- Linker, A., Hoffman, P., Sampson, P. & Meyer, K. (1958). *Biochim. biophys. Acta*, **29**, 443.
- Masamune, H. & Yosizawa, Z. (1956). *Tohoku J. exp. Med.* **65**, 57.
- Mathews, M. E. & Dorfman, A. (1953). *Arch. Biochem. Biophys.* **42**, 41.
- Meyer, K. (1955). *Polysaccharides in Biology*, p. 31. Ed. by Springer, G. New York: Josiah Macy Foundation.
- Meyer, K., Davidson, E., Linker, A. & Hoffman, P. (1956). *Biochim. biophys. Acta*, **21**, 506.
- Meyer, K., Hoffman, P. & Linker, A. (1959). In *Connective Tissue, Thrombosis Atherosclerosis*, p. 181. Ed. by Page, I. H. New York: Academic Press Inc.
- Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
- Mori, T. (1953). *Advanc. Carbohydr. Chem.* **8**, 315.
- Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
- Pasternak, C. A. & Kent, P. W. (1952). *Research*, **5**, 486.
- Richardson, W. D. & Dewar, E. T. (1958). *Nature, Lond.*, **182**, 1779.
- Ricketts, C. R. (1952). *Biochem. J.* **51**, 129.
- Schiller, S., Mathews, M. B., Jefferson, H., Ludowieg, J. & Dorfman, A. (1954). *J. biol. Chem.* **211**, 717.
- Shashoua, V. E. & Kwart, H. (1959). *J. Amer. chem. Soc.* **81**, 2899.
- Soda, T. & Tereyama, H. (1948). *J. chem. Soc. Japan*, **69**, 65. Quoted in *Chem. Abstr.* (1953), **47**, 3246.
- Stacey, M. (1946). *Advanc. Carbohydr. Chem.* **2**, 161.
- Vasseur, E. (1948). *Acta chem. scand.* **2**, 900.
- Vasseur, E. (1950). *Acta chem. scand.* **4**, 1144.
- Werner, I. (1953). *Acta Soc. Med. Upsalien.* **58**, 1.
- Wood, J. W. & Mora, P. T. (1958). *J. Amer. chem. Soc.* **80**, 3700.

Metabolism of Nicotinamide and Nicotinic Acid in *Corcyra cephalonica* St.

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(Received 1 June 1959)

Sundaram, Radhakrishnamurty & Sarma (1954) and Sundaram & Sarma (1954) reported that in the larva of the rice moth, *Corcyra cephalonica* St., *N*-methylnicotinamide is not an excretory product of nicotinic acid and nicotinamide as it is in many higher animals. The end products of the vitamin metabolism in this insect are principally nicotinic acid and to a lesser but significant extent nicotinuric acid. It was inferred that the deamidation of nicotinamide is an important pathway in the metabolism of the vitamin in *Corcyra*. Somewhat similar observations have been recently made for the mould *Neurospora crassa*, which neither methylates the vitamin nor conjugates it with glycine but disposes of it predominantly as free nicotinic acid, deamidating nicotinamide in the process (Sundaram, Rajagopalan & Sarma, 1958).

The results of a study of the metabolism of ¹⁴C-labelled nicotinic acid and nicotinamide in *Corcyra* and the enzymes mediating the deamidation and the conjugation reactions are presented in this paper.

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EXPERIMENTAL

The stock colony of *Corcyra cephalonica* St. was maintained on whole wheat-flour diet as described earlier (Sarma, 1943).

Determination of nicotinuric acid excreted by Corcyra larvae. The experimental diet consisted of salt-extracted wheat flour, salts mixture and vitamins (Sarma, 1943) and was supplemented with nicotinic acid or nicotinamide (30 mg./100 g.). Larvae (10–12 days old), hatched on a whole wheat-flour diet, were maintained on the experimental diet until the major portion of the diet had been consumed. The excreta were then separated from diet particles. A portion (100 mg.) of the powdered excreta was autoclaved with 5 ml. of water at 15 lb./in.² for 1 hr. The mixture was then adjusted to pH 4.5, made to volume and filtered. The filtrate was brought to pH 7.0 and assayed microbiologically for nicotinic acid and its tertiary derivatives with *Lactobacillus arabinosus* 17–5 (Snell, 1951) and *Leuconostoc mesenteroides* 9135 (Krehl, De La Huerge, Elvehjem & Hart, 1946). *L. arabinosus* responds to both nicotinic acid and nicotinuric acid, whereas *L. mesenteroides* 9135 specifically measures only free nicotinic acid (Johnson, 1945). When the supplement to the larval diet was nicotinamide, a second 100 mg. lot of the larval excreta was autoclaved with 0.6N-H₂SO₄. Nicotinamide, but not nicotinuric acid, is hydrolysed to nicotinic acid under these conditions (Johnson, 1945). An assay of the hydrolysed sample with *L. mesenteroides* would therefore give the combined nicotinic acid and nicotinamide content of the excreta.

Metabolism of [¹⁴C]nicotinic acid and [¹⁴C]nicotinamide in Corcyra. Batches (1 g.) of the experimental diet were supplemented with nicotinic acid or nicotinamide at a

physiological (25 $\mu\text{g./g.}$) as well as an extraphysiological (400 $\mu\text{g./g.}$) level, each dish containing 1 μC of the corresponding ^{14}C -labelled compound. *Corcyra* larvae were placed on the diets and a concentrate of the excreta, prepared as described by Sundaram *et al.* (1954), was chromatographed by the ascending method on Whatman no. 1 filter paper with butan-1-ol saturated with aq. 3% (w/v) ammonia. The air-dried chromatogram was radioautographed on Ilford Ilflex film, the period of exposure being 4–6 weeks. The R_F values were: nicotinamide, 0.78; nicotinic acid, 0.23; nicotinuric acid, 0.13 and *N*'-methyl-nicotinamide, 0.07.

Deamidation of nicotinamide by Corcyra larval homogenate in vitro. *Corcyra* larvae from the stock colony were picked out, cleaned free of adhering food particles, washed with ice-cold water and chilled. The chilled larvae were ground with sand in a porcelain mortar surrounded by a freezing mixture of ice and salt. A homogenate in ice-cold water was prepared so as to contain the equivalent of 120 mg. of fresh larval tissue/ml. and centrifuged at 1000 *g* for 10 min. to sediment the sand and the cell debris. The supernatant was used as the enzyme source in the incubation system, consisting of 0.5 ml. of 0.2 *M*-sodium phosphate buffer, pH 7.0, 0.5 ml. of nicotinamide solution (4 μmoles) and 1 ml. of enzyme preparation. In the blank, 0.5 ml. of water replaced the nicotinamide solution. After incubation at 37° for 2 hr. the ammonia and the free nicotinic acid present in the incubation mixture were determined as described by Sundaram *et al.* (1958).

The intracellular localization of the nicotinamide deamidase in *Corcyra* was studied by centrifuging a cell-free homogenate of the larvae, in 0.25 *M*-sucrose, prepared in the Potter-Elvehjem homogenizer (Teflon-pestle type; Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.), at 18 000 *g* for 1 hr. on an International Refrigerated Centrifuge (model PR 2) and by assaying the clear supernatant and the particulate matter.

pH optimum of Corcyra nicotinamide deamidase. The experimental details were as described by Sundaram *et al.* (1958). The enzyme preparation was the supernatant obtained by centrifuging a 10% larval homogenate in 2.5% (w/v) NaCl at 18 000 *g* for 1 hr.

Properties of nicotinamide deamidase of Corcyra. In these experiments the supernatant obtained on centrifuging a 20% cell-free homogenate of *Corcyra* larvae at 18 000 *g* for 1 hr. was used. The incubation system consisted of 0.5 ml. of 0.4 *M*-2-amino-2-hydroxymethylpropane-1:3-diol-HCl buffer, pH 7.5, 0.5 ml. of solution containing nicotinamide (4 μmoles) and supplements if any, and 1 ml. of enzyme extract. The enzyme activity was measured after incubation at 37° for 2 hr. in terms of the nicotinic acid released (Sundaram *et al.* 1958).

Conjugation of nicotinic acid with glycine in vitro. The complete incubation system (4 ml.) contained, in addition to 2 ml. of a 20% homogenate of *Corcyra* larval tissue in 0.9% KCl, 0.01 *M*-glycine, 2.5 mm-nicotinic acid (containing 1 μC of [carboxy- ^{14}C]nicotinic acid), 5 mm-DL-malic acid, 6 mm-adenosine triphosphate (ATP), 12 μM -cytochrome *c*, 0.02 *M*- K_2HPO_4 and KCl (0.9%). ATP and cytochrome *c* were products of the Sigma Chemical Co. Incubation was conducted in a Dubnoff metabolic shaker (Precision Scientific Co., Chicago, Ill., U.S.A.) at 37° for 3–4 hr. in air. At the end of the incubation, the mixture was treated with 0.4 ml. of 40% (w/v) trichloroacetic acid and centrifuged. The supernatant was extracted thrice with ether to remove the trichloroacetic acid and evaporated to dryness in a vacuum desiccator. The residue was extracted with 1 ml. of 95% (w/v) ethanol and the extract centrifuged. The clear supernatant was chromatographed and radioautographed with nicotinic acid and nicotinuric acid as standards.

RESULTS

The radioautographs of the excreta of *Corcyra* larvae dosed with [^{14}C]nicotinic acid and [^{14}C]nicotinamide at the physiological and the extraphysiological levels revealed that the main product excreted of either form of the vitamin was free nicotinic acid. Nicotinuric acid was also excreted to a smaller but significant extent, particularly when the insect had ingested the vitamin in extraphysiological amounts. In neither case was *N*'-methylnicotinamide excreted. These observations bear out the earlier conclusion that deamidation of nicotinamide is the chief chemical reaction operating in the vitamin metabolism in *Corcyra* and that *N*'-methylnicotinamide, which is the main metabolic product in many of the higher animals (Lin & Johnson, 1953; Reddi & Kodicek, 1953), is absent in the larval faeces. Although mammals excrete nicotinuric acid only as a metabolite of nicotinic acid administered in massive amounts (Lin & Johnson, 1953), the data in Table 1 shows that when nicotinamide is administered to *Corcyra* the glycine conjugate is excreted to about the same extent as when nicotinic acid is ingested.

The results of a typical experiment, presented in Table 2, demonstrate the presence of an enzyme system in *Corcyra* larval tissue which can deamidate nicotinamide *in vitro* with the formation of nicotinic acid and ammonia in stoichiometric

Table 1. *Excretion of nicotinuric acid by Corcyra cephalonica*

Total nicotinic acid (A) was determined with *Lactobacillus arabinosus* 17-5, and free nicotinic acid, before (B) and after (C) hydrolysis, with *Leuconostoc mesenteroides* 9135. Results are expressed as $\mu\text{g./g.}$ of larval excreta.

Addition to diet (30 mg./100 g.)	Total nicotinic acid (A)	Free nicotinic acid (B)	Nicotinic acid + nico- tinamide (C)	Nicotinuric acid (D)	100 D/A
Nicotinic acid	364	264	—	100	27.5
Nicotinamide	420	270	315	105	25.0

Table 2. Deamidation of nicotinamide by cell-free extract of *Corcyra* larvae

A cell-free extract of *Corcyra* larvae was incubated with nicotinamide, and the ammonia and the nicotinic acid in the incubation mixture were estimated. At the end of incubation, an amount of nicotinamide was added to the blank equal to that added to the test system before incubation. For experimental details see text. Results are expressed as μ moles/100 ml. of incubation mixture.

Experimental system	Ammonia	Ammonia liberated by deamidation (A)	Nicotinic acid	Nicotinic acid released by deamidation (B)	A/B
Blank	17	17	Trace	16	1.06
Test	34		16

Fig. 1. pH optimum of *Corcyra* nicotinamide deamidase. A portion (1 ml.) of a 10% larval homogenate in 2.5% NaCl was incubated with 0.5 ml. of 0.4M-NaOH-KH₂PO₄ buffer of appropriate pH and 0.5 ml. of a solution containing nicotinamide (4 μ moles) for 2 hr. at 37°. Nicotinic acid liberated was determined microbiologically.

proportion. The existence of nicotinamide deamidase in the larval system has been shown also by paper-electrophoretic analysis of the incubation mixture, followed by the application of Koenig's reaction (Sundaram, Rajagopalan & Sarma, 1959). The pH optimum of the *Corcyra* deamidase is 7.0–7.5 (Fig. 1) as against the optimum of pH 8.0–9.5 of the *Neurospora* enzyme (Sundaram *et al.* 1958). Separation of cell-free homogenate of the larval tissue into the particulate and supernatant components by differential centrifuging and subsequent assay of the fractions for enzymic activity showed that the enzyme could be recovered in the supernatant to the extent of more than 90%, indicating that the intracellular locale of the enzyme in

Table 3. Effect of various compounds and inhibitors on nicotinamide deamidase of *Corcyra cephalonica*

The enzyme activity in 1 ml. of supernatant from a 15% homogenate of larval tissue was determined in the presence of each of the compounds at the concentration indicated. For experimental details see text.

Compound or inhibitor	Concn. (mm)	Percentage of activity of control
None (control)	—	100
Hg ²⁺ ions	1	0
Cu ²⁺ ions	1	23
Mg ²⁺ ions	1	100
Mn ²⁺ ions	1	100
<i>o</i> -Iodosobenzoate	0.1	100
<i>p</i> -Chloromercuribenzoate	0.1	97
<i>p</i> -Chloromercuribenzoate + mm-cysteine	0.1	99
8-Hydroxyquinoline	10	73
$\alpha\alpha'$ -Dipyridyl	10	15
Ethylenediaminetetra-acetate	10	83

Table 4. Effect of various metal ions on the inhibition of *Corcyra* nicotinamide deamidase by $\alpha\alpha'$ -dipyridyl

Experimental details are similar to those given in Table 3.

Concn. of $\alpha\alpha'$ -dipyridyl (mm)	Metal ion	Concn. of metal ion (mm)	Percentage of activity of control
None	None	—	100
10	None	—	12
10	Mg ²⁺	10	13
10	Mn ²⁺	10	15
10	Zn ²⁺	10	4
10	Co ²⁺	10	14
10	Fe ²⁺	10	68
5	None	—	26
5	Fe ²⁺	5	84
5	Fe ²⁺	10	99

Corcyra tissue, as in *N. crassa*, is the soluble cytoplasmic matter. In the tissues of the pigeon and the chick the enzyme is distributed predominantly in the particulate subcellular components (Rajagopalan, Sundaram & Sarma, 1958).

The effects of certain metal ions, metal-chelating agents and sulphydryl reagents on the nicotinamide deamidase of *Corcyra* are summarized in

Table 3. The dipyriddy inhibition is not overcome by Mg^{2+} , Mn^{2+} , Co^{2+} or Zn^{2+} ions but is largely counteracted by Fe^{2+} ions (Table 4), showing a specific requirement for Fe^{2+} ions for enzymic activity. Repetition of these experiments gave results which, though quantitatively different, conformed to the same pattern. The results of the inhibition studies conform more or less to the pattern observed earlier for *N. crassa* deamidase (Sundaram *et al.* 1958).

The presence of an enzyme system which mediates the conjugation reaction in a cell-free homogenate of *Corcyra* larvae was shown as described above. The autoradiograph revealed a spot corresponding to nicotinuric acid when the complete system was used but not when glycine or ATP was omitted. The requirement for ATP, the probable involvement of coenzyme A in the biosynthesis of nicotinuric acid (Sundaram & Sarma, 1956), the activation of nicotinic acid by glycine *N*-acylase (Schachter & Taggart, 1954) and the report of the formation of nicotinuric acid by rat-kidney and -liver mitochondria under conditions supporting oxidative metabolism (Jones & Elliot, 1954) point to the similarity in mechanism between the reactions leading to the formation of hippuric acid and nicotinuric acid.

DISCUSSION

Among the vitamins of the B complex, nicotinic acid and nicotinamide and more recently thiamine are the only members whose metabolic fate has been investigated in detail. Studies with nicotinic acid and nicotinamide have mostly been confined to mammals. The present investigation with *Corcyra cephalonica* has shown that whereas the excretion of nicotinuric acid is a characteristic of both insect and mammals, the deamidation of nicotinamide figures predominantly in the insect in contrast to its methylation, which is the metabolic pathway recognized as the most important in a majority of the mammals.

Nicotinamide deamidase has been shown to be present in lactic acid bacteria (Hughes & Williamson, 1953), yeast (Oka, 1954), mycobacteria (Halpern & Grossowicz, 1957) and *Neurospora* (Sundaram *et al.* 1958). Quagliariello, Guerritore & Porcellati (1952) report its occurrence in the tissues of the rat and the hog; but the validity of their conclusions has been questioned by Hughes & Williamson (1953). Pigeon liver and chick kidney and, to a markedly lesser extent, rabbit liver contain nicotinamide deamidase (Rajagopalan *et al.* 1958). Further, it appears more than fortuitous that the organisms which are capable of deamidating nicotinamide, namely *Corcyra*, *N. crassa*, the pigeon, the chick and the rabbit, do not methylate

it (Sundaram *et al.* 1958; Cantoni, 1951; Chang & Johnson, 1957; Ellinger & Abdel Kader, 1949). The methylation and the deamidation reactions may thus be alternative pathways in the vitamin metabolism.

Recent reports (Preiss & Handler, 1958) on the biosynthesis of the pyridine nucleotide coenzymes establish more or less unequivocally that in certain instances, under physiological conditions, nicotinic acid rather than nicotinamide is an intermediate in the synthesis of diphosphopyridine nucleotide (DPN). According to this scheme, nicotinic acid is first incorporated into the nicotinic acid analogue of DPN, termed deamido-DPN, which is then amidated to DPN. It has also been suggested that free nicotinamide present in tissues is formed as a degradation product of DPN and not by the amidation of nicotinic acid (Preiss & Handler, 1958). In the light of these findings, the deamidation of nicotinamide assumes additional significance not merely as a means of disposal of the vitamin from the living system but as a possible mechanism for the conversion of nicotinamide into nicotinic acid, which may then be utilized for the formation of the pyridine nucleotide coenzymes.

SUMMARY

1. The metabolism of [^{14}C]nicotinic acid and [^{14}C]nicotinamide in the insect larva *Corcyra cephalonica* St. has been investigated. With either form of the vitamin the predominant product excreted is free nicotinic acid. Nicotinuric acid is also excreted to a significant extent, particularly when the vitamin has been ingested in extra-physiological amounts.

2. The presence in *Corcyra* larval tissues of an enzyme deamidating nicotinamide to nicotinic acid and ammonia *in vitro* has been demonstrated. The intracellular locale of the enzyme is the soluble cytoplasmic matter.

3. The nicotinamide deamidase of *Corcyra* has an optimum pH of 7.0–7.5 and is inhibited by Hg^{2+} and Cu^{2+} ions and by metal-chelating agents, particularly $\alpha\alpha'$ -dipyridyl. The dipyriddy inhibition is largely reversed by Fe^{2+} but not by Mn^{2+} , Mg^{2+} , Co^{2+} or Zn^{2+} ions.

4. The conjugation of nicotinic acid with glycine has been effected by a cell-free homogenate of *Corcyra* larval tissues in the presence of adenosine triphosphate and under oxidative conditions.

5. The results and the significance of the biological deamidation of nicotinamide have been discussed.

We wish to acknowledge the kind courtesy of Dr E. Kodicek who sent us the sample of nicotinuric acid and of Dr B. C. Johnson who generously provided the cultures of *Leuconostoc mesenteroides* and *Lactobacillus arabinosus* and

the ^{14}C -labelled nicotinic acid and nicotinamide used in this investigation. We are grateful to the Indian Council of Medical Research for financial assistance.

REFERENCES

- Cantoni, G. L. (1951). *J. biol. Chem.* **189**, 203.
 Chang, M. L. W. & Johnson, B. C. (1957). *J. biol. Chem.* **226**, 799.
 Ellinger, P. & Abdel Kader, M. M. (1949). *Biochem. J.* **44**, 77, 627.
 Halpern, Y. S. & Grossowicz, N. (1957). *Biochem. J.* **65**, 716.
 Hughes, D. E. & Williamson, D. H. (1953). *Biochem. J.* **55**, 851.
 Johnson, B. C. (1945). *J. biol. Chem.* **159**, 227.
 Jones, K. M. & Elliot, W. H. (1954). *Biochim. biophys. Acta*, **14**, 586.
 Krehl, W. A., De La Huerge, J., Elvehjem, C. A. & Hart, E. B. (1946). *J. biol. Chem.* **166**, 53.
 Lin, P. H. & Johnson, B. C. (1953). *J. Amer. chem. Soc.* **75**, 2974.
 Oka, Y. (1954). *J. Biochem., Tokyo*, **41**, 89, 101.
 Preiss, J. & Handler, P. (1958). *J. biol. Chem.* **233**, 488.
 Quagliariello, G., Gueritore, D. & Porcellati, G. (1952). *Abstr. 2nd int. Congr. Biochem., Paris*, p. 227.
 Rajagopalan, K. V., Sundaram, T. K. & Sarma, P. S. (1958). *Nature, Lond.*, **182**, 51.
 Reddi, K. K. & Kodicek, E. (1953). *Biochem. J.* **53**, 286.
 Sarma, P. S. (1943). *Indian J. med. Res.* **31**, 165.
 Schachter, D. & Taggart, J. V. (1954). *J. biol. Chem.* **208**, 263.
 Snell, E. E. (1951). Quoted by Gyorgy, P. in *Vitamin Methods*, p. 360. New York: Academic Press Inc.
 Sundaram, T. K., Radhakrishnamurty, R. & Sarma, P. S. (1954). *Curr. Sci.* **23**, 92.
 Sundaram, T. K., Rajagopalan, K. V. & Sarma, P. S. (1958). *Biochem. J.* **70**, 196.
 Sundaram, T. K., Rajagopalan, K. V. & Sarma, P. S. (1959). *J. Chromat.* **2**, 531.
 Sundaram, T. K. & Sarma, P. S. (1954). *Curr. Sci.* **23**, 398.
 Sundaram, T. K. & Sarma, P. S. (1956). *Biochim. biophys. Acta*, **22**, 547.

Glutathione Reductase of *Thiobacillus thio-oxidans**

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(Received 3 June 1959)

In the course of study of adenosine triphosphate formation from adenosine diphosphate by a cell-free extract of *Thiobacillus thio-oxidans* with hexokinase and glucose 6-phosphate dehydrogenase, the presence of reduced glutathione always resulted in the re-oxidation of reduced triphosphopyridine nucleotide formed. It was suspected that glutathione was oxidized to oxidized glutathione by the extract, and that a glutathione reductase present in the extract reduced it again, oxidizing reduced triphosphopyridine nucleotide (TPNH) to triphosphopyridine nucleotide (TPN) at the same time. The presence of glutathione reductase in this sulphur-oxidizing, chemoautotrophic bacterium was thought of interest in connexion with the mechanism of sulphur oxidation by the organism. Glutathione reductase was found in yeast (Meldrum & Tarr, 1935; Racker, 1955), plant tissues (Mapson & Goddard, 1951; Conn & Vennesland, 1951), mammalian tissues (Rall & Lehninger, 1952; Racker, 1955) and in *Escherichia coli* (Asnis, 1955). Oxidized glutathione (GSSG) is reduced to glutathione (GSH) according to the following equation:

A triphosphopyridine nucleotide-dependent glu-

tathione reductase was found in the extract of *T. thio-oxidans* and some characteristics of the enzyme were investigated.

MATERIALS AND METHODS

Reagents. GSSG was obtained from Nutritional Biochemicals Corp. TPNH, flavinadenine dinucleotide (FAD), riboflavin 5'-phosphate and *p*-chloromercuribenzoate were obtained from the California Foundation for Biochemical Research.

Organism. *Thiobacillus thio-oxidans*, obtained from the American Type Culture Collection, was grown in a mineral-salt medium in Fernbach flasks (Suzuki & Werkman, 1958a).

Enzyme preparation. The cell-free extract of *T. thio-oxidans* was prepared and dialysed according to Suzuki & Werkman (1958b). The extract contained 8.5 mg. of protein/ml., as determined by the biuret method (Layne, 1956). Acid treatment of the extract was carried out by dialysing 0.5 ml. of the extract in a collodion bag against six 150 ml. portions of 0.1 M-sodium acetate-HCl buffer, pH 3.0. The bag was connected to a slow-moving motor, and the dialysis was carried out at 4° for 3 hr. The bag was then suspended in 150 ml. of 0.02 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7.4, and the dialysis was continued for another hour.

Spectrophotometry. Glutathione reductase activity was measured spectrophotometrically after the oxidation of TPNH at 340 mμ. A Beckman DU spectrophotometer with 1 cm. cells was used.

* This work was supported in part by funds of the U.S. Public Health Service.