 3 vol. of distilled acetone cooled to 2°, in a Waring Blendor for 45 sec. and immediately filtering off the acetone on a Büchner funnel. The last traces of acetone were removed from the powder under vacuum. Operations were carried out in a room at 2°. The powder was stored in a desiccator at 2° and was used for the isolation of the enzyme (transaminase); the powder retained its full activity for about 6 months.

Assay of enzyme activity. Transaminase activity of various preparations was determined by the method of Tonhazy, White & Umbreit (1950). Reaction mixtures contained 0·1 ml. of enzyme, 20 μ-moles of DL-aspartic acid, 20 μ-moles of α-oxoglutaric acid and buffer to a volume of 2 ml. Both the substrates were brought to pH 7·4 by addition of dilute alkali. Phosphate buffer (0·081 M-K_2HPO_4–0·019 M-KH_2PO_4), pH 7·4, was first used but later 0·05 M-2 amino-2-hydroxymethylpropane-1:3 diol (tris)–HCl buffer, pH 8·5, was used. Tubes containing buffer, enzyme and aspartic acid were incubated for 5 min. at 37°, after which α-oxoglutaric acid was added. The tubes were immediately placed under nitrogen and incubated for a further 30 min. Enzyme blank consisted of buffer, enzyme and α-oxoglutaric acid. The reaction was stopped by the addition of 0·2 ml. of 10% metaphosphoric acid (Sall, Richards, Harrison & Myerson, 1957). The oxaloacetic acid that was formed as one of the reaction products was then converted into pyruvic acid with aniline citrate and the pyruvate formed was measured as its 2:4-dinitrophenylhydrazone as described by Tonhazy *et al.* (1950), with filter no. 540 in a Klett–Summerson photoelectric colorimeter. Activity of the enzyme is expressed as μg. of pyruvate formed in 30 min./mg. of protein at 37°. When the reaction was studied in the reverse direction, with glutamic acid and oxaloacetic acid as reactants, the aspartic acid formed was first separated with the help of descending chromatograms, with phenol–water (70:30, v/v) in the presence of 3% ammonia in the chamber as the developing solvent. Chromatograms were developed at 23°. Spots were developed with 0·1% ninhydrin in butanol and the colour was measured by extracting it into 75% ethanol, with filter no. 560 in the same instrument as mentioned above. The other product, α-oxoglutaric acid, was estimated as its hydrazone by chromatography. The hydrazone was prepared by the method of Isherwood & Cruickshank (1954). Butanol–ethanol–water (50:10:40, by vol.) was used as the developing solvent. The ascending chromatograms were developed for 18 hr. at 23°. The yellow spots were extracted with 2 ml. of 10% sodium carbonate. The red colour developed after addition of 4 ml. of 2 N-sodium hydroxide was read with filter no. 540 (Tulpule & Patwardhan, 1952).

Protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Iron. Iron determinations were carried out by the method of Mahler & Elowe (1954). For this estimation, all the steps involved in enzyme purifications as well as preparation of various reagents were carried out with glass-distilled water. All glassware used for iron determination was Pyrex.

Chemicals. All chemicals used were of reagent grade. DL-Aspartic acid was dissolved in hot water and then precipitated with ethanol. The glutamic acid and aspartic acid employed were found to be chromatographically pure. α-Oxoglutaric acid was precipitated from ethyl acetate by light petroleum. Oxaloacetic acid was kindly supplied by Dr P. S. Sarma.

Solutions of sodium salt of L-thyroxine and of 3:5-diiodotyrosine (British Drug Houses Ltd.) were prepared immediately before use by dissolving the compounds in a minimum amount of alkali and making up to volume with CO_2-free distilled water (Wolff & Wolff, 1957).

Pyridoxal phosphate and bovine serum albumin (Armour and Co.) were kindly supplied by (the late) Dr K. V. Giri. Ethylenediaminetetra-acetic acid (disodium salt) was a gift from Glyco Products, N.Y. For purification studies, Dowex-2 × 8 (20–50 mesh), chloride form, was used. This was washed thoroughly with 6 N- and 3 N-HCl and then with water before use.

Purification of the enzyme

I. *Water extraction.* Acetone-dried powder (1 g.) was extracted with 10 ml. of ice-cold water for 10 min. This and all the subsequent operations were carried out at 2°. The extract was then centrifuged in a refrigerated centrifuge at 2°, at 1000 *g* for 5 min. The supernatant, which was slightly turbid, was collected and the residue discarded.

II. *Treatment with Dowex-2* (Cl *form*). The supernatant was treated with the anion-exchange resin (1 g. of resin for 1 g. of the original powder). After being stirred continuously for 10 min. it was allowed to stand for another 10 min. The resin and the white precipitate that formed were centrifuged off at 1000 *g*, and the supernatant, which was still slightly turbid, was used for the next step. Almost all of the activity of the original extract was recovered after this treatment.

When Dowex-50 in hydrogen form was used, the enzyme extract lost all the activity, although the protein content of the extract was the same as that of the solution treated with anion-exchange resin; it was not possible to restore the activity by the addition of boiled juice of the active enzyme or pyridoxal phosphate or iron, or both the last-named, and no activity was present in the resin eluate. With the anion-exchange resin, similar increases in specific activity of the enzyme were obtained with Dowex-2 in chloride form or with the weak resin IR-4 B.

III. *Treatment with alumina-Cγ.* At this stage the enzyme solution (pH 6·9) contained 37 mg. of total protein. To this solution was added 1 ml. of alumina-Cγ gel (1 ml. of gel equivalent to 90 mg. dry wt.). Before addition of the gel, excess of water from the gel was always centrifuged off to avoid dilution of the enzyme solution. After stirring

Table 1. *Purification of the enzyme*

Reactants: aspartic acid, 20 μmoles; α-oxoglutaric acid, 20 μmoles; enzyme, 0·1 ml.; buffer (0·081 M-K_2HPO_4–0·019 M-KH_2PO_4), pH 7·4; incubation was for 0·5 hr. at 37° under nitrogen. All the operations of purification were carried out at 2°.

Purification stage	Protein (mg./ml.)	Specific activity (μg. of pyruvate/ mg. of protein)	Pyruvate formed (μg.)	Total protein (mg.)
I. Water extract of acetone-dried powder	15·8	86	10 600	123
II. Dowex-2 (Cl form) treatment	5·1	290	10 600	37
III. Supernatant from alumina-Cγ treatment	1·5	616	5 800	9·4
IV. Calcium phosphate gel eluate	0·4	1 420	3 550	2·5
V. Ammonium sulphate fraction between 45 and 75% saturation	0·4	1 525	1 990	1·3
VI. Alumina-Cγ gel eluate	—	2 000	1 494	0·7

with the gel for 10 min. the gel was separated by centrifuging and the clear solution was taken for further treatment.

IV. *Calcium phosphate gel eluate.* Enzyme solution from the step III, which now contained about 10 mg. of protein, was treated with 1 ml. of calcium phosphate gel (1 mg. of gel equivalent to 28 mg. dry wt.). At the end of 15 min. the gel was separated. The supernatant had very little activity. The gel was washed twice with 5 ml. portions of water and then eluted with 5 ml. of 0·1 M-phosphate buffer (K_2HPO_4–KH_2PO_4), pH 7·4.

V. *Treatment with ammonium sulphate.* The eluate (4·2 ml.) was made up to 5 ml. by addition of the last-mentioned buffer and then fractionated with ammonium sulphate at 0°. Solid ammonium sulphate (1·4 g.) was added over a period of 5 min. to bring the solution to 45% saturation. After 15 min. the precipitate formed was removed by centrifuging in the refrigerated centrifuge. The supernatant (5 ml.) was brought to 75% saturation by addition of 1·03 g. of solid ammonium sulphate. After keeping for 30 min., the precipitate formed was collected. This was taken up in 3 ml. of 0·1 M-potassium phosphate buffer, pH 7·4, and dialysed for 2 hr. at 2° against pre-cooled distilled water to remove traces of ammonium sulphate.

VI. *Second treatment with alumina-Cγ gel.* After dialysis, the enzyme solution (protein content 1·3 mg.), pH 7·4, was treated with 0·5 ml. of gel. After 10 min. the solution was centrifuged and the gel eluted with 2 ml. of 0·1 M-potassium phosphate buffer, pH 7·4.

Results of purification are given in Table 1. The enzyme preparation used for most of the studies was purified to stage IV. Attempts to purify the enzyme after stage VI did not meet with any success.

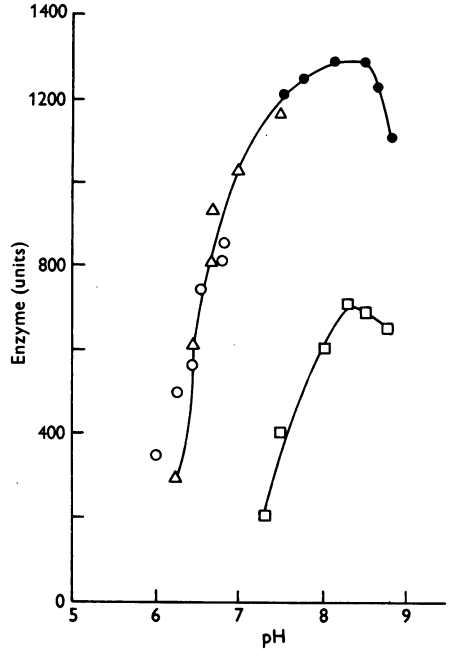

Fig. 1. Effect of various buffers on transaminase. ○, Citric acid–Na_2HPO_4 (0·1 M); △, KH_2PO_4–K_2HPO_4 (0·1 M); ●, 0·05 M-tris–HCl; □, tris–0·05 M-maleic acid–0·05 M-NaOH. Incubation was for 0·5 hr. under nitrogen at 37°.

RESULTS

Effect of pH. Behaviour of the enzyme in the presence of various buffers was studied over a range pH 5·0–9·0. The optimum pH of the enzyme was found to lie between 8·2 and 8·5. Cruickshank & Isherwood (1958) have also reported the pH optimum for glutamic acid–aspartic acid transaminase from wheat germ to be between pH 8·0 and 8·5. Lower enzyme activity was obtained when sodium phosphate buffer (Na_2HPO_4–NaH_2PO_4) was used than when the potassium phosphate buffer was employed.

The enzyme activity over the same range of pH in tris–HCl buffer was significantly higher than with tris–maleic acid–NaOH buffer (Fig. 1). This may have been due either to the presence of Na^+ ions or to the maleic acid. In tris–maleic acid–NaOH buffer, the pH optimum was about 8·2, whereas in tris–HCl buffer it was 8·5.

To find whether this inhibitory effect was due to the presence of maleic acid, the effect of addition of various dicarboxylic acids (oxalic, malonic,

succinic, malic, maleic, fumaric and *o*-phthalic) on the transaminase activity was studied. All the acids were added as their potassium salts with final concentration at 5 mM. It was observed that whereas all the other acids gave 10–12 % inhibition, maleic acid and *o*-phthalic acid gave about 30 % inhibition. As maximum activity was observed with tris–HCl buffer, pH 8·5, this has been employed in most of the studies. It was noticed that over the same range of pH, activity in citrate–phosphate buffer was almost equal to that in phosphate buffer.

Effect of cations on transaminase activity. Preliminary studies indicated that calcium, copper, lithium and zinc did not significantly affect the activity, whereas nickel, manganese and magnesium lowered it considerably. Mercury completely inhibited the activity. Iron in the ferrous state gave a slight increase whereas in the ferric state it was not active. Further studies were therefore continued with ferrous iron. Earlier studies had shown that much activity is lost after dialysis and could be restored to a great extent by the addition of iron as ferrous sulphate (Patwardhan, 1958). Complete restoration could not be obtained. This was suspected to be due to the difference in duration of dialysis of various preparations. It was noticed that when the dialysis was carried out against 8-hydroxyquinoline (5 mM) only for 18 hr., addition of iron restored the activity. After prolonged dialysis (36 hr.) against phosphate buffer, addition of pyridoxal phosphate gave an increase in activity (Table 2). When the enzyme preparation was dialysed, first for 36 hr. against phosphate buffer and then against 8-hydroxyquinoline buffer, it was observed that separate additions of iron and pyridoxal phosphate were not sufficient, but the presence of both was necessary to restore activity (Table 3). This indicates the requirement for both pyridoxal phosphate and iron. It was not possible in the last stage completely to restore the activity to that of the original enzyme.

It is known that metals can catalyse the oxidative decarboxylation of some α-oxodicarboxylic acids (Krebs, 1942; Speck, 1949). Thus it was possible that iron might have decarboxylated non-

Table 2. *Activation of transaminase by pyridoxal phosphate*

The enzyme purified up to stage IV was dialysed for 36 hr. against potassium phosphate buffer, pH 7·4, in a cold room. Reaction buffer: tris–HCl, pH 8·5; incubation was for 0·5 hr. at 37° under nitrogen. Iron was added as $FeSO_4,7H_2O$. Order of additions: enzyme, iron, pyridoxal phosphate, aspartic acid, α-oxoglutaric acid. Amounts of Fe^{2+} ions and pyridoxal phosphate used were 10 μg. each.

Treatment of enzyme	Addition	Units (μg. of pyruvate/ mg. of protein)
1. Dialysed	Nil	285
2. Dialysed	Fe^{2+} ions	450
3. Dialysed	Pyridoxal phosphate	830
4. Dialysed	Fe^{2+} ions + pyridoxal phosphate	900
5. Undialysed	Nil	1200

Table 3. *Effect of pyridoxal phosphate and ferrous iron on transaminase activity*

The enzyme preparation employed in the experiments of Table 2 was, at the end of 36 hr., further dialysed for 18 hr. against aqueous 5 mM-8-hydroxyquinoline in the cold. Reaction buffer: tris–HCl, pH 8·5; incubation was for 0·5 hr. under nitrogen. Order of additions was the same as that given in Table 2. Amounts of Fe^{2+} ions and pyridoxal phosphate used were 10 μg. each.

Treatment of enzyme	Addition	Units (μg. of pyruvate/ mg. of protein)
1. Dialysed	Nil	166
2. Dialysed	Pyridoxal phosphate	261
3. Dialysed	Fe^{2+} ions	309
4. Dialysed	Fe^{2+} ions + pyridoxal phosphate	725

Table 4. *Absence of formation of pyruvate in the enzyme reaction*

Treatment of enzyme	Addition	Units (μg. of pyruvate/ mg. of protein)
1. Dialysed	Nil	400
2. Dialysed	Fe^{2+} ions (10 μg.)	880
3. Dialysed (no aniline citrate was added during colour development)	Fe^{2+} ions (10 μg.)	50
4. Undialysed	Nil	987

enzymically the oxaloacetic acid (one of the reaction products), converting it into pyruvic acid and thus shifting the equilibrium so as to give an apparent increase in the enzyme activity. To test this, an experiment was carried out, the results of which are described in Table 4. It is observed that in the absence of aniline citrate (used in the method of Tonhazy, White & Umbreit, 1950) to convert oxaloacetic acid into pyruvic acid, no pyruvate formation could be demonstrated.

The participation by iron in the enzymic transamination was further substantiated by employing another method. This time, the reaction was studied in the reverse direction. Glutamic acid and oxaloacetic acid were added to the reaction mixture and, at the end of the incubation period, the reaction products aspartic acid and α-oxoglutaric acid were determined. The same effect of iron on the enzymic transamination was noticed. Dialysis of the enzyme decreased the formation of both α-oxoglutaric acid and aspartic acid and addition of iron restored it to a considerable extent (Table 5).

When oxaloacetic acid alone was incubated with the enzyme, it was observed that only about 8% of oxaloacetic acid was converted into pyruvic acid at the end of 30 min. Pyruvic acid thus formed can transaminate with glutamic acid, which is another reactant, to give alanine and thus disturb the equilibrium of the glutamic acid–aspartic acid transaminase reaction. Although the enzyme preparation used in the above-mentioned experiment was purified to stage IV, it had about 15% of glutamic acid–alanine transaminase activity in the presence of added pyruvic acid compared with glutamic acid–aspartic acid transaminase activity. When, however, glutamic acid and oxaloacetic acid were incubated in the presence of enzyme, we could not detect (on paper chromatograms) any alanine formation in the absence of externally added pyruvic acid. Under our experimental conditions any interference due to glutamic acid–pyruvic acid transaminase in our reaction was therefore thought to be small.

Effect of thyroxine on transaminase activity. While this work was in progress, a report appeared indicating that the hormone thyroxine inhibited the various dehydrogenases which require zinc for their activity (Wolff & Wolff, 1957). Lardy (1954) has shown that some bivalent cations, such as Cu^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+} and Mg^{2+}, can form complexes with thyroxine under appropriate conditions. In the light of this, we thought that the effect of thyroxine in our present studies might give us more indication of the role of iron in transaminase. The thyroxine effect was studied by preincubating the enzyme with thyroxine before the addition of aspartic acid and α-oxoglutaric acid (Table 6). It was observed that 0·1 mM-thyroxine decreased the activity of transaminase to about 75%. When L-thyroxine and iron are added together to the enzyme system so that there is competition for thyroxine between the iron of the enzyme and the externally-added metal, there is less inhibition than in presence of thyroxine only and the inhibition is still further lowered when the

Table 5. *Effect of iron on glutamic acid–aspartic acid transaminase reaction studied in the reverse direction*

Reactants added: glutamic acid 20 μmoles; oxaloacetic acid 20 μmoles; enzyme 0·1 ml.; potassium phosphate buffer (0·1 M), pH 7·4. Incubation was under nitrogen at 37° for 0·5 hr.

Treatment of enzyme	Addition	Aspartic acid formed (μmoles)	α-Oxoglutaric acid formed (μmoles)
1. Dialysed	Nil	5·1	5·4
2. Dialysed	Fe^{2+} ions (20 μg.)	7·7	8·2
3. Undialysed	Nil	9·0	11·2

Table 6. *Effect of thyroxine on transaminase*

L-Thyroxine (sodium salt) was dissolved in a minimum amount of dilute alkali. Iron was added as $FeSO_4,7H_2O$.

Tube no.	Thyroxine (μg.)	Fe^{2+} ions (μg.)	Molar ratio (thyroxine: iron)	Units (μg. of pyruvate/ mg. of protein)
1	Nil	Nil	—	1320
2*	200	Nil	—	543
3†	200	14	1	675
4†	200	28	0·5	806
5‡	200	14	1	950
6‡	200	28	0·5	996

* Thyroxine was added before addition of aspartic acid.
† Thyroxine and iron were added simultaneously.
‡ Thyroxine and iron were pre-incubated for 10 min. and then enzyme and other reactants were introduced.

Table 7. *Iron content and the specific activity of transaminase*

Reactants: aspartic acid 20 μmoles; α-oxoglutaric acid 20 μmoles; enzyme 0·1 ml.; buffer, tris–HCl, pH 8·5; incubation was for 0·5 hr. at 37° under nitrogen.

Treatment	Specific activity (μg. of pyruvate/ mg. of protein)	Iron content (μmg./mg. of protein)	Specific activity/ iron content
1. Water extract of acetone-dried powder	135	38	3·55
2. Dowex-2 (Cl form) treatment	427	120	3·55
3. Alumina-Cγ supernatant	1300	428	3·05
4. Calcium phosphate gel eluate	1393	440	3·16

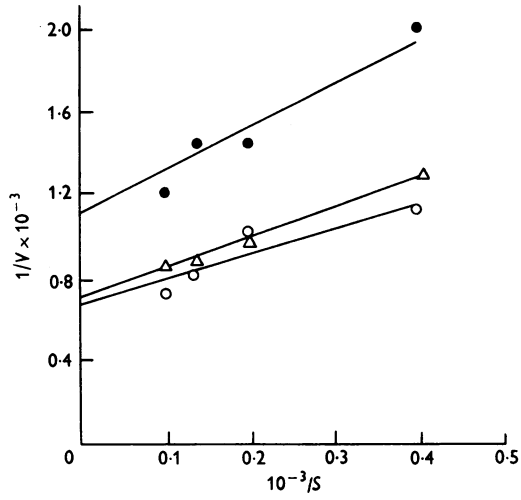

Fig. 2. Inhibition of transaminase by L-thyroxine. ○, Control; △, L-thyroxine (sodium salt), final concentration 25 μmoles; ●, L-thyroxine (sodium salt), final concentration 50 μmoles; aspartic acid and thyroxine were added simultaneously. Buffer, tris–HCl; incubation was for 0·5 hr. under nitrogen at 37°. The lines are drawn by the method of least squares.

amount of iron added is doubled. If thyroxine is pre-incubated with the same amount of iron, and then both are added to the enzyme the inhibition is less and it appears as if thyroxine has been bound by the added iron so that the available thyroxine to react with iron of the enzyme is considerably decreased. We were not able to show any shift in the absorption peak (325 mμ) of thyroxine when the hormone was incubated with the enzyme, although a slight depression in the thyroxine-absorption spectrum was noted. 3:5-Di-iodotyrosine did not have any effect on the activity. When the results are plotted as $1/V$ against $1/S$ it appears that the inhibition is of a non-competitive nature (Fig. 2).

Estimation of iron. Iron estimation was carried out in the presence of hydroxylamine hydrochloride. The results indicate that the iron content increases as purification proceeds; the ratio of iron (μmg.) to specific activity of the enzyme is almost

Table 8. *Inhibition of transaminase activity by citrate*

Reactants: aspartic acid 20 μmoles; α-oxoglutaric acid 20 μmoles; enzyme purified to stage IV and citrate were pre-incubated in the presence of buffer for 15 min. before addition of substrates. Incubation was under nitrogen at 37° for 0·5 hr.

	Buffer	pH	Final concn. of citrate (mM)	Inhibition (%)
1.	KH_2PO_4–K_2HPO_4	6·4	5	Nil
2.		6·8	5	13
3.		7·4	5	40
4.	Tris–HCl	7·6	5	33
5.	Tris–HCl	8·2	5	40

constant during the various stages of purification (Table 7).

Effect of metal-binders. The effect of various iron-binders and chelating agents on transaminase activity was studied and a wide range of inhibition was demonstrated by various compounds. The inhibitors were added at a final concentration of 5 mM and the reaction was carried out in potassium phosphate buffer, pH 7·5. The enzyme was pre-incubated with the inhibitor for 10 min. at 37° before the reactants, aspartic acid and α-oxoglutaric acid, were added. In general, chelating agents such as 8-hydroxyquinoline and $\alpha\alpha$-dipyridyl did not show much inhibition whereas chelating agents such as pyrophosphate and citrate gave between 25 and 40% inhibition. Cyanide almost completely destroyed the activity. Next to cyanide, citrate appeared to be the most effective inhibitor. It is known that trisodium and disodium citrate chelate Fe^{2+} ions powerfully to form a complex (Bobtelsky & Jordan, 1945). The complex is stable on the alkaline side of neutrality and breaks down as the acid side is approached. The results presented in Table 8 also indicate that no inhibition by citrate is observed below pH 6·8, but between pH 6·8 and 7·4 the inhibition increased.

No inhibition due to citrate could be observed when the reaction was studied in the range pH 6·0–7·2 with citrate–phosphate and phosphate buffers. The final concentration of citrate in citrate–phosphate buffer varied from 0·01 M at

pH 6·0 to 5 mM at pH 7·2, whereas in the inhibition studies mentioned above, final citrate concentration was 5 mM. This observation of citrate inhibition can thus be taken as additional evidence for participation of iron in the enzymic transamination.

DISCUSSION

This study gives sufficient evidence to suggest the involvement of a metal in enzymic transamination. Participation of pyridoxal phosphate in this reaction has already been established by other workers. In the present study it was possible to remove the metal by dialysis against 8-hydroxyquinoline, when the enzyme activity was reduced and it could be restored to a considerable extent by adding the iron again. This effect of iron has been demonstrated by studying its influence on the formation of three different reaction products, i.e. oxalacetic acid, aspartic acid and α-oxoglutaric acid, when the reaction was run in the other direction. Chemical analysis for iron indicated that iron accompanied the enzyme through the various stages of purification studied and the ratio of iron (μmg./mg. of protein) to the specific activity was also constant. Thyroxine, which is capable of binding metals (Lardy, 1954), showed inhibition of transaminase activity. These observations indicate that iron is the most likely participant in transamination.

The addition of certain iron-chelating agents to the reaction mixture did not significantly lower the activity of the enzyme, suggesting that the metal bound to the enzyme is not equally accessible to all metal-binders.

Metzler *et al.* (1954) have suggested the mode of participation of metal in this transamination. The first step in transamination is known to be a Schiff's-base formation between the donor amino acid and the pyridoxal phosphate that is already on the enzyme, attached probably through its phosphate group and the ring nitrogen. The presence of metal can be expected to promote the formation of the Schiff's base and the maintenance of planarity of the conjugated system through chelate-ring formation (Metzler *et al.* 1954). The chelated-metal ion also provides an additional electron-attracting group that operates in the same direction as the heterocyclic nitrogen atom, thus increasing the electron displacement from the α-carbon atom. If this is true, it would mean that the role of metal is somewhat secondary to that of pyridoxal phosphate in that the presence of metal helps to initiate or speed the reaction which can potentially take place even in the presence of pyridoxal phosphate alone. It is also possible, as suggested by Williams & Nielands (1954), that combination of the carbonyl group of amino acid with the metal will weaken the base strength of the amino group so that it readily condenses with the formyl group of pyridoxal phosphate.

The present study has given an indication of the role of metal in plant transaminase. It would be interesting if the same effect can also be demonstrated in transaminase from bacterial and animal sources. Quite recently the involvement of iron in another pyridoxal phosphate-dependent enzyme system, namely the biosynthesis of δ-aminolaevulinic acid, has been suggested by Brown (1958), in chicken-erythrocyte preparations.

SUMMARY

1. Glutamic acid–aspartic acid transaminase has been purified about 23-fold from a plant source, beans of *Dolichos lablab*.

2. Iron has been shown to participate as a co-factor with pyridoxal phosphate in the enzymic transamination. Iron estimations were carried out during the various stages of purification and the ratio of iron to specific activity was found to be constant.

3. The participation of iron has been demonstrated by studying its influence on the formation of oxaloacetic acid, α-oxoglutaric acid and aspartic acid respectively when the reaction was studied in both directions.

4. The optimum pH of the enzyme was found to be between 8·2 and 8·5.

5. Thyroxine inhibited the transaminase activity. Thyroxine incubated with iron before addition to the enzyme system inhibited the activity to a considerably less extent than when only thyroxine was added.

I wish to express my thanks to Dr M. Srinivasan, Assistant Director, for helpful discussions and suggestions during the course of this investigation. I am also grateful to Dr V. Subrahmanyan, Director, for his keen interest in the work. Thanks are also due to Glyco Products Co. Inc., U.S.A., for a kind gift of ethylenediaminetetra-acetic acid (disodium salt).

REFERENCES

Bobtelsky, M. & Jordan, J. (1945). *J. Amer. chem. Soc.* **67**, 1824.

Brown, E. G. (1958). *Nature, Lond.*, **182**, 313.

Cruickshank, D. H. & Isherwood, F. A. (1958). *Biochem. J.* **69**, 189.

Eggleston, L. V. (1958). *Biochem. J.* **68**, 557.

Happold, F. C. & Struyvenberg, A. (1954). *Biochem. J.* **58**, 379.

Happold, F. C. & Turner, J. M. (1957). *Nature, Lond.*, **179**, 155.

Isherwood, F. A. & Cruickshank, D. H. (1954). *Nature, Lond.*, **173**, 121.

Jakoby, W. B. & Bonner, D. M. (1953). *J. biol. Chem.* **205**, 699.

Krebs, H. A. (1942). *Biochem. J.* **36**, 303.
Lardy, H. A. (1954). *Brookhaven Symp. Biol.* **7**, 90. Cited in Wolff, J. & Wolff, E. C. (1957), *Biochim. biophys. Acta*, **26**, 387.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Mahler, H. R. & Elowe, D. G. (1954). *J. biol. Chem.* **210**, 165.
Metzler, D. E., Ikawa, M. & Snell, E. E. (1954). *J. Amer. chem. Soc.* **76**, 648.
Patwardhan, M. V. (1958). *Nature, Lond.*, **181**, 187.
Sall, J., Richards, H. K., Harrison, E. & Myerson, R. M. (1957). *J. Lab. clin. Med.* **50**, 297.
Speck, J. F. (1949). *J. biol. Chem.* **178**, 315.
Srinivasan, M. (1957). *Lancet*, ii, 317.
Tonhazy, N. E., White, N. G. & Umbreit, W. W. (1950). *Arch. Biochem.* **28**, 36.
Tulpule, P. G. & Patwardhan, V. N. (1952). *Nature, Lond.*, **169**, 671.
Wijesundera, S. & Woods, D. D. (1953). *J. gen. Microbiol.* **9**, iii.
Williams, V. R. & Nielands, J. B. (1954). *Arch. Biochem. Biophys.* **53**, 56.
Wolff, J. & Wolff, E. C. (1957). *Biochim. biophys. Acta*, **26**, 387.
Yanofsky, C. (1952). *J. biol. Chem.* **198**, 343.

Biochem. J. (1960) **75**, 408

Regulation of Glucose Uptake by Muscle

4. THE SPECIFICITY OF MONOSACCHARIDE-TRANSPORT SYSTEMS IN RAT-DIAPHRAGM MUSCLE*

BY F. C. BATTAGLIA†‡ AND P. J. RANDLE
Department of Biochemistry, University of Cambridge

(*Received* 16 *November* 1959)

In previous papers (Randle & Smith, 1958*a*, *b*) evidence was presented which showed that the transport of D-glucose and D-xylose across the muscle-cell membrane of isolated rat diaphragm is accelerated by insulin, anoxia and substances which inhibit oxidative phosphorylation (transport is defined as a process in which substances move across the cell membrane in combination with a specific constituent of the cell). These factors were also shown to accelerate transport of D-glucose and L-arabinose in the perfused isolated rat heart (Morgan, Randle & Regen, 1959). The conclusion was drawn by these workers that the transport of sugars across the muscle-cell membrane is inhibited by an energy-rich phosphate compound; that factors such as anoxia, which inhibit oxidative phosphorylation, accelerate transport by lowering intracellular levels of high-energy phosphate compounds; and that insulin accelerates transport by interfering with a reaction between high-energy phosphate and the transport system (Randle & Smith, 1958*a*, *b*).

In the present study the specificity of monosaccharide-transport systems in rat diaphragm has been defined by investigating the extent to which different sugars compete for transport. Evidence has been sought for the nature of the transport system by investigating the effect of some enzyme inhibitors on the transport of sugars. Finally we have attempted to determine whether sugars may pass directly from the transport system into muscle polysaccharides and whether the effect of insulin on sugar transport is secondary to an effect of the hormone on the formation of maltose and glycogen.

* Part 3: Morgan, Randle & Regen (1959).

† U.S. Public Health Service Postdoctoral Fellow.

‡ Present address: Department of Physiology, Yale University, New Haven, Connecticut, U.S.A.

METHODS AND PROCEDURE

Incubation and perfusion media. Diaphragms were incubated in the bicarbonate-buffered medium of Gey & Gey (1936) and hearts were perfused with the bicarbonate-buffered medium of Krebs & Henseleit (1932). D-Glucose, D-galactose, D-xylose, D-arabinose, L-arabinose and raffinose were obtained from T. Kerfoot and Co. Ltd., D-mannose, D-fructose and α-methyl-D-glucoside from British Drug Houses Ltd., D-lyxose from California Corporation for Biochemical Research, Los Angeles, U.S.A. and D-3-*O*-methylglucose from Ayerst, McKenna and Harrison, N.Y., U.S.A. These compounds were used without further purification. D-3-*O*-Methylglucose was subjected to chromatography (15 μl. of 1%) on Whatman no. 1 paper with pyridine–ethyl acetate–water (1:2:2) (Jermyn & Isherwood, 1949). Only one substance was detected when the chromatogram was developed with aniline hydrogen phthalate reagent (Partridge, 1949). β-Methyl-D-glucoside was prepared in this Laboratory by Mr B. R. Slater by the Koenig–Knorr reaction.

D-[$^{14}C_6$]Glucose (5 mc/m-mole), D-[I-^{14}C]mannose (1 mc/m-mole) and D-[$^{14}C_6$]sorbitol (6·2 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.