Since serum proteins constitute only 2% of the rat's total proteins this serum contribution of about 1% of the total glycine required in globin synthesis appears to be significant.

SUMMARY

1. Rats were injected intravenously with rat serum globulin or serum albumin obtained from donor animals injected with [2-¹⁴C]glycine. The incorporation of serum-protein-bound glycine into haem and globin was followed for 15 days.

2. Serum-protein-bound glycine is utilized efficiently both as a source of glycine by the protein globin and as a precursor in the formation of the non-protein moiety, haem.

3. The specific activities of haem and globin glycine indicate that the injected protein is broken down to free amino acids before the incorporation of glycine into globin takes place.

4. On breakdown of serum protein, the extent of utilization of serum-protein-bound glycine in the formation of globin and haem is less than or equal to that of injected free glycine, a finding in contrast with those previously reported for other systems in which free amino acids may not be the intermediates involved.

5. About 1.4% of serum-protein-bound glycine is utilized as a source of globin glycine. This represents about 1% of the total glycine required in globin synthesis. Since serum proteins constitute only 2% of the rat's total proteins, this contribution of serum-protein-bound glycine to globin biosynthesis appears to be significant.

REFERENCES

- Anson, M. L. & Mirsky, A. E. (1930). *J. gen. Physiol.* **13**, 469.
 Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **52**, 87.
 Cohen, S., Holloway, R. C., Matthews, C. & McFarlane, A. S. (1956). *Biochem. J.* **62**, 143.
 Drabkin, D. L. & Wise, C. D. (1960). *Science*, **132**, 1491.
 Creskoff, A. J., Fitz-Hugh, T., jun. & Farris, E. J. (1949). *The Rat in Laboratory Investigation*, 2nd ed., p. 406. Ed. by Farris, E. J. & Griffith, J. Q., jun. Philadelphia: J. B. Lippincott.
 Haurowitz, F. (1950). *Chemistry and Biology of Proteins*, p. 207. New York: Academic Press Inc.
 Lee, N. D., Anderson, J. T., Miller, R. & Williams, R. H. (1951). *J. biol. Chem.* **192**, 733.
 Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
 Levy, A. L. & Chung, D. (1955). *J. Amer. chem. Soc.* **77**, 2899.
 Levy, L. M., Walter, H. & Sass, M. D. (1959). *Nature, Lond.*, **184**, 643.
 Loftfield, R. B. & Harris, A. (1956). *J. biol. Chem.* **219**, 151.
 Muir, H. M., Neuberger, A. & Perrone, J. C. (1952). *Biochem. J.* **52**, 87.
 Owen, J. A. (1957). *Advanc. clin. Chem.* **1**, 237.
 Perrone, J. C. (1951). *Nature, Lond.*, **167**, 513.
 Shemin, D. (1946). *J. biol. Chem.* **162**, 297.
 Shemin, D. (1956). *Harvey Lect.* **50**, 258.
 Spector, W. S. (Ed.) (1956). *Handbook of Biological Data*, p. 90. Philadelphia: W. B. Saunders.
 Walter, H. (1960). *Nature, Lond.*, **188**, 643.
 Walter, H., Haurowitz, F., Fleischer, S., Lietze, A., Cheng, H. F., Turner, J. E. & Friedberg, W. (1957). *J. biol. Chem.* **224**, 107.
 Walter, H. & Mahler, H. R. (1958). *J. biol. Chem.* **230**, 241.
 Walter, H. & Zipper, H. (1961). *Nature, Lond.*, **192**, 1292.
 Whipple, G. H. (1956). *The Dynamic Equilibrium of Body Proteins*, p. 34. Springfield, Ill.: C. C. Thomas.

Biochem. J. (1962) **84**, 536

L-Threonine Ethanolamine Phosphate: A Compound Newly Isolated from Fish

By H. ROSENBERG, A. H. ENNOR, D. D. HAGERMAN* AND SADAOKO SUGAI†

Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia

(Received 10 January 1962)

Preliminary observations on the distribution of both stereoisomers of serine ethanolamine phosphate (2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate, I) have been reported (Rosen-

berg & Ennor, 1961). In further experiments, a hitherto undescribed ninhydrin-positive phosphorus-containing compound was detected in protein-free extracts of fish tissues. This compound, initially detected as a spot on a two-dimensional paper chromatogram, has been isolated and positively identified as L-threonine ethanolamine phosphate (2-aminoethyl L-2-amino-2-carboxy-1-methylethyl hydrogen phosphate, II).

* John Simon Guggenheim Memorial Fellow and Fulbright Research Scholar, 1961-62, while on leave from Harvard University Medical School, Boston, Mass., U.S.A.

† Australian National University Research Scholar.

This paper describes the isolation, identification and chemical synthesis of L-threonine ethanolamine phosphate.

MATERIALS AND METHODS

Chemicals. All reagents used were of analytical grade. The solvents used for chromatography were purified by fractional distillation.

Fishes. Two species of fish were used for these experiments: One, the Australian lungfish (*Neoceratodus*) was collected in North Queensland, packed in damp seaweed and transported to Canberra by air. The animals arrived in excellent condition and were kept in a large aerated aquarium for 2 days before use. They were then stunned by a blow on the head and the organs removed and extracted as described below. The other species, rainbow trout (*Salmo irideus*), was obtained from the New South Wales State Hatcheries at Jindabyne. The fish were killed immediately after removal from ponds, the organs frozen in liquid air and stored in solid CO₂ for transport to Canberra.

Ion-exchange resins and chromatography. The following ion-exchange resins were used: Amberlite CG-120 (X8; H⁺ form; mesh 100–200); Dowex 50 (X4; H⁺ form; mesh 50–100); Dowex 50 (X4; NH₄⁺ form; mesh 200–400). Before use all resins were repeatedly recycled by passage through the Na⁺ and H⁺ forms and exhaustively washed with water.

Fractionation of amino acids on columns of Amberlite CG-120 with an increasing concentration gradient of HClO₄ was carried out essentially as described by Hurlbert, Schmitz, Brumm & Potter (1954). The column measured 5.3 cm. × 60 cm. The mixing vessel contained 1.0 l. of water. The reservoir, placed 120 cm. above the top of the column for gravity feed, initially contained 2.0 l. of 0.25N-HClO₄. When this solution was exhausted it was replaced successively by 2.0 l. each of 0.5N-, 0.75N-, 1.0N- and 1.25N-HClO₄, and finally by 1.0 l. of 1.5N-HClO₄. The effluent was collected in 35 ml. fractions at a rate of 45 ml./min.

Ascending two-dimensional paper chromatography was performed on Whatman no. 3 papers in ethanol-formic acid (98–100%, w/v; British Drug Houses Ltd.)–water (7:1:2, by vol.; solvent 1) and in water-saturated phenol (solvent 2). The chromatograms were thoroughly dried in air at 30°, dipped in a 0.2% (w/v) solution of ninhydrin in acetone and heated for 10 min. at 80°.

Orthophosphate was identified according to Rosenberg (1959) and estimated by the method described by King (1932).

Determination of amino acids. Samples of eluates were made up to 0.5 ml. with water and mixed with 0.5 ml. of 1% (w/v) ninhydrin in pyridine. To each tube 0.05 ml. of 5N-HCl was added and the tubes were heated in a steam bath for 5 min. The volume was brought to 10.0 ml. with water and the colour densities were measured in a Hilger Spekker photometer with a spectrum-yellow filter.

Optical rotation in water was measured in a 1.0 ml., 1.0 dm. cell in a Bellingham and Stanley polarimeter reading to 0.01°, with the sodium D line.

Melting points. All melting points given are uncorrected.

EXPERIMENTAL AND RESULTS

Examination by two-dimensional paper chromatography of protein-free extracts of the viscera of the lungfish demonstrated the presence of a ninhydrin-reacting material with *R_F* slightly different from that of serine ethanolamine phosphate. The *R_F* values of this material and serine ethanolamine phosphate were 0.29 and 0.24 in solvent 1 and 0.38 and 0.24 in solvent 2, respectively. A 'desalted extract' was prepared by passage of a neutralized-perchloric acid extract through Dowex 50 (H⁺ form), elution of adsorbed material with aq. 2.5N-ammonia and evaporation of the eluate under reduced pressure. This material was streaked on Whatman no. 3 paper and the chromatogram was developed in solvent 1, which separated serine ethanolamine phosphate and the unknown compound from other ninhydrin-reacting materials with much higher *R_F* values. The band containing the unknown compound was cut out and eluted with water, and the eluate again streaked on paper. Development was performed in solvent 2 and complete separation of serine ethanolamine phosphate and the unknown compound was achieved. The unknown compound was eluted with water and the eluate tested for homogeneity by two-dimensional chromatography in solvents 1 and 2. Only one ninhydrin-reacting spot was detected. A portion of the eluate was heated at 100° for 16 hr. in 5N-hydrochloric acid and the hydrolysate chromatographed after removal of hydrochloric acid. Threonine, ethanolamine and inorganic phosphate were the principal hydrolysis products. The compound was therefore tentatively assumed to be threonine ethanolamine phosphate. Insufficient material was available for large-scale isolation of the new compound from the lungfish; but, since preliminary experiments had indicated the presence of the compound in the trout, this fish was selected for further study.

Isolation of L-threonine ethanolamine phosphate from trout. Liver, intestinal tract, kidneys, spleen and gonads were removed immediately after death and frozen. The pooled material (1050 g.) was extracted in a large-capacity Waring Blendor with 2.0 l. of 1.5N-perchloric acid. After

centrifuging, the residue was re-extracted with 1.0 l. of *N*-perchloric acid. The combined extracts were adjusted to pH 7.2 by the addition of 10*N*-potassium hydroxide, cooled and kept overnight at 3°. The precipitated potassium perchlorate was filtered off and the filtrate was passed through a column (5.5 cm. × 40 cm.) of Dowex 50 (H^+ form; mesh 50–100). The column was washed with 1.5 l. of water and the adsorbed material eluted with aq. 1.25*N*-ammonia. The eluate was collected in 25 ml. fractions at a rate of 16 ml./min. About 5 μ l. of each fraction was tested for ninhydrin-reacting material by spotting on paper; the paper was dried, dipped in ninhydrin solution (0.2%, w/v, in acetone) and heated for 10 min. at 80°. All ninhydrin-positive fractions (nos. 60–100) were pooled and evaporated under reduced pressure in a rotary evaporator until free of ammonia. The resultant solution (pH 7.5, 250 ml.) was stored overnight at 4°. The slight precipitate which formed was removed by centrifuging.

The clear solution was applied to a column (5 cm. × 60 cm.) of Amberlite CG-120 (H^+ form; mesh 100–200), and the adsorbed material eluted by an increasing gradient of perchloric acid as described in the Materials and Methods section. The elution pattern of ninhydrin-positive material and the acid concentration of the eluted fractions are shown in Fig. 1.

Preliminary experiments showed that both serine ethanolamine phosphate and threonine ethanolamine phosphate emerged only in the second major peak (fraction C, Fig. 1) after the acid concentration of the effluent reached 0.8*N*. This result was confirmed by chromatographic examination of the eluate; fractions A, B and D were discarded and the contents of tubes 213–262 (fraction C) pooled, adjusted to pH 7.2 by the addition of 10*N*-potassium hydroxide and cooled to 0°. After filtration the solution was freed from traces of potassium perchlorate by adsorption on a small (4 cm. × 14 cm.) column of Dowex 50 (H^+ form; mesh 50–100) and elution with aq. 2.5*N*-ammonia. The eluate (50 ml.) was reduced in volume to about 10 ml. on a rotary evaporator, applied to a column (4.5 cm. × 45 cm.) of Dowex 50 (NH_4^+ form; mesh 200–400), and eluted with water at a rate of 11 ml./min.

Samples of all of the ninhydrin-positive fractions were spotted on paper and chromatographed in solvent 2. The chromatogram (Fig. 2) showed that this step resulted in nearly complete separation of serine ethanolamine phosphate and threonine ethanolamine phosphate from the bulk of the amino acids in fraction C, as well as a partial separation of the two compounds from each other. Two more similar passages of the overlapping fractions through Dowex 50 (NH_4^+ form) resulted in a complete separation of threonine ethanolamine phosphate, as judged by paper chromatography in solvent 2.

All of the fractions containing threonine ethanolamine phosphate only were pooled and the volume of the combined solution was reduced to 1 ml. on a rotary evaporator. The solution was diluted with 5 ml. of methanol and filtered. The compound failed to crystallize after the addition of a further 15 ml. of methanol; methanol-acetone (1:4, v/v) was then added slowly at room temperature. A fine precipitate appeared immediately and the addition was continued until 100 ml. of the mixture had been added. The solid was collected by centrifuging, washed several times with acetone and dried *in vacuo* over potassium hydroxide. The yield was 90 mg. The *threonine*

ethanolamine phosphate had m.p. 176° (decomp.) after preliminary frothing at 151° (Found for material dried at 70°/0.005 mm.: C, 29.9; H, 6.1; N, 11.5; P, 12.9. $C_6H_{13}N_2O_6P$ requires C, 29.8; H, 6.2; N, 11.6; P, 12.8%).

The product was examined by two-dimensional chromatography in solvents 1 and 2 and was free from contaminating ninhydrin-positive material.

Synthesis of L-threonine ethanolamine phosphate. L-Threonine ethanolamine phosphate has not been previously synthesized. In the present work the synthesis followed that described for serine ethanolamine phosphate by Jones & Lipkin (1956). The procedure involved the reaction of *N*-benzyloxycarbonyl-L-threonine benzyl ester with phenyl phosphorodichloridate followed by the addition of *N*-benzyloxycarbonylethanolamine and the removal of the protecting groups by catalytic hydrogenation, with catalysts prepared as described by Linstead & Thomas (1940). *N*-Benzyloxycarbonylethanolamine was prepared according to Rose (1947) with the modifications introduced by Jones & Lipkin (1956). Phenyl phosphorodichloridate

Fig. 1. Elution pattern of ninhydrin-positive material obtained by gradient elution from a column of Amberlite CG-120 (H^+ form). Details of the gradient-elution technique and methods of estimation are given in the text. Full line: extinction of ninhydrin-reacting material; broken line: acid concentration of effluent.

Fig. 2. Ninhydrin-treated paper chromatogram of samples from fractions collected from a column of Dowex 50 (NH_4^+ form). The solvent was water-saturated phenol. Serine ethanolamine phosphate (X) and threonine ethanolamine phosphate (Y) denote reference standards. Areas outlined by thin lines denote faint spots.

was a commercial product (Aldrich Chemical Co., Milwaukee, U.S.A.). *N*-Benzyloxycarbonyl-L-threonine benzyl ester has not been previously synthesized. It was prepared according to the method described by Baer & Maurukas (1955) for the preparation of the corresponding serine compound. By this procedure 10.5 g. of *N*-benzyloxycarbonyl-L-threonine benzyl ester was obtained from 6 g. of L-threonine (yield 62%), m.p. 81.5° (Found: C, 66.6; H, 6.3; N, 4.1. $C_{19}H_{21}NO_5$ requires C, 66.5; H, 6.1; N, 4.1%).

Chromatographic examination of the product obtained after coupling and hydrogenation showed the presence of threonine ethanolamine phosphate as well as seven other compounds, including ethanolamine and threonine. The mixture was fractionated on Amberlite CG-120 (H^+ form) and Dowex 50 (NH_4^+ form) in a manner similar to that described for the isolation of the natural compound. The solution of chromatographically pure L-threonine ethanolamine phosphate obtained was treated with methanol and acetone as described above and the crystalline material dried *in vacuo* over potassium hydroxide. The yield was 400 mg., equivalent to 5.5%. The threonine ethanolamine phosphate had m.p. 176° (decomp.) with preliminary frothing at 155° (Found for material dried at 70°/0.005 mm.: C, 30.5; H, 6.1; N, 11.3; P, 12.9. $C_6H_{15}N_2O_8P$ requires C, 29.8; H, 6.2; N, 11.6; P, 12.8%).

Two-dimensional chromatography of the product in solvents 1 and 2 showed it to be free from ninhydrin-positive contaminants and to yield a single spot when chromatographed with the natural material.

Comparison of synthetic and natural L-threonine ethanolamine phosphate. The m.p. (decomp.) of a mixture of the natural and synthetic compounds was 176° with preliminary frothing at 157°.

The $[\alpha]_D^{25.0}$ of the synthetic and natural materials was -36.9° (*c* 0.70 in water) and -37.3° (*c* 0.94 in water) respectively. The threonine in the natural compound is thus of the L-configuration.

The infrared spectra of the two compounds were identical (Fig. 3).

Partial acid hydrolysis of natural and synthetic threonine ethanolamine phosphate. About 4 mg. of each sample was heated in sealed tubes with 0.2 ml. of 5*N*-hydrochloric acid for 50 min. at 100°. The hydrochloric acid was removed under reduced pressure and the residues were dried overnight *in vacuo* over potassium hydroxide. Both of the dry residues were dissolved in 0.2 ml. of water, and 10 μ l. of the solution was spotted on paper and developed two-dimensionally in solvents 1 and 2. A mixture of authentic reference compounds which were considered to be probable hydrolysis products (threonine, threonine *O*-phosphate, ethanolamine and ethanolamine *O*-phosphate), with synthetic threonine ethanolamine phosphate, were similarly chromatographed. The patterns of spots produced by the two samples (Figs. 4A and 4B) were identical. A comparison with the marker map (Fig. 4C) shows that the five major components of the hydrolysates were threonine, ethanolamine, the

two corresponding *O*-phosphate esters and some residual threonine ethanolamine phosphate. The chromatograms of the hydrolysates also contained a spot (6, Fig. 4A), corresponding in position to

Fig. 3. Infrared spectra. Upper and lower curves represent the spectra of isolated threonine ethanolamine phosphate and synthetic L-threonine ethanolamine phosphate respectively. The spectra were obtained with a potassium bromide disk in a Perkin-Elmer model 21 double-beam infrared spectrophotometer. The absorption maxima (775, 917, 963, 1032, 1074, 1226, 1400, 1523, 1639, 2980 and 3415 cm^{-1}) of both compounds occur at identical frequencies.

Fig. 4. Two-dimensional chromatograms (solvent 1 in horizontal direction and solvent 2 in vertical direction) of the products of partial hydrolysis of isolated threonine ethanolamine phosphate (A), synthetic L-threonine ethanolamine phosphate (B) and of a mixture of reference compounds (C). Details of the hydrolysis procedure are given in the text. 1, Threonine ethanolamine phosphate (synthetic); 2, ethanolamine *O*-phosphate; 3, threonine *O*-phosphate; 4, threonine; 5, ethanolamine; 6, (?) chloroethylamine; 7, unidentified.

Table 1. *Distribution of serine ethanolamine phosphate and threonine ethanolamine phosphate in some tissues of the trout and lungfish*

Experimental details are given in the text. Values are expressed as μ moles/100 g. of tissue.

Tissue	Trout		Lungfish	
	Serine ethanolamine phosphate	Threonine ethanolamine phosphate	Serine ethanolamine phosphate	Threonine ethanolamine phosphate
Muscle	2.4	7.4	2.0	3.7
Liver	93	102	3.2	13.6
Gut	37	96	6.3	53.8
Kidney	97	361	5.3	20
Gonads	31	73	3.5	5.2

2-chloroethylamine, previously reported to be present in hydrolysates of compounds containing ethanolamine in ester linkage with phosphoric acid (Rosenberg & Ennor, 1959). Another spot (7, Fig. 4A) was not identified.

Distribution of L-threonine ethanolamine phosphate in the organs of the lungfish and trout. Some experiments have been carried out to determine quantitatively the amounts of L-threonine ethanolamine phosphate and L-serine ethanolamine phosphate present in various tissues of both the trout and lungfish. Samples of 'desalted extracts' representing known amounts of tissue were applied to sheets of Whatman no. 3 paper and serine ethanolamine phosphate and threonine ethanolamine phosphate separated from amino acids and from each other by two-dimensional chromatography in solvents 1 and 2. The compounds were located on the chromatograms with ninhydrin and the spots cut out and eluted with water according to Reith (1957). The eluates were wet-ashed with sulphuric acid and nitric acid, and the phosphate content was determined.

The L-threonine ethanolamine phosphate and L-serine ethanolamine phosphate content of the organs examined is given in Table 1. Various tissues of the chicken, river turtle and frog were similarly examined but no threonine ethanolamine phosphate was detected.

DISCUSSION

Until recently phospholipids were the only phosphate diesters of amino acids known to occur in animal tissues. The discovery of L-serine ethanolamine phosphate in reptilian muscle by Roberts & Lowe (1954) and of D-serine ethanolamine phosphate in the earthworm by Rosenberg & Ennor (1959) and Ennor, Rosenberg, Rossiter, Beatty & Gaffney (1960) extended the list of this type of diester. Other experiments (Rosenberg & Ennor,

1961) have suggested that D-serine ethanolamine phosphate is restricted to the terrestrial and freshwater oligochaetes and that the L-enantiomorph is found only in birds, fish, amphibians and reptiles. In each of these groups it appears to be synthesized only in the kidneys.

The discovery and isolation of a hitherto unrecognized phosphate diester in fish tissues adds still another compound to the class. The experiments provide unequivocal proof of the identity of the new compound with synthetic L-threonine ethanolamine phosphate. The distribution of L-threonine ethanolamine phosphate in the tissues of the trout is generally similar to that of L-serine ethanolamine phosphate in the same species, although the amounts of threonine ethanolamine phosphate found in any one organ are greater than those of serine ethanolamine phosphate. A similar distribution is found in the lungfish, but in this animal the actual amounts of both compounds present are much smaller than in the trout. Rosenberg & Ennor (1961) suggested that the unusual species distribution of serine ethanolamine phosphate may be of evolutionary significance, and similar remarks may apply to threonine ethanolamine phosphate. This compound has so far been detected in two species of fish where the amounts found greatly exceed those of serine ethanolamine phosphate. Examination of some of the more primitive classes of fish and other chordates might therefore reveal species in which threonine ethanolamine phosphate alone is found.

Nothing is known about the metabolic role of either threonine ethanolamine phosphate or serine ethanolamine phosphate, but their structural similarity suggests that they may be concerned in closely related metabolic reactions. The chemical structure of serine ethanolamine phosphate is related to that of certain phospholipids and might indicate a metabolic interrelationship. Threonine ethanolamine phosphate may be similarly related to the metabolism of phosphatidylthreonine which has been isolated from tuna fish (Igarashi, Zama & Katada, 1958; Katada, Zama & Igarashi, 1959).

SUMMARY

1. L-Threonine ethanolamine phosphate has been synthesized and certain of its chemical and physical properties were determined.

2. A compound identical in all respects with L-threonine ethanolamine phosphate has been identified in, and isolated from, the tissues of the freshwater trout (*Salmo irideus*). The compound is also present in the lungfish (*Neoceratodus*).

3. The distribution of L-threonine ethanolamine phosphate has been determined in various organs of both species of fish. The compound could not be

detected in tissues of the chicken, river turtle or frog, all of which contain serine ethanolamine phosphate.

We are indebted to the Chief Secretary, New South Wales, and to the Chief Inspector of Fisheries, Queensland, for permission to collect trout and lungfish respectively, and to Mr Herbert Butler for provision of facilities at Jindabyne Hatcheries. We are also indebted to Mr Brian Thorpe for skilled technical assistance, and to Dr Joyce Fildes and Dr E. Spinner for elementary and infrared analyses. This work was supported by grants from the Rockefeller Foundation and from the National Heart Foundation of Australia.

REFERENCES

- Baer, E. & Maurukas, J. (1955). *J. biol. Chem.* **212**, 25.
 Ennor, A. H., Rosenberg, H., Rossiter, R. J., Beatty, I. M. & Gaffney, T. J. (1960). *Biochem. J.* **75**, 179.
 Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 23.
 Igarashi, H., Zama, K. & Katada, M. (1958). *Nature, Lond.*, **181**, 1282.
 Jones, E. E. & Lipkin, D. (1956). *J. Amer. chem. Soc.* **78**, 2408.
 Katada, M., Zama, K. & Igarashi, H. (1959). *Bull. Jap. Soc. sci. Fish.* **24**, 735.
 King, E. J. (1932). *Biochem. J.* **26**, 292.
 Linstead, R. P. & Thomas, S. L. S. (1940). *J. chem. Soc.* p. 1127.
 Reith, W. S. (1957). *Nature, Lond.*, **179**, 580.
 Roberts, E. & Lowe, R. P. (1954). *J. biol. Chem.* **211**, 1.
 Rose, W. G. (1947). *J. Amer. chem. Soc.* **69**, 1384.
 Rosenberg, H. (1959). *J. Chromat.* **2**, 487.
 Rosenberg, H. & Ennor, A. H. (1959). *Biochem. J.* **73**, 521.
 Rosenberg, H. & Ennor, A. H. (1961). *J. Biochem., Tokyo.* **50**, 81.

Biochem. J. (1962) **84**, 541

Properties of a New Glyoxylate Reductase from Leaves

BY I. ZELITCH AND A. M. GOTTO*

Department of Biochemistry, The Connecticut Agricultural Experiment Station, New Haven, Conn., U.S.A., and Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford

(Received 22 February 1962)

Observations of the effect of glycollate and glyoxylate added in catalytic quantities in stimulating the rate of oxygen uptake by homogenates of spinach leaves first led to the discovery of a specific NADH₂-linked glyoxylate reductase (Zelitch, 1953, 1955). This enzyme (glycollate-NAD oxidoreductase, EC 1.1.1.26) catalyses the reduction of glyoxylate to glycollate (reaction 1) and of hydroxypyruvate to D-glycerate (Stafford, Magaldi & Vennesland, 1954).

On the addition of the flavoprotein glycollate oxidase, glyoxylate reductase can transfer hydrogen from NADH₂ to oxygen through a series of reactions which constitute a glycollate-oxidation cycle (Zelitch & Ochoa, 1953; Zelitch, 1953).

Although crude preparations of glyoxylate reductase from both spinach and tobacco leaves react with both NADH₂ and NADPH₂ in the presence of glyoxylate, the highly purified crystalline enzyme from tobacco leaves (Zelitch, 1955) and an enzyme of similar specific activity from spinach leaves (Holzer & Holldorf, 1957) do not function with NADPH₂.

Evidence has been presented that NADH₂-linked glyoxylate-reductase activity is associated with chloroplasts in spinach leaves (Zelitch & Barber, 1960), and that glycollic acid plays an important role in the carbon metabolism and respiration of leaves in sunlight (Zelitch, 1958, 1959). These considerations, together with the demonstration of the role of these acids in synthetic reactions of micro-organisms (Kornberg & Gotto, 1961; Kornberg & Sadler, 1961), have stimulated our further interest in the glyoxylate-reductase reaction. We have now found an enzyme in both tobacco and spinach leaves which catalyses the reduction of glyoxylate to glycollate in the presence of NADPH₂. This new enzyme has been partially purified and shown to have properties different from the NADH₂-linked glyoxylate reductase previously isolated from the same tissues. A preliminary account of this investigation has been presented (Zelitch & Gotto, 1961).

MATERIALS AND METHODS

Leaf extracts. Tobacco (*Nicotiana tabacum*) leaves weighing about 10 g. each from plants grown outdoors, and spinach (*Spinacea oleracea*) leaves purchased locally, were used. The leaves were washed in tap water and stored at 5° in a polythene bag for several hours before extraction.

* Present address: Department of Biochemistry, Vanderbilt School of Medicine, Nashville, Tenn., U.S.A.