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.

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Glutathione Reductase of *Thiobacillus thio-oxidans**

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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.

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RESULTS

Demonstration of glutathione reductase in the extract. When TPNH was incubated with the cell-free extract of *T. thio-oxidans* in the presence of GSSG, there was a rapid oxidation of TPNH (Fig. 1). The cell-free extract was essential for the

Fig. 1. Demonstration of glutathione reductase activity. The complete system contained GSSG (4 mg.), TPNH (0.2 μ mole), the cell-free extract (0.1 ml.), tris, pH 7.4 (100 μ moles), and water to make a total volume of 3 ml. The oxidation of TPNH was followed spectrophotometrically at 340 m μ . O, Complete system; ▲, cell-free extract omitted; △, GSSG omitted.

Fig. 2. Effect of pH on the activity. Each system contained GSSG (4 mg.), TPNH (0.3 μ mole), the cell-free extract (0.1 ml.), a buffer (100 μ moles) and water to make a total volume of 3 ml. O, Potassium acid phthalate buffer; ●, tris buffer.

oxidation and the activity was easily destroyed by boiling for 2 min. at pH 7.4. TPNH was oxidized slowly by the extract in the absence of GSSG, but the rate was very low and the oxidation was negligible in comparison with the rapid oxidation in the presence of glutathione. L-Cystine in the same molar concentration did not replace GSSG. Reduced diphosphopyridine nucleotide (DPNH) was not oxidized by the extract in the presence of GSSG, even after the addition of phosphate. Thus the glutathione reductase of *T. thio-oxidans* is a TPN-linked enzyme and the presence of a DPN-linked glutathione reductase found in baker's yeast by Racker (1955) is excluded.

Effect of pH. The activity of the glutathione reductase was determined at various H⁺ ion concentrations and the results are shown in Fig. 2. The pH optimum was around 7.0 and was similar to the value reported for the glutathione reductase of *E. coli* (Asnis, 1955).

Sulphydryl nature of the enzyme. The activity of the extract of *T. thio-oxidans* was completely inhibited by 0.1 mM-*p*-chloromercuribenzoate and the inhibition was partially reversed by L-cysteine (Fig. 3). The result indicates dependence of the enzymic activity on sulphydryl groups.

Fig. 3. Inhibition of glutathione reductase by *p*-chloromercuribenzoate. The complete system contained GSSG (4 mg.), TPNH (0.3 μ mole), the cell-free extract (0.1 ml.), tris, pH 7.4 (100 μ moles), and water to make a total volume of 3 ml. x, Complete system; △, 0.3 μ mole of *p*-chloromercuribenzoate was added at zero time and 3 μ moles of cysteine were added after 10 min. O, 0.3 μ mole of *p*-chloromercuribenzoate was added at zero time and 30 μ moles of cysteine were added after 10 min.

Flavoprotein nature of the enzyme. FAD was shown to be the prosthetic group of the enzyme of *E. coli* (Asnis, 1955). Precipitation of the enzyme with ammonium sulphate at pH 0.6, the method Asnis used to dissociate the prosthetic group from the protein part, destroyed the activity of the extract of *T. thio-oxidans* completely, and the addition of FAD did not reactivate it. A moderate acid treatment, described in the previous section, was found satisfactory to show the nature of the prosthetic group of the enzyme. The activity of the acid-treated extract was stimulated by the addition of FAD, as shown in Fig. 4. Riboflavin 5'-phosphate did not stimulate the activity. The result suggests that the glutathione reductase of *T. thio-oxidans* is a flavoprotein with FAD as the prosthetic group. The activity of the extract dialysed at pH 7.4 instead of 3.0 was not stimulated by FAD.

Stimulation by phosphate. The glutathione reductase activity of an undialysed extract was not influenced by the addition of phosphate, but that of dialysed extracts was stimulated by the presence of phosphate ions both when dialysed at pH 3.0 and

at pH 7.4. The result with the extract dialysed at pH 3.0 is shown in Fig. 4. It is apparent from this figure that a rather high concentration of phosphate is required to stimulate the activity. Arsenate at the same concentration stimulated the activity to a lesser extent.

DISCUSSION

The glutathione reductase of *T. thio-oxidans* resembles in its pH dependence, substrate specificity, sulphhydryl nature and prosthetic group that of *E. coli* (Asnis, 1955), although the inhibition of the latter enzyme by 0.1 mM-*p*-chloromercuribenzoate could be completely reversed by mM-cysteine, whereas the former enzyme was only partially reactivated even by 10 mM-cysteine. Stimulation by phosphate was observed with a DPN-linked glutathione reductase of baker's yeast, but the activity linked with TPN was not stimulated by the addition of phosphate (Racker, 1955). Thus the stimulation of the TPN-linked glutathione reductase of *T. thio-oxidans* by the addition of phosphate indicates a difference between the enzymes of these two organisms.

Starkey (1937) observed a small amount of hydrogen sulphide produced from elemental sulphur by *T. thio-oxidans* and suggested that it was formed from sulphur by sulphhydryl groups present in the cells through a mechanism similar to that proposed by Sluiter (1930). Sluiter showed a reduction of elemental sulphur by GSH according to the following equation:

This non-enzymic reaction, coupled with glutathione reductase (equation 1) and a TPN-reducing system, will reduce elemental sulphur continuously to hydrogen sulphide. The ability of the cell-free extract of *T. thio-oxidans* to oxidize sulphide rapidly and its inability to attack elemental sulphur unless GSH is present (Suzuki & Werkman, 1959) supports this hypothesis that elemental sulphur is reduced to sulphide before oxidation by this organism. Glutathione reductase will play a major role in this hypothesis, supplying GSH from GSSG, although the nature of the system which could reduce TPN continuously is totally unknown and will remain unknown until the mechanism of energy transfer in this chemotrophic sulphur oxidation is elucidated.

SUMMARY

1. The presence of triphosphopyridine nucleotide-linked glutathione reductase was demonstrated in a cell-free extract of *Thiobacillus thio-oxidans*.

Fig. 4. Stimulation of the acid-treated extract by FAD and phosphate. Each system contained GSSG (4 mg.), TPNH (0.3 μmole), the acid-treated extract (0.05 ml.), tris, pH 7.4 (100 μmoles), and water to make a total volume of 3 ml. Additions were made as follows: x, none; □, 0.05 μmole of riboflavin 5'-phosphate; ▲, 0.05 μmole of FAD; ○, 0.05 μmole of FAD and 10 μmoles of phosphate (pH 7.4); Δ, 0.05 μmole of FAD and 50 μmoles of phosphate (pH 7.4); ●, 0.05 μmole of FAD and 50 μmoles of arsenate (pH 7.4).

2. The pH optimum was found to be near 7.0. The activity was completely inhibited by 0.1 mm-*p*-chloromercuribenzoate and the inhibition was partially reversed by L-cysteine. The activity of an acid-treated extract was stimulated by flavin-adenine dinucleotide, but not by riboflavin 5'-phosphate. Phosphate and, to a lesser extent, arsenate stimulated the enzymic action.

3. A possible role of the enzyme in the oxidation of elemental sulphur by the organism is discussed.

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A Comparative Study of the Seed Polyphenols of the Genus *Theobroma*

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The importance of the polyphenolic constituents of the cacao bean in determining the flavour character of processed cacao has long been recognized. Earlier studies have, however, been largely limited to the clone, Imperial College Selection 1 (Forsyth, 1952, 1955; Griffiths, 1958) and West African Amelonado cacao (Rohan, 1958). During the past 30 years a collection of varieties of *T. cacao* and related species has been established at the Imperial College of Tropical Agriculture and more recently plants of the related genus *Herrania* have also been established for studies on their suitability for hybridization. The study of the distribution of the polyphenols within this group of plants appeared desirable for several reasons.

First, it is important to establish whether the flavour of a clone or variety of cacao is related to the type and quantity of the polyphenols present. Since it has been suggested (Forsyth, 1951) that not only the astringency but also the chocolate-flavour principle derives from the phenolic constituents of the cacao bean, it was of interest to determine whether the characteristic polyphenols of cacao are restricted to the seeds of the species used in cocoa manufacture or whether they are also found in the seeds of related species which do not yield chocolate-flavoured products.

Secondly, the suitability of material for inter-specific crossing will largely depend upon the cotyledon containing similar phenolics to those of *T. cacao* and lacking phenolic constituents which would give rise to a decrease in palatability or to undesirable pharmacological properties.

Thirdly, it was of interest to observe the distribution pattern of polyphenols in relation to the taxonomy of the group. Although the phenolic constituents of leaves (Bate-Smith, 1957; Roberts, Wight & Wood, 1958) and woody tissues (Erdtman, 1958) have been extensively studied from the taxonomic viewpoint, the literature relating to the comparative study of the phenolics of seeds is sparse.

Mature seeds of the genus *Theobroma* are relatively large (1–3 cm. in length) and soft in texture. The cotyledons, which are white or purple, are closely invested by a fibrous testa to which mucilaginous material, the 'pulp' of the cacao bean, adheres. The ripe seeds of *Theobroma* spp. are particularly suitable for comparative studies since the relative proportions of the seed phenolics remains constant at maturity. Comparative studies on leaf material are complicated by the complex changes undergone by the phenolics during development and senescence (Griffiths, 1958).

EXPERIMENTAL

Source of plant material. All seeds employed in these studies were obtained from ripe pods harvested from trees growing at the Imperial College of Tropical Agriculture, Trinidad, B.W.I.

Extraction and separation. The polyphenols were extracted by homogenizing 5 g. of peeled cotyledons in 25 ml. of cold, absolute methanol or methanolic 1% HCl (where indicated), in a M.S.E. micro-blender for 5 min. A volume (0.05 ml.) of the filtered extract was then applied to the chromatogram (Whatman Chromatography Paper no. 1).