- Holt, A. & Wold, F. (1961). *J. biol. Chem.* **236**, 3227.
 Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 450. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Malmström, B. G. (1957). *Arch. Biochem. Biophys.* **70**, 58.
 Malmström, B. G. (1961). In *The Enzymes*, 2nd ed., vol. 5, p. 471. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
 Rosenberg, A. (1963). *Fed. Proc.* **22**, 346.
 Westhead, E. W. & Malmström, B. G. (1957). *J. biol. Chem.* **228**, 655.
 Wold, F. & Ballou, C. E. (1957a). *J. biol. Chem.* **227**, 301.
 Wold, F. & Ballou, C. E. (1957b). *J. biol. Chem.* **227**, 313.
 Wood, T. (1961). *J. Chromat.* **6**, 142.
 Wood, T. (1962). *Biochem. J.* **85**, 31 p.
 Wood, T. (1963). *Biochem. J.* **87**, 453.

Biochem. J. (1964) **91**, 460

The Reduction of Pantethine by an Extract of Camel Intestine

BY I. F. DURR AND N. CORTAS

Department of Biochemistry, School of Medicine, American University of Beirut, Beirut, Lebanon

(Received 21 October 1963)

Thompson, Bird & Peterson (1954) have reported that pantethine was as active as pantotheine in promoting chick growth when administered orally or intraperitoneally. This finding suggests that pantethine may be readily reduced to pantotheine. Since several mechanisms are known for the reduction of disulphides, it was decided to determine the nature of this reductive reaction.

The results presented below indicate that the reduction of pantethine occurs by the coupling of reactions (1) and (2):

Reaction (1) proceeds non-enzymically, the mercaptide ion of GSH being the reactive species. Reaction (2) is catalysed by glutathione reductase which has been partially purified from the small intestine of the dromedary camel.

MATERIALS AND METHODS

Chemicals. Pantethine, NADPH, NADP⁺, GSH, GSSG, cystine, oxidized 6,8-dimercapto-octanoic acid (α -lipoic acid) and tris were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. DEAE-cellulose (mesh no. 40) was obtained from the Brown Co., Berlin, N.H., U.S.A. Other reagents were purchased from E. Merck, Darmstadt, Germany.

Buffers. 1 M-Potassium phosphate buffer, pH 7.5, and 1 M-tris-HCl buffer were prepared as described by Gomori (1955). 0.1 M-Ammonium formate buffer, pH 5.5, was prepared by titrating 0.1 M-formic acid with 0.1 N-ammonia to pH 5.5 by using a glass electrode.

Preparation of diethylaminoethylcellulose. DEAE-cellulose (mesh no. 40) (100 g.) was suspended in 2.5 l. of distilled water. The suspension was adjusted to pH 7.5 by the addition of 130 ml. of 1.5 M-KH₂PO₄ and 150 ml. of N-KOH. The mixture was thoroughly stirred for 1 hr., then left for the sediment of DEAE-cellulose to form overnight.

The supernatant layer was decanted, and the slurry was then filtered under suction to remove residual buffer solution. The DEAE-cellulose was washed with 6 l. of distilled water and finally suspended in 2 l. of 1 mM-potassium phosphate buffer containing EDTA (1 mM). A 25 ml. portion of this suspension is equivalent to 1 g. of the dry DEAE-cellulose.

Determination of the concentrations of proteins, mercaptans and reduced nicotinamide-adenine dinucleotide phosphate. The concentration of proteins was determined spectrophotometrically (Warburg & Christian, 1941). Mercaptans were determined according to the method of Grunert & Phillips (1951), crystalline GSH being used to prepare the reference curve. The NADPH and NADH were assayed spectrophotometrically at 340 m μ assuming a molecular extinction coefficient of 6220.

Assay of enzymic activity. The reduction of disulphides was spectrophotometrically determined in the presence of NADH or NADPH by following the decrease in the extinction at 340 m μ at 30°. Each cuvette contained 2.4 μ -moles of the disulphide to be tested, 0.2–1 mg. of protein, 0.3 μ mole of NADPH or NADH and 100 μ moles of potassium phosphate buffer, pH 7.5, in a final volume of 2.9 ml. Readings were taken at 1 min. intervals. A specific activity of 1 represents the oxidation of 1 μ mole of NADPH or NADH/mg. of protein/min. under the above conditions of assay.

Preparation of the enzyme. (1) Preparation of extract. Unless otherwise indicated all operations were carried out at 7°. The small intestine (34 m.) of a freshly slaughtered camel *Camelus dromedarius* was cut into about 1 m. pieces opened lengthwise and thoroughly washed with chilled 0.1 M-KCl. The mucosa was scraped with a microscope slide and 980 g. of tissue was collected. When the mucosa was frozen at -10° the glutathione-reductase activity of the tissue was retained for at least 1 month. A portion (36 g.) of frozen mucosa was placed in a Waring Blender with 90 ml. of 0.1 M-KCl and blended at top speed for 2 min. Then another 90 ml. of 0.1 M-KCl was added and the suspension was further blended for 1 min. Centrifugation at 4000g for 20 min. yielded 193 ml. of a clear solution (9.45 mg. of protein/ml.).

Table 1. *Steps in the purification of glutathione reductase from camel intestine*

Experimental details are given in the Materials and Methods section.

Fraction	Total protein (mg.)	Specific activity	Total units
(1) Extract	1824	0.012	22
(2) From ammonium sulphate fractionation	561	0.05	28
(3) From chromatography on DEAE-cellulose			
(a) 45 ml.	372	0.06	22
(b) 10 ml.	16	0.28	4
(c) 20 ml.	64	0.12	7
(4) From rechromatography of (a) + (c) on DEAE-cellulose	110	0.17	19

(2) Ammonium sulphate fractionation. To each 100 ml. of the above extract 1 ml. of 0.2M-EDTA and 24.3 g. of finely powdered ammonium sulphate were gradually added with continuous stirring. Then 15 min. after the additions were complete the suspension was centrifuged at 14000g for 20 min. The precipitate was discarded and to each 100 ml. of the supernatant solution 28.5 g. of solid ammonium sulphate was slowly added and the suspension centrifuged as described above. The precipitate was collected and homogenized in a tight-fitting Potter-Elvehjem homogenizer for 30 sec. with 20 ml. of 1 mM-potassium phosphate buffer, pH 7.5, containing EDTA (1 mM). The solution was dialysed for 5 hr. against 4 l. of the same buffer. The dialysed solution (21 ml.) contained 26.7 mg. of protein/ml. This fraction could be stored unfrozen at 10° for 1 week without any apparent loss of glutathione-reductase activity.

(3) Chromatography on DEAE-cellulose. DEAE-cellulose suspension (150 ml.) was poured into a glass column (cross-sectional area 2 cm.²) and allowed to settle by gravity. Then 250 ml. of 1 mM-potassium phosphate buffer, pH 7.5, containing EDTA (1 mM), was passed through the column under a pressure equivalent to a water head of 1 m. A portion (20 ml.) of the ammonium sulphate fraction (26.7 mg. of protein/ml.) was diluted with the same buffer to 60 ml. and passed through the column at a flow rate of 2 ml./min. The first 30 ml. contained no protein and was discarded. The next 30 ml. was collected, and more buffer was added. Fractions were collected as indicated in Table 1. The fractions having a low specific activity were combined and rechromatographed on a new column of the same dimensions. The first 35 ml. was discarded and buffer was passed through the column to collect another 45 ml. Table 1 shows that up to 23-fold purification was obtained.

RESULTS

Reduction of pantethine. Table 2 shows that pantethine was reduced by the dialysed crude extract of the intestinal mucosa. It is also evident that dialysis did not completely remove the endogenous thiols. Further, Table 2 shows that the extract had an active glutathione reductase. In view of the ubiquitous distribution of GSH and the reductase, as well as their role in the reduction of disulphides (Pihl, Eldjarn & Bremer, 1957), it was

Table 2. *Reduction of pantethine and oxidized glutathione by an extract from camel intestine*

The complete reaction mixture contained 5 mg. of protein (crude extract), 2 μ moles of either pantethine or GSSG as indicated, 0.79 μ mole of NADPH, and 100 μ moles of tris buffer, pH 8, in a final volume of 1.6 ml. Incubation was for 30 min. at 37°. The crude extract (10 ml.) was dialysed for 20 hr. against 2 $\times$ 4 l. of water before it was used in this experiment.

Reaction mixture	Thiol liberated (μ moles)
Complete system with pantethine	0.44
NADPH omitted	0.25
Substrate (pantethine or GSSG) omitted	0.21
Complete system with GSSG	1.47

decided to determine whether GSH was involved in the reduction of pantethine.

Purification of the crude extract failed to show a protein fraction capable of reducing pantethine in the presence of either NADH or NADPH. However, as shown in Fig. 1 (curve 1), the pantethine-dependent oxidation of NADPH occurs in the presence of GSH and glutathione reductase. Further consideration of Fig. 1 (curve 1) shows a lag before pantethine accelerated the rate of NADPH oxidation. This lag is expected if a preliminary interaction between GSH and pantethine occurs to produce GSSG (reaction 1). Preincubation of GSH and pantethine completely eliminated the lag and greatly increased the rate of NADPH oxidation, as indicated in Fig. 1 (curve 2). The observed oxidation of NADPH in the presence of GSH alone may be due to a slight contamination of GSH with GSSG. Neither cysteine nor reduced α -lipoic acid replaced GSH in the reduction of pantethine.

Non-enzymic occurrence of reaction (1). To determine whether reaction (1) was catalysed by an enzyme, pantethine and GSH were preincubated with and without the crude extract. In addition all the protein fractions obtained during purification were also tested. As shown in Fig. 1 (curves 2

and 3) no significant difference in the rate of NADPH oxidation was observed. The slower rate of the reaction when the enzyme was preincubated with pantethine and GSH shown in Fig. 1 (curve 3) was the result of a slight inactivation of glutathione reductase. When this enzyme was assayed under the conditions of the preincubation experiment it lost 27% of its activity.

Participation of mercaptide ions in reaction (1). Fig. 2 shows the effect of pH on the rate of pantethine reduction as measured by the oxidation of NADPH in the presence of GSH and glutathione reductase. At pH 5.5 the rate of the reaction was equal to that of the control even when pantethine was preincubated with GSH. However, at pH 8 the overall rate of pantethine reduction was greatly increased, suggesting that the reactive species in reaction (1) is the mercaptide ion of GSH.

Effect of pantethine:reduced glutathione ratio on reaction (1). Eldjarn & Pihl (1957) have demonstrated that thiol-disulphide interactions (e.g. reaction 1) proceed in two steps, with the equilibrium of both reactions being in favour of the intermediate mixed disulphide. Consequently, at a constant concentration of GSH, increasing the concentration of pantethine will eventually decrease the overall reduction of pantethine, since GSH will be mainly bound as a mixed disulphide with pantethine. This is borne out by the results of the following experiment. A reaction mixture was

Fig. 1. Pantethine-GSH interaction. The test cuvette contained 0.7 mg. of enzyme, 7.2 μ moles of pantethine, 6 μ moles of GSH, 0.25 μ mole of NADPH and 100 μ moles of phosphate buffer, pH 7.5, in a final volume of 3 ml. The blank cuvette contained everything except NADPH. The control cuvette contained everything except pantethine. Corrections for dilutions were made. Readings were recorded at 1 min. intervals. $\circ$, Curve 1, no preincubation; GSH and pantethine were added at points A and B respectively. $\triangle$, Curve 2, preincubation without enzyme; enzyme was added at point C. $\bullet$, Control for curve 2. $\blacksquare$, Curve 3, preincubation with enzyme; at point D, NADPH was added. $\blacktriangle$, Control for curve 3. Preincubations (where done) were for 15 min. at 30°.

prepared containing 3.6 μ moles of GSH, 8 mg. of protein, 0.3 μ mole of NADPH and 100 μ moles of potassium phosphate buffer, pH 7.5, in a final volume of 2.9 ml. When 3.6 μ moles of pantethine were added to this reaction mixture, 0.124 μ mole of pantethine was reduced/min. However, the addition of 7.2 μ moles of pantethine to a similar reaction mixture resulted in the reduction of only 0.079 μ mole of pantethine/min. The rate of reduction was calculated from the initial ΔE_{340} value during the first minute after the addition of pantethine.

Properties of intestinal glutathione reductase.

(a) Substrate specificity. Of the disulphides tested, namely, GSSG, cystine, pantethine and oxidized lipoic acid, only GSSG was active. NADPH was required as a cofactor, and could not be replaced by NADH.

(b) Stoichiometry and reversibility of the reaction. Table 3 indicates that for every mole of NADPH oxidized 2 moles of GSH are produced. Attempts to demonstrate the reversibility of the reaction were not successful. Under different conditions of temperature, pH and concentrations of

Fig. 2. Dependence of pantethine-GSH interaction on pH. The test cuvette contained 0.7 mg. of enzyme, 7.2 μ moles of pantethine, 6 μ moles of GSH, 0.25 μ mole of NADPH and buffer as indicated below, in a final volume of 3 ml. NADPH and pantethine were omitted from the blank and the control cuvettes respectively. The reaction mixture was preincubated for 15 min. at 30°. Subsequently the enzyme was added at point A. Corrections for dilutions were made, and readings were recorded at 1 min. intervals. $\triangle$, Test cuvette, 100 μ moles of ammonium formate buffer, pH 5.5. $\blacktriangle$, Control cuvette for pH 5.5 test. $\circ$, Test cuvette, 100 μ moles of tris-HCl buffer, pH 8.0. $\bullet$, Control cuvette for pH 8.0 test.

Table 3. *Stoichiometry of reduction of oxidized glutathione by an extract of camel intestine*

The complete reaction mixture contained 6.5 mg. of enzyme, 100 μ moles of tris buffer, pH 8, 2.1 μ moles of GSSG and 1.1 μ moles of NADPH, in a final volume of 2.1 ml. Incubation was for 30 min. at 37°. Positive values indicate formation and negative values indicate disappearance. Samples for the determination of thiols and NADPH were taken at zero time and 30 min. later.

Reaction mixture	Δ GSH (μ moles)	Δ NADPH (μ moles)
Complete system	+1.96	-1.08
GSSG omitted	+0.02	-0.09
NADPH omitted	+0.04	—

GSH and NADP⁺, neither the reduction of NADP⁺ nor the disappearance of GSH was observed.

(c) Optimum pH. The enzyme was active between pH 5.5 and 8. The rates of GSSG reduction at pH 5.5 and at pH 8 were 72 % and 38 % respectively of the rate of reduction at pH 7.5.

DISCUSSION

The present results indicate that pantethine is reduced by the coupling of reactions (1) and (2). Reaction (1) involves a non-enzymic exchange between pantethine and GSH. No specific pantethine reductase was found. Reaction (2) is catalysed by glutathione reductase.

Although the thermodynamics of the thiol-disulphide exchange reactions do not allow a complete reduction of the disulphide (Pihl & Eldjarn, 1958), nevertheless the complete reduction of pantethine is possible in the presence of glutathione reductase and catalytic amounts of GSH. It was shown that the overall rate of pantethine reduction expressed in terms of GSH formed was 0.12 μ mole/min. at pH 7.5. Assuming that GSH has a pK_{SH} of about 9 (Martin & Edsall, 1958), then approx. 2 % of GSH will be in the form of the mercaptide ion, GS⁻. Under more alkaline conditions the rate of pantethine reduction is expected to increase as the concentration of GS⁻ rises. The effect of pH on pantethine reduction was shown (Fig. 2). Whereas at pH 5.5 the reaction was completely arrested, significant reduction occurred at pH 8.0. The lack of exchange at pH 5.5 was not limited by the action of glutathione reductase. In fact this enzyme is almost twice as active at pH 5.5 as at pH 8.0. Under comparable conditions pantethine apparently reacts with GSH more rapidly than the disulphides reported by Pihl *et al.* (1957). For example, it can be calculated from their data that, of the fast-reacting disulphides,

NNN'N'-tetraethylecystamine reacted with GSH at a rate of 0.04 μ mole/min., which is one-third of the rate obtained with pantethine.

The general properties of intestinal glutathione reductase appear to be similar to those of the enzyme obtained from other sources. However, whereas the intestinal enzyme is twice as active at pH 5.5 as at pH 8, the reverse is true of hepatic glutathione reductase (Mize & Langdon, 1962). Our studies also indicate that the reaction is essentially irreversible. However, Mapson & Isherwood (1963) could demonstrate the slight reversibility of GSSG reduction only by the application of fluorimetric analysis.

SUMMARY

1. The reduction of pantethine occurs by a coupling of two reactions. The first reaction is a non-enzymic exchange between pantethine and GSH to produce pantetheine and GSSG. The second reaction involves a regeneration of GSH from GSSG by glutathione reductase.

2. The pantethine-GSH exchange is dependent on alkaline pH, suggesting that the mercaptide ion, GS⁻, is the reactive species of GSH.

3. Glutathione reductase has been partially purified from the intestinal mucosa of the camel. The enzyme is specific to GSSG and NADPH. Reversibility could not be demonstrated.

This investigation was supported by a grant from the Medical Research Committee of the American University of Beirut. N.C. was supported by a grant from the Anna Fuller Foundation to medical students. We thank Dr U. Khalidi for suggesting the experimental animal.

REFERENCES

- Eldjarn, L. & Pihl, A. (1957). *J. biol. Chem.* **225**, 499.
- Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 138. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Grunert, R. & Phillips, P. (1951). *Arch. Biochem. Biophys.* **30**, 217.
- Mapson, L. W. & Isherwood, F. A. (1963). *Biochem. J.* **86**, 173.
- Martin, R. B. & Edsall, J. T. (1958). *Bull. Soc. Chim. biol., Paris*, **40**, 1763.
- Mize, C. E. & Langdon, R. G. (1962). *J. biol. Chem.* **237**, 1589.
- Pihl, A. & Eldjarn, L. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, p. 43. Ed. by Hoffmann-Ostenhof, O. London: Pergamon Press Ltd.
- Pihl, A., Eldjarn, L. & Bremer, J. (1957). *J. biol. Chem.* **227**, 339.
- Thompson, R. Q., Bird, O. D. & Peterson, F. E. (1954). *J. Nutr.* **53**, 365.
- Warburg, O. & Christian, W. (1941). *Biochem. J.* **310**, 384.