and an accumulation of fat. Methionine levels are unaffected. Depletion of liver glycogen is pronounced and lasting and vitamin B_{12} apparently does not protect against this metabolic disturbance.

4. Protection by vitamin B_{12} is non-specific and may be due to an effect on the maintenance of mitochondrial integrity.

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Biosynthesis of Glutathione by Rat Erythrocytes

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Studies on the turnover of reduced glutathione in the blood of several species *in vivo* have indicated that this tripeptide is in a dynamic state (Burwell, Brickley & Finch, 1953; Dimant, Landsberg & London, 1955; Grinstein, Kamen & Moore, 1949; Jope, 1946; London, Shemin, West & Rittenberg, 1949; Mortensen, Haley & Elder, 1956; Shemin & Rittenberg, 1946). The half-life of glutathione, as determined from a disappearance of the label in glutathione after a single injection of [^{15}N]glycine, has been shown to be much shorter in comparison with the time for 50% survival of the erythrocytes, as evaluated from the ^{15}N concentration in haemin (Dimant *et al.* 1955). These observations raised speculation about the origin of glutathione in mammalian erythrocytes.

Anderson & Mosher (1951) detected ^{35}S in erythrocyte glutathione after administration of

labelled cystine to rats by stomach intubation and suggested that glutathione is synthesized in the liver and subsequently incorporated into erythrocytes during the circulation of blood through the liver. Glutathione in mammalian blood, however, is known to be exclusively in the erythrocytes (Ling & Chow, 1953; Woodward & Fry, 1932) and leucocytes (McKinney, 1953); there is no measurable amount of glutathione present in plasma, nor is there any evidence for the permeability of the erythrocyte membrane to glutathione of the surrounding medium. The suggested hepatic origin for erythrocyte glutathione is therefore unlikely. Other workers have suggested that glutathione may be formed in the immature cells, and that it may subsequently undergo turnover in the circulating erythrocytes as a result of exchange of one or more of the constituents of glutathione (Lemberg & Legge, 1949). However, the variation in glutathione levels of erythrocytes after supplementation or withdrawal of dietary methionine (Mortensen, 1953) strongly suggested the possibility of synthesis of glutathione afresh in erythrocytes.

Incorporation of labelled glycine into human and duck erythrocytes has been demonstrated *in vitro* by Dimant *et al.* (1955). Elder & Mortensen (1956) made similar observations with rat erythrocytes. However, mere incorporation of the label into glutathione is no direct evidence for synthesis of this metabolite afresh, as has already been suggested by these workers; the label in glutathione could arise entirely by an exchange of the component amino acids with preformed glutathione. This paper includes more direct evidence for the presence in rat erythrocytes of an enzyme system which catalyses the synthesis of glutathione from its constituent amino acids. An attempt has been made to determine the conditions required for maximal activity of the enzyme(s). Finally, the effect of folic acid and vitamin B_{12} deficiency on synthesis of glutathione *in vitro* has been investigated.

EXPERIMENTAL

Albino rats (Wistar strain) weighing 200–250 g. and fed on a laboratory stock diet (Kasbekar, Lavate, Rege & Sreenivasan, 1959) were used for the studies on glutathione synthesis.

Folic acid-deficient animals were obtained as follows (Fatterpaker, Marfatia & Sreenivasan, 1955): young growing rats, 80–100 g., were fed on the following synthetic diet (in percentages): ethanol-extracted casein 15, sesame oil 6, vitamin A preparation 1·6 (5000 i.u.), salt mixture (U.S. Pharmacopoeia IV) 4·0, sucrose 9·0, vitamin mixture 1·0 and starch to 100. The diet contained (mg./kg.) the following vitamins: thiamine hydrochloride 6, riboflavin 10, nicotinic acid 30, pyridoxine hydrochloride 6, calcium pantothenate 20, biotin 1, *p*-aminobenzoic acid 100, inositol 500, choline chloride 500, vitamin K 10, vitamin E 50 and vitamin B_{12} 0·150. Iodinated casein (0·15%) was incorporated into the diet during the first 10 days to deplete the animals of their folic acid reserves. After this period, succinyl sulphathiazole (1·5%) was substituted for iodinated casein for about 5 weeks until the animals showed signs of folic acid deficiency, including reduced blood-cell counts. The folic acid-supplemented group was fed on an identical diet supplemented with 20 mg. of folic acid/kg. of diet.

Vitamin B_{12} deficiency was produced similarly by omission of the vitamin in the basal diet and substitution of folic acid (5 mg./kg.). Vitamin B_{12}-protected animals received intraperitoneally 10 μg. of the vitamin/animal every alternate day.

Blood was obtained from the animals by venepuncture under ether anaesthesia. It was immediately heparinized to prevent clotting. Incubation was carried out either with whole blood or with separated erythrocytes; for the latter, the blood was immediately centrifuged at 8500 *g* for 15 min. in an International refrigerated centrifuge, and the plasma and whitish buffy coat covering the erythrocyte sediment were discarded. The cells were finally washed and dispersed in the same suspension media as were used for incubation.

Assay of glutathione. All incubations were carried out in Warburg vessels at 37° in a final volume of 4 ml. of isotonic media (composition described in Results). At the beginning as well as at the end of incubation for 3 hr., unless otherwise stated, portions (1 ml.) were withdrawn, the cells were centrifuged at 8500 *g* and washed with suspension medium until the washings were free of cysteine, as determined by the nitroprusside colour reaction. This was essential to eliminate the small interference by cysteine. To the cell sediment was then added 4 ml. of water, and haemolysis was allowed to proceed to completion at 0°. The haemolysate was deproteinized with 1 ml. of 25% metaphosphoric acid. Sufficient sodium chloride (B.P. quality) was added to saturate the solution. The resulting protein precipitate was centrifuged and portions (1 ml.) of the clear supernatant were used for determinations of glutathione by the alloxan '305' method (Kasbekar *et al.* 1959). The difference between the initial and final values was taken as a measure of net synthesis of glutathione by the cells.

In each case, the cell count of the incubation mixture was determined on a haemocytometer after the usual dilutions with Hayem's solution. Since the glutathione in blood is entirely in the cells, the values are expressed as μg. of glutathione synthesized in 3 hr./flask and calculated to 10^6 cells/mm.^{3} of medium.

RESULTS

In preliminary experiments, whole blood (0·5 ml.) or erythrocytes (equivalent volume) separated by centrifuging were incubated in isotonic saline (0·154 M) containing 100 mg. of glucose/100 ml., both aerobically (air as the gas phase) and anaerobically (nitrogen as the gas phase) for 3 hr. at 37° in a Warburg assembly. The system consisted of 0·025 M-glycine, 0·01 M-glutamate and 0·01 M-cysteine hydrochloride, dissolved in sodium chloride solution and adjusted to pH 7·4, which was also the pH of the incubation mixture. Total volume was 4 ml. The results are given in Table 1.

No synthesis of glutathione was observed with whole blood under aerobic conditions, and with erythrocytes the synthesis was small. It is possible that the synthesis is masked by destruction of reduced glutathione in the presence of air (Lyman & Barron, 1937). Under anaerobic conditions a definite though small synthesis was evident. There was a slight inhibition of synthesis with whole blood; similar inhibition in the incorporation of labelled glycine into erythrocyte glutathione in the presence of plasma has been reported by Elder & Mortensen (1956). These observations are, however, in contrast with the accelerating effect of plasma on incorporation of amino acid into reticulocyte proteins (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1952).

Effect of potassium and magnesium ions on formation of glutathione. A distinct stimulation of the conjugating ability was observed with Krebs–Ringer solution (Umbreit, Burris & Stauffer, 1949) as the incubation medium (Table 2). Omitting either sodium chloride or calcium chloride had no effect on glutathione formation, but there was a sharp decline in the synthetic activity when either potassium chloride or magnesium sulphate was omitted.

The composition of Krebs–Ringer solution was therefore modified so as to contain 100 parts of KCl (0·154 M), 7 parts of $MgSO_4,7H_2O$ (0·154 M) and 1 part of KH_2PO_4 (0·154 M). A fivefold concentrated stock solution was prepared; 20 ml. of this was diluted to 100 ml. together with 10 ml. of 0·1 M-phosphate buffer, pH 7·4, and used in subsequent experiments. Comparative data obtained with the original and modified Krebs–Ringer solutions on the synthesis of glutathione are given in Table 3.

Effect of pH. The effect of pH on glutathione synthesis by erythrocytes was studied by incubating the cells in the modified Krebs–Ringer solution containing 0·1 M-phosphate buffers from pH 5·8 to 7·8. The cells were isolated and washed with the respective suspension medium. The amino acids were dissolved in the Krebs–Ringer solution and their final H^+ ion concentrations adjusted to those of incubation mixtures. A fairly sharp pH maximum is observed at pH 6·8 (Fig. 1).

Table 1. *Glutathione synthesis by erythrocytes incubated in sodium chloride solution*

Whole blood (0·5 ml.) or equivalent of erythrocytes was incubated for 3 hr. in isotonic (0·154 M) NaCl soln. containing 0·025 M-glycine, 0·01 M-glutamate and 0·01 M-cysteine hydrochloride. Total volume was 4 ml. and the pH was adjusted to 7·4. Glutathione values (ranges in four experiments) are per flask, calculated to 10^6 cells/mm.[3]

	Glutathione synthesized (μg.)	
	Aerobic	Anaerobic
Whole blood	Negligible	12·5–16·5
Erythrocytes	<7	16·0–21·5

Table 2. *Effect of potassium and magnesium ions on glutathione synthesis by rat erythrocytes*

For details, see Table 1 and text. Krebs–Ringer solution was used instead of isotonic (0·154 M) NaCl soln. and incubation was under anaerobic conditions.

Composition of suspension medium	Glutathione synthesized (μg.)
Krebs–Ringer solution	29·0–34·0
NaCl omitted	32·5–37·5
$CaCl_2$ omitted	29·5–35·0
KCl and KH_2PO_4 omitted	21·0–24·5
$MgSO_4$ omitted	17·5–22·0

Table 3. *Effect of modified Krebs–Ringer solution on glutathione synthesis*

For details see Table 2 and text. The modified Krebs–Ringer phosphate soln. contained only KCl, KH_2PO_4 and $MgSO_4$ together with phosphate buffer, pH 7·4.

	Glutathione synthesized (μg.)
Original Krebs–Ringer soln.	29·5–33·5
Modified Krebs–Ringer phosphate soln.	39·0–42·5

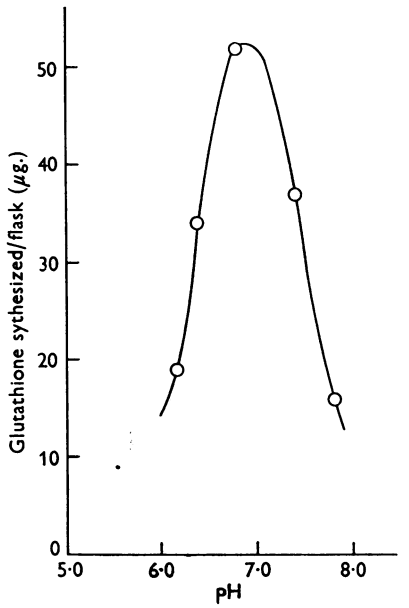

Fig. 1. Effect of pH on synthesis of glutathione. Incubation conditions were as described in Table 3 with modified Krebs–Ringer phosphate solution with 0·1 M-phosphate buffers of appropriate pH.

Effect of concentration of amino acids. Fig. 2 shows the effect of amino acid concentrations on glutathione synthesis. The experiments were planned by varying the concentration of one amino acid while those of the other two were kept constant. Thus while the influence of cysteine concentration was ascertained the concentrations of glutamate and glycine were maintained at 0·01 and 0·025 M respectively. No glutathione appeared in the incubation mixture in the absence of cysteine. Maximal synthesis of glutathione was found to occur with 0·03 M-cysteine; this concentration was maintained in subsequent studies.

About 30% of the glutathione that could be synthesized was formed in the absence of glutamate, suggesting either the presence of endogenous glutamate in the erythrocytes or its formation during incubation. In general, glutamate concentrations did not appear to influence the synthesis as much as did cysteine. Maximal synthesis occurred with 0·03 M-glutamate.

With 0·03 M-concentrations each of glutamate and cysteine, the rate of glutathione synthesis was

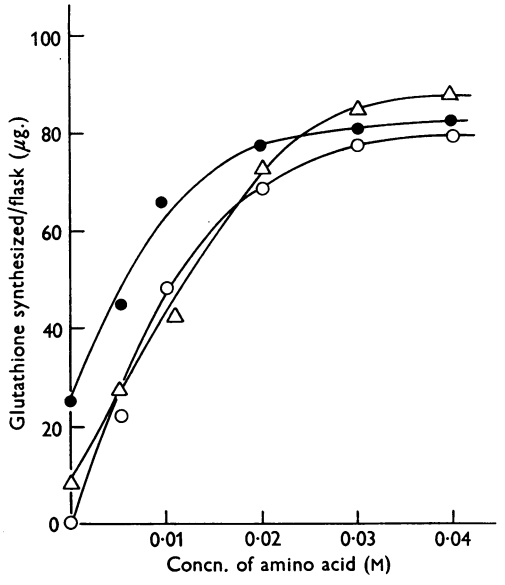

Fig. 2. Effect of amino acid concentration on synthesis of glutathione. For conditions of incubation see Table 3 and text. The concentrations of glutamate and glycine were 0·01 and 0·025 M respectively when cysteine concentration was varied (○). The concentrations of cysteine and glycine were 0·03 and 0·025 M respectively when glutamate concentration was varied (●). The concentrations of cysteine and glutamate were 0·03 M each when glycine concentration was varied (△).

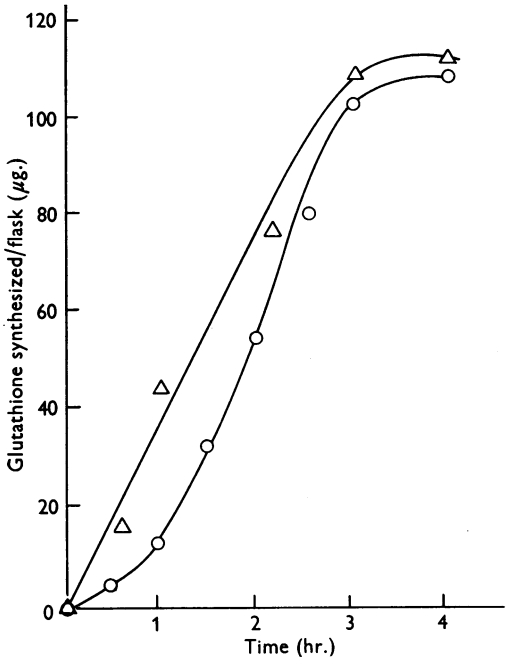

Fig. 3. Rate of glutathione synthesis. The system contained (each 0·03 M) cysteine, glutamic acid and glycine, 8 μmoles of adenosine monophosphate and erythrocytes equivalent to 0·5 ml. of blood in the modified Krebs–Ringer phosphate solution (see Table 3 and text), pH 6·8. Final volume: 4 ml. Incubations were at 37°, anaerobically. The pre-incubation with glutamic acid and cysteine was for 1 hr. △, With free amino acids; ○, pre-incubated with glutamic acid and cysteine.

Table 4. *Effect of adenosine and adenine nucleotides on glutathione synthesis by erythrocytes*

Incubations were for 3 hr. in modified Krebs–Ringer phosphate medium (see Table 3 and text), pH 6·8. Concentrations of cysteine, glutamic acid and glycine were 0·03 M each. With no additions to the system 89·5 μg. of glutathione was synthesized.

Additions	Glutathione synthesized (μg.)					
Amount added/flask (μmoles) ...	2	4	6	8	10	70
Adenosine	94	99	107	111	114	115
Adenosine monophosphate	99	110	116	120	*	*
Adenosine diphosphate	91	92	*	*	*	*
Adenosine triphosphate	*	*	*	*	*	*

* Values were not determined owing to haemolysis of cells.

linear with respect to glycine up to 0·02 M. Very little glutathione was formed in the absence of glycine. With 0·03 M-glycine maximum synthesis of glutathione was observed. Since the level of enzyme(s) in the incubation mixture was maintained fairly constant by limiting the number of cells/mm.^{3} to between 1 and 2×10^6, the saturation of the enzyme by one or more amino acids at 0·03 M-concentration appears to limit further synthesis of glutathione.

Effect of adenine nucleotides. Since the formation of peptidic bonds requires energy (Cohen & McGilvery, 1946; Elliott, 1948; Lipman, 1945; McGilvery & Cohen, 1950; Speck, 1947) and involves participation of adenylic system, it was of interest to study their effect on glutathione synthesis. The results given in Table 4 indicate that both adenosine and adenosine 5′-monophosphate enhanced glutathione synthesis considerably. Whereas large quantities of adenosine had little effect on the synthesis, adenosine 5′-monophosphate in concentration higher than 8–10 μmoles/flask caused haemolysis of the erythrocytes. Adenosine diphosphate and adenosine triphosphate caused irregular haemolysis even at low concentrations.

Rate of glutathione synthesis. Fig. 3 shows a typical progress curve of the reaction. The system contained (each 0·03 M) glycine, cysteine and glutamate, and 8 μmoles of adenosine 5′-monophosphate in modified Krebs–Ringer phosphate solution, pH 6·8. The erythrocytes were incubated anaerobically at 37°. An initial induction period similar to that reported by Snoke, Yanari & Bloch (1953) in the soluble extracts of acetone preparations of pigeon livers was observed when the synthesis was studied with free amino acids. A lag phase in the incorporation of labelled glycine into erythrocyte glutathione has also been reported by Elder & Mortensen (1956). Glutathione appears to be formed considerably faster from glutamylcysteine and glycine than from the free amino acids, with acetone preparations of livers. These findings indicate a direct utilization of glutamylcysteine and suggest that the induction period in glutathione synthesis from the free amino acids reflects consecutive reactions of which glutamylcysteine formation is the first. This interpretation is supported by the fact that if the addition of glycine is delayed until glutamic acid and cysteine have been allowed to react by pre-incubation for 1 hr., the induction period disappears and the rate curve becomes linear. In either case, the rate of synthesis declines after 3 hr. and may be due to a change of pH, lack of high-energy phosphate generation, a gradual inactivation of the enzyme or a combination of all these factors.

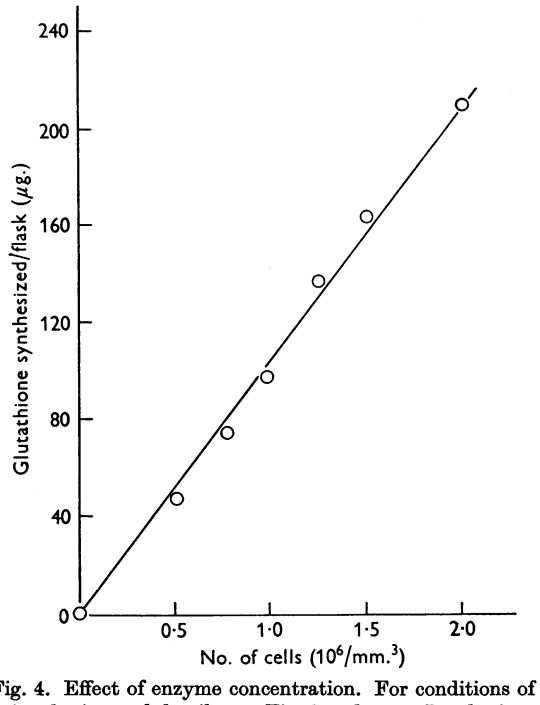

Fig. 4. Effect of enzyme concentration. For conditions of incubation and details, see Fig. 3 and text. Incubations were for 3 hr.

Fig. 4 shows that the amount of glutathione formed is proportional, within experimental error, to the number of cells present.

Inhibition of synthesis by fluoride. Fluoride in a final concentration of 0·015 M or more completely inhibits glutathione synthesis (Fig. 5), a result which would be obtained if peptide-bond formation was dependent on the energy derived from glycolytic reactions.

The inhibition of glutathione biosynthesis by plasma was confirmed under conditions which have yielded maximal glutathione. Thus the amount of glutathione synthesized in 3 hr. under nitrogen in the Krebs–Ringer phosphate medium was lowered, on addition of plasma, from the normal range of 119–130 to 95–101 μg./flask. The corresponding values under aerobic conditions were 56–68 and 51–59 μg./flask respectively.

Effects of folic acid and vitamin B_{12} deficiencies. Erythrocytes isolated from the deficient and protected animals were incubated with modified Krebs–Ringer solution containing each of the constituent amino acid (each 0·03 M) and 8 μmoles of adenosine 5′-monophosphate anaerobically for 3 hr. Preliminary observations indicate that folic acid deficiency apparently did not influence glutathione formation *in vitro* (Table 5). Even though there was a small but significant lowering of glutathione synthesis by erythrocytes from hyperthyroid animals not receiving vitamin B_{12}, no

definite effect of this vitamin in the system could be recorded because of an observed slight haemolysis.

DISCUSSION

The work of Johnston & Bloch (1951) on glutathione synthesis by fresh-liver homogenates and soluble fractions of acetone-dried pigeon-liver powders has shown a stimulation of synthesis in the presence of K^+ and Mg^{2+} ions. Since Krebs–Ringer solution is so constituted as to closely approximate the ionic composition of mammalian serum, thus providing a near-physiological extracellular environment, and since it also contains K^+ and Mg^{2+} ions, it was of interest to find out its effect on glutathione synthesis by erythrocytes.

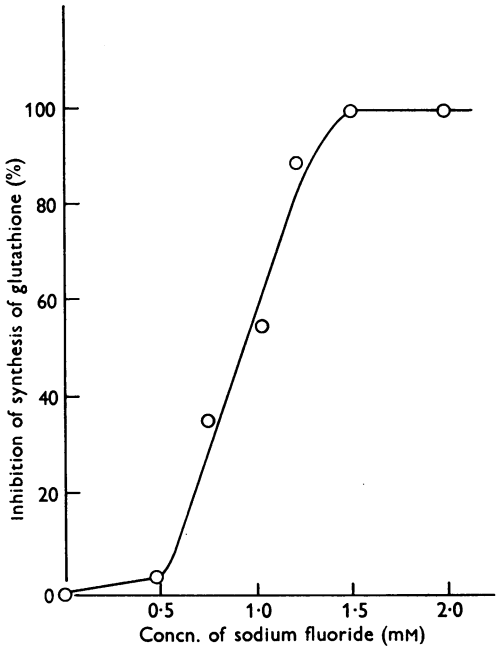

Fig. 5. Effect of fluoride. Incubations were for 3 hr. under the conditions described in Fig. 3 with additions of sodium fluoride in final concentrations as shown.

Table 5. *Effect of folic acid and vitamin B_{12} deficiencies on glutathione synthesis*

Incubations were for 3 hr. anaerobically under conditions described for Fig. 3 with free amino acids. Erythrocytes were from folic acid- or vitamin B_{12}-deficient animals and corresponding controls. For methods of induction of deficiencies, see text.

Group	Glutathione synthesized (μg.)
Fed with stock diet	119±7
Folic acid-deficient	115±3
Folic acid-fed	110±6
Vitamin B_{12}-deficient	87±10
Vitamin B_{12}-fed	114±4

The enhanced synthesis of glutathione in the presence of K^+ and Mg^{2+} ions appears to be a combined effect of these ions on glycolysis and peptide-bond synthesis. The effect of K^+ ion is of particular interest since decrease in blood glutathione has been reported in potassium deficiency by Grunert & Phillips (1951). Johnston & Bloch (1951) have demonstrated that K^+ ions specifically activate glutathione formation with purified liver-enzyme preparations when adenosine triphosphate is used as a source of energy for peptide-bond synthesis. Steward & Preston (1941) also reported that added K^+ ions increase the rate of protein synthesis in plant tissues and suggested a direct effect of these ions on the formation of peptide bonds. The synthesis of the peptide bond in pantothenic acid in bacteria has also been shown to be enhanced by K^+ ions (Mass, 1952).

The demonstration of the protective effects of adenosine and adenylic acid (muscle) (Gabrio, Donohue & Finch, 1955; Gabrio, Hennessey, Thomasson & Finch, 1955; Harris & Prankerd, 1955; Klebanoff, 1957; Overgaard-Hansen, Jorgenson & Praetbrius, 1957; Prankerd, 1956; Prankerd & Altman, 1954) against changes occurring *in vitro* during the storage of blood or ageing of erythrocytes at 37° for short periods of time, e.g. decreased utilization of glucose, loss of organic phosphates and a rapid breakdown of intracellular adenosine triphosphate to adenosine diphosphate, adenosine 5′-monophosphate and hypoxanthine, afforded additional reason for a study of the effect of these factors. The presence of adenosine has been demonstrated to lead to the expulsion by erythrocytes of sodium and to the maintenance of intracellular potassium levels (Gabrio, Donohue & Finch, 1955; Harris & Prankerd, 1955). The results given in Table 4 indicate that both adenosine and adenosine 5′-monophosphate enhanced glutathione synthesis considerably.

The observed stimulation of glutathione synthesis in the presence of adenosine or adenosine 5′-monophosphate may be due to the maintenance of organic phosphate levels of erythrocytes, increased consumption of glucose and therefore of increased regeneration of adenosine triphosphate for peptide-bond synthesis.

Fig. 5 indicates the inhibitory effect of fluoride on the synthesis of glutathione by erythrocytes. A similar inhibition of glutathione formation by soluble extracts of acetone preparations from pigeon liver by fluoride has been reported by Johnston & Bloch (1951); the inhibition was observed only when glycolytic intermediates other than phosphopyruvic acid served as energy source (Snoke *et al.* 1953), indicating a specific inhibitory effect of fluoride on glycolysis. The formation of glutamine from glutamate and ammonia is also inhibited by

fluoride when a glycolytic intermediate is used as a sparking system (Speck, 1949).

Since erythrocyte glutathione is low in thyrotoxic rat blood (Kasbekar *et al.* 1959) the observed haemolysis of erythrocytes from hyperthyroid animals after incubation is of particular interest in considering the possible role of glutathione in maintaining the structural integrity of erythrocytes. Similar observations which tend to relate glutathione and sulphydryl groups to haemolysis have been reported in studies with horse erythrocytes by Fegler (1952), who found that exposure of the cells to oxygen or iodine resulted in an increased rate of haemolysis, and that the rate of haemolysis increased rapidly after the glutathione content of the cells fell below 40% of the initial value. Benesch & Benesch (1954) observed that the haemolytic effect of organic mercurials was abolished by prior addition of an equivalent amount of glutathione. Labarre (1945) has reported that the haemolytic effect of cobra venom injected into rabbits can be abolished by the simultaneous or prior injection of glutathione. The dynamic character of mammalian-erythrocyte glutathione may be of some consequence in the maintenance of the structural integrity of erythrocytes; the viability of the cell will depend, at least in part, on a continued capacity of the cell to replace glutathione and to maintain it in a reduced state.

Lowering in glutathione synthesis *in vitro* by erythrocytes from vitamin B_{12}-deficient animals has to be evaluated in terms of the extent to which this is attributable to haemolysis during incubation. It is significant in this connexion that in the vitamin B_{12}-protected rats fed with iodinated-protein there is a near-normal maintenance of blood-glutathione level (Kasbekar *et al.* 1959).

SUMMARY

1. The existence in rat erythrocytes of an enzyme system capable of conjugating the constituent amino acids to form glutathione has been shown.

2. An absolute requirement for magnesium and potassium ions for glutathione formation is indicated. Addition of adenosine or of adenosine 5′-monophosphate has a favourable effect on the synthesis.

3. The effects of dietary folic acid and vitamin B_{12} deficiencies on synthesis of glutathione *in vitro* have been studied. Folic acid has little effect whereas vitamin B_{12} deficiency causes a decrease in glutathione synthesis by erythrocytes although there is interference due to haemolysis under this condition.

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