

The Reduction of Disulphides by Human Erythrocytes

By L. ELDJARN, J. BREMER* AND H. C. BÖRRESEN

Institute of Clinical Biochemistry, Rikshospitalet, University of Oslo, Norway

(Received 9 June 1961)

It is known that disulphides can be reduced by the mammalian organism with the appearance of the corresponding thiol in blood and urine (Eldjarn, 1950; Fischer & Goutier-Pirotte, 1954). Reduction of cystine and homocystine *in vivo* may also be inferred from the fact that they can replace cysteine and homocysteine in food (White & Beach, 1938).

Few experiments have been published on the biochemical reactions underlying these reductions. Cell-free rat-liver preparations reduce homocystine and the reduction was ascribed to an enzymic 'transhydrogenation' between homocystine and GSH (Racker, 1955). A crude glutathione-reductase preparation reduces a number of disulphides. In this case spontaneous exchange reactions between the disulphides and GSH were found to be part of the reaction mechanism (Pihl, Eldjarn & Bremer, 1957). A similar reaction mechanism has been assumed for the reduction of insulin S·S groups in the presence of rat-liver extracts. Two enzymes were required for the insulin reduction, one of which was glutathione reductase (Narahara & Williams, 1959).

We now report on the ability of human erythrocytes to reduce disulphides, with the appearance of the SH form of the substrate in the suspending medium. Preliminary experiments with rat tissues demonstrated that erythrocytes, and brain and kidney slices are particularly active in this regard. In all cases, higher concentrations of certain disulphides inhibited the reductive ability of the tissues. Owing to the limited but well-studied metabolic activities of erythrocytes, these cells were chosen for further studies.

MATERIALS AND METHODS

Cysteamine (2-mercaptoethylamine) and cysteamine derivatives were obtained from Fluka A.-G. Chemische Fabrik, Buchs, Switzerland. The corresponding disulphides were prepared by oxidation with I_2 and purified by recrystallization from ethanol-HCl-ether. The acetylcysteamine was prepared as described by Eldjarn, Pihl & Sverdrup (1956). All other chemicals used were commercial products of high purity.

Heparinized freshly drawn human blood was obtained from the hospital blood bank. The cells were isolated by

centrifuging and washed twice with a buffered solution, pH 7.4, containing the following (m-equiv./l.): Na^+ , 153; K^+ , 2.2; Cl^- , 143; HPO_4^{2-} , 11. The number of cells/ml. was determined by visual counting or by determining the haematocrit value. In most experiments the cells were suspended in a concentration of approximately 0.4×10^6 cells/mm.³ (i.e. 1 ml. of packed cells to 19 ml. of suspending medium). The incubation medium (pH 7.4) contained the following (m-equiv./l.): Na^+ , 142; K^+ , 6.7; Cl^- , 110; HPO_4^{2-} , 33.4; EDTA, 1. In all experiments the incubations were performed at 37° in air.

In order to follow the reduction of disulphides, samples of the incubation mixture were pipetted off at suitable intervals and centrifuged as quickly as possible. The SH content of the cell-free supernatant was determined, either by I_2 titration or by electrometric titration with the rotating Pt electrode (Kolthoff, Stricks & Morren, 1954). The samples were ready for titration 3–4 min. after the withdrawal from the incubation bath.

In the experiments where radioactive compounds were used as substrates, the cells and the incubation medium were separated by centrifuging in capillary test tubes. Samples of the supernatant as well as of the lysed cells were subjected to paper electrophoresis after acidification to pH 2.

The electrophoresis was performed according to Eldjarn & Pihl (1957) both with and without 0.02 M-HgSO₄ in the acid phthalate electrophoresis buffer. This method permits the separation of the SH and S·S forms of the disulphides, as well as of their mixed disulphides with proteins and glutathione. The distribution and amounts of radioactive components on the paper electrophoretograms were determined in a Frieske & Hoepfner strip counter. The results obtained with lysed cells were taken as intracellular radioactive components and were not corrected for the small amount of incubation medium contaminating the cells before lysis.

A number of disulphides did not give rise to the appearance of SH groups in the suspending medium. In these instances the disappearance of the disulphide from the medium was measured by electrolytical reduction of the disulphide (Dohan & Woodward, 1939), followed by titration of the SH groups as described above.

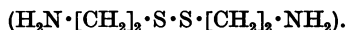
Experiments were undertaken to study the effect of disulphides on uptake of O₂ in erythrocytes in the presence of methylene blue. In these instances packed cells (1 ml.) were suspended in 5 ml. of medium, and uptake of O₂ was measured by the usual Warburg method with air as the atmosphere.

Under specified conditions the reduction rate was proportional to the cell number. All results are therefore given as μ moles of SH formed/hr./10¹¹ cells. All results must be considered minimum values, as a partial spontaneous reoxidation of SH groups could not be avoided, even in the presence of EDTA.

* Fellow of The Norwegian Cancer Society.

RESULTS

Fig. 1 shows the extracellular appearance of iodine-titratable material during the incubation of erythrocytes with cystamine



Both low and high concentrations of cystamine give rise to a rapid initial rise in iodine consumption, a rise which also appears in the absence of glucose or other reducing substrate. When a low concentration of cystamine is used (less than approx. 3 mM) with glucose as the reducing substrate, this initial rapid rise is followed by a slower steady increase in iodine-titratable material until an iodine consumption corresponding to a quantitative reduction of the added disulphide is obtained. The rate of this increase in iodine-titratable material was found in experiments where the haematocrit value was varied from 1/5 to 1/40, to be proportional to the number of cells present. When higher concentrations of cystamine are used with glucose as the reducing substrate, no such increase follows the initial rapid appearance of SH groups.

In order to prove that the iodine consumption is caused by a reduction of cystamine S·S groups, the experiments shown in Figs. 2 and 3 were undertaken. In these experiments [³⁵S]cystamine was used, and the intra- and extra-cellular metabolites were isolated and estimated as described by Eldjarn & Pihl (1957). These experiments verified that with the low concentration of cystamine

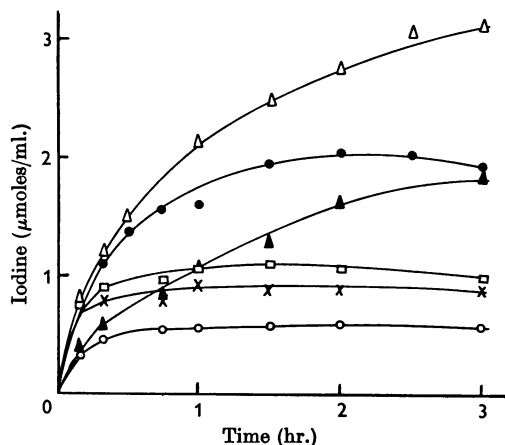


Fig. 1. Time curves for the appearance of extracellular iodine-titratable SH groups during the incubation of cystamine with erythrocytes and different reducing substrates (5.5 mM). ○, Cystamine (1 mM); ●, cystamine (1 mM) + glucose; □, cystamine (10 mM); ×, cystamine (10 mM) + glucose; ▲, cystamine (10 mM) + adenosine; △, cystamine (10 mM) + inosine.

(1 mM) a quantitative reduction took place, whereas with the higher concentration (8 mM) only the initial rise of SH groups could be demonstrated. Further, from Fig. 3 it is evident that the initial and rapid appearance of cysteamine corresponds to a reduction caused by the formation of mixed disulphides with glutathione and with protein SH groups. These mixed disulphides were also temporarily observed in the experiment shown in

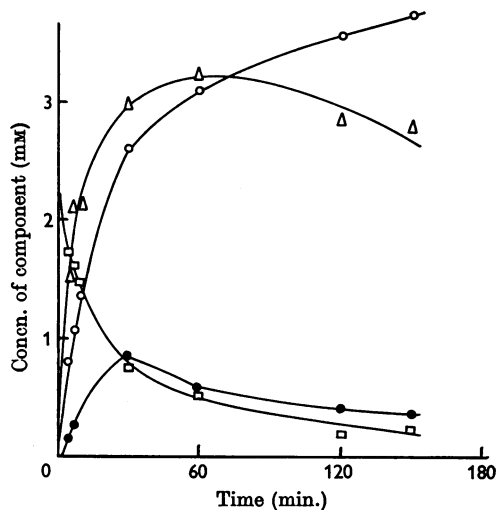


Fig. 2. Time curves showing the appearance of intra- and extra-cellular radioactive components when [³⁵S]cystamine (1.5 mM) is incubated with erythrocytes in the presence of glucose (5.5 mM). ○, Extracellular RSSR; □, extracellular RSH; △, intracellular RSSR; ●, intracellular haemoglobin-bound radioactivity.

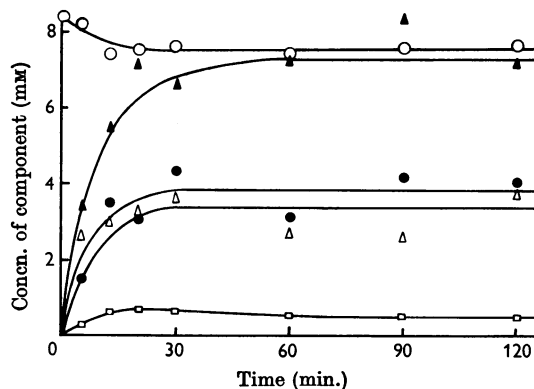


Fig. 3. Time curves showing the appearance of intra- and extra-cellular radioactive components when [³⁵S]cystamine (8 mM) is incubated with erythrocytes in the presence of glucose (5.5 mM). ○, Extracellular RSSR; ▲, intracellular RSSR; □, extracellular RSH; △, intracellular GSSR; ●, intracellular haemoglobin-bound radioactivity.

Fig. 2, but here they again disappeared as the reduction proceeded. Fig. 2 shows that cystamine in the disulphide form could not be demonstrated intracellularly, indicating that the compound became reduced as fast as it diffused into the cells. The steady-state concentrations obtained in Fig. 3 demonstrate that identical concentrations of cystamine are found intra- and extra-cellularly. The haemoglobin-bound radioactivity corresponds to a mixed disulphide formation with one SH group per haemoglobin molecule (mol.wt. 67 000). The concentrations of the mixed disulphides between glutathione and cysteamine corresponds to a glutathione concentration of 3 mM, in agreement with the known glutathione content of human erythrocytes (Henneman, Altschule & Gonez, 1954).

To obtain further insight into the nature of the reduction and the mechanism of the inhibition, the requirement for reducing substrates was studied. From Table 1 it is apparent that glucose, mannose and to a very small extent galactose and fructose 1,6-diphosphate support the reduction. The reduction rate was not influenced by variations in

glucose concentrations between 0.5 mM and 50 mM. A number of nucleosides (adenosine, xanthosine and inosine) were particularly effective. Of particular significance is the observation that, with these nucleosides as reducing substrate, no inhibition of the S·S reduction with high S·S concentration was observed (Fig. 1). A series of phosphorylated glycolytic intermediates (6-phosphogluconate, phosphoglyceric acid, ribose 5-phosphate, fructose 1,6-diphosphate, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate) gave little or no reduction, in agreement with the observation that erythrocytes are nearly impermeable to phosphorylated intermediates (Frankerd, 1961).

The above results were obtained with *NN'*-diethylcystamine as substrate for the reduction. In Table 2 are given the results when a number of other disulphides were tested. It is apparent that the disulphides may be divided into three groups:

Cystamine and a series of cystamine derivatives are reduced at high rate. When used in higher concentrations, they inhibit their own reduction as well as the oxygen consumption in the presence of methylene blue when glucose is used as substrate.

Sodium tetrathionate, cystine diethyl ester and the disulphides of thioglycollic acid and thioethanol are reduced, but at a considerably lower rate than the cystamine derivatives. When used in higher concentrations, these disulphides do not interfere with their own reduction, or with the oxygen consumption in the presence of methylene blue when glucose is used as substrate.

The naturally occurring disulphides, GSSG, cystine and homocystine, do not become reduced and do not interfere with the oxygen consumption. Since it is known that these compounds are reduced by cell-free preparations of glutathione reductase when glutathione is present (Pihl *et al.*

Table 1. *Effect of different substrates on the reduction of NN'-diethylcystamine by erythrocytes*

The substrates were 5 mM and the diethylcystamine was 1 mM.

Reducing substrate	SH produced (μ moles/hr./ 10^{11} cells)
None	0-10
Glucose	208
Fructose 1,6-diphosphate	15
Mannose	104
Galactose	14
Adenosine	~185
Xanthosine	~180
Inosine	~500

Table 2. *Reduction of different disulphides by erythrocytes in the presence of glucose (5.5 mM)*

Disulphide	SH produced (μ moles/hr./ 10^{11} cells) with mM-disulphide	Inhibiting effect with 10 mM-disulphide	
		On its own S·S-reduction	On O ₂ consumption in the presence of methylene blue
Cystamine	175	+	+
<i>NN'</i> -Dimethylcystamine	235	+	+
<i>NN'</i> -Diethylcystamine	208	+	+
<i>NN'</i> -Tetramethylcystamine	162	+	+
<i>NN'</i> -Tetraethylcystamine	214	+	+
<i>NN'</i> -Diacetylcystamine	168	+	+
Sodium tetrathionate	75	-	-
Thioglycollic acid disulphide	33	-	-
Thioethanol disulphide	102	-	-
Cystine diethyl ester	25	-	-
GSSG	0	Non-permeable	.
L-Cystine	0	Non-permeable	.
L- and D-Homocystine	0	Non-permeable	.

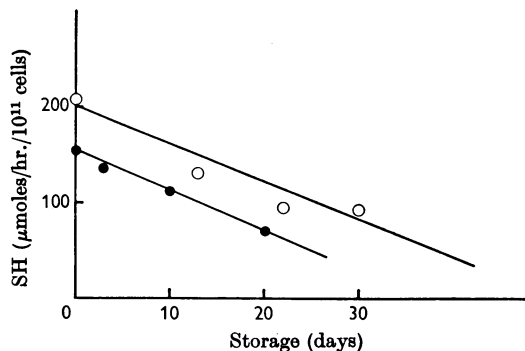


Fig. 4. Effect of blood storage (4°; acid citrate-glucose buffer) on the ability of erythrocytes to reduce cystamine (1 mM) in the presence of glucose (5.5 mM). The two curves give the results obtained with blood from two individuals.

1957), a probable explanation for our observation would be that these disulphides do not penetrate the erythrocytes at a measurable rate. That this is indeed the case was demonstrated in experiments where the disulphide concentration in the suspending medium was found to stay unchanged after mixing with an equal volume of washed erythrocytes. For cystine, the use of ³⁵S-labelled compound verified this finding and demonstrated the impermeability even when serum was used as suspending medium. However, the reduction of GSSG as well as of cystine and homocystine could be brought about when small amounts of cystamine (0.2 mM) were added. Obviously the cystamine was reduced by the cells, and the resulting cystamine reduced the disulphides in the medium by spontaneous exchange reactions.

Because of the practical implications, the changes brought about in erythrocytes by storage at 4° in acid citrate-glucose buffer has attracted considerable interest. A number of changes have been reported, but no direct evidence for a loss of glutathione or glutathione reduction has been described (Pranker, 1961). Fig. 4 demonstrates the changes in the S-S-reducing capacity of erythrocytes after storage at 4° in acid citrate-glucose buffer. The capacity is reduced to 50% in about 17 days.

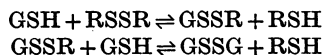
DISCUSSION

From the results reported above it is evident that intact human erythrocytes reduce a number of disulphides with the appearance of the corresponding thiols in the suspending medium. Disulphide reduction has previously been studied in cell-free preparations from animal tissues by Racker (1955), Narahara & Williams (1959) and Pihl *et al.* (1957). In all these studies glutathione and glutathione reductase were found to be part of the reductive

system. In the reduction of homocystine (Racker, 1955) and insulin (Narahara & Williams, 1959) a second enzyme was stated to be required for the transfer of hydrogen (or electrons) from GSH to the disulphide.

The disulphide reduction has also been studied in yeast (Nickerson & Falcone, 1956; Black, Harte, Hudson & Wartofsky, 1960) and in plant tissues (Hatch & Turner, 1960). In these organisms no requirement for glutathione reductase and glutathione was observed. However, animal tissues have not been reported to contain any disulphide reductase other than glutathione reductase.

The disulphide reduction by intact erythrocytes observed by us is therefore most probably brought about by glutathione reductase. Whether the intermediary exchange reactions:



are spontaneous or are catalysed by transhydrogenases cannot be decided. At the intracellular pH of erythrocytes (approximately 7.1) the spontaneous exchange reaction is sufficiently rapid to account for the velocity of reduction observed (Pihl & Eldjarn, 1958).

The formation of mixed disulphide with GSH in erythrocytes incubated with high concentrations of cystamine might offer an explanation for the complete inhibition of the disulphide reduction observed under these conditions, since this mixed disulphide is known not to serve as substrate for glutathione reductase (Pihl *et al.* 1957). In the disulphide equilibrium:



the equilibrium constant at pH 7.4 and 37° is known to be 14.7 (Eldjarn & Pihl, 1957). The experiment shown in Fig. 3 demonstrates that the erythrocytes are freely permeable to cystamine, but impermeable to GSSG and GSSR. The intracellular concentration of GSSR and of RSSR were found to be approximately 3.5 and 7.2 mM. On the basis of these figures the intracellular GSSG concentration can be calculated to be approximately 0.1 mM. The Michaelis constant of glutathione reductase for GSSG is reported to be 1.4 mM (Asnis, 1955). Thus a substantial inhibition of the reductive system might be expected, dependent on the enzyme concentration. On the other hand, in cell-free preparations high reduction rates have been observed with such low GSSG concentrations (Pihl *et al.* 1957). It seems difficult therefore to maintain that the complete inhibition of disulphide reduction can be explained by the mixed disulphide formation. This conclusion is also supported by the finding that high cystamine concentrations do not inhibit reduction of cystamine when adenosine or

inosine is used as the reducing substrate. Thus there seems to be other metabolic effects of cystamine in erythrocytes.

Studies on cystamine metabolism in rabbit erythrocytes have been reported by Fischer & Goutier-Pirotte (1954), who could not demonstrate any reduction and concluded that rabbit erythrocytes were impermeable to cystamine. This discrepancy may possibly be explained by species variations. Differences in permeability have been observed with other compounds in erythrocytes from different species (Pranker, 1961).

Our results on the reduction of different disulphides by human erythrocytes also indicate that the penetration into the cell usually is the rate-limiting step. This can be inferred from the following observations: (a) The lack of reduction of a number of physiological disulphides could be ascribed to impermeability of the cell. (b) Fig. 2 demonstrates that, in cystamine reduction, the cystamine proper could not be demonstrated intracellularly, indicating that it is reduced as fast as it diffused into the cell. (c) It can be shown that the disappearance of cystamine in Fig. 3 corresponds fairly well to a 'one-way' diffusion according to the equation:

$$\frac{-dc}{dt} = kc.$$

However, the validity of such a relationship must be limited since variations in the concentration of the disulphide of thioethanol and sodium tetrathionate between 1 and 10 mM did not influence the reduction velocity as given in Table 2.

In preliminary studies we have observed that cystamine is reduced by brain, kidney and testicular tissues from rat, but not by rat-liver slices in spite of the fact that a cell-free rat-liver preparation does reduce cystamine (Pihl *et al.* 1957). Thus permeability differences may also exist between different tissues from the same species.

Ingram (1955) found that native human haemoglobins contain four SH groups as two pairs of closely neighbouring groups, when titrated with silver nitrate and mercuric chloride. In contrast, our findings (Fig. 3) demonstrate that only one of these SH groups is freely reactive to the extent that it can enter into exchange reaction with cystamine and form a mixed disulphide. The possibility should also be considered that two additional SH groups might have reacted, but with the formation of an intramolecular disulphide, as has previously been demonstrated for catalase (Pihl & Eldjarn, 1958).

The fact that cystamine derivatives, in contrast with a number of other disulphides, inhibit the S-S reduction of erythrocytes, probably is related to differences in the ability to form mixed disul-

phides with protein SH groups with consequent inhibition of enzyme activities (Eldjarn & Pihl, 1959). The ubiquitous occurrence of glutathione has been ascribed to its ability to 'keep the enzymes in a happy state either by preventing their oxidation or by protecting them against toxic heavy metals' (Racker, 1954). Few studies with intact cells have appeared on this problem. The cystamine derivatives may prove to be powerful means to study this problem.

Sulphydryl groups have repeatedly been postulated to be of importance for the viability of erythrocytes. However, no fall in GSH concentration on storage has been reported. On the other hand, our results demonstrate a relatively rapid decline in the ability of the stored erythrocytes to reduce disulphides. The mechanism for this decline is now under investigation.

SUMMARY

1. The reduction of a number of disulphides has been studied in human erythrocytes.
2. Cystamine and a number of cystamine derivatives are reduced at a rapid rate. The reduction most likely proceeds via spontaneous exchange reactions with intracellular glutathione, the oxidized glutathione formed being reduced by glutathione reductase.
3. Tetrathionate, cystine ethyl ester and the disulphides of mercaptoethanol and of thioglycollic acid are reduced, but at a much slower rate than cystamine.
4. Physiological disulphides (oxidized glutathione, cystine and homocystine) are not reduced at all, owing to an impermeability of the erythrocytes to these compounds.
5. When human erythrocytes are stored in the conventional acid citrate-glucose buffer, a rapid decline in disulphide-reducing capability is observed.

This work has been supported by United States Public Health Service (grant no. A 3891) and by The Norwegian Research Council for Science and the Humanities. We wish to thank Miss Gesche Torp for valuable technical assistance.

REFERENCES

- Asnis, R. E. (1955). *J. biol. Chem.* **213**, 77.
 Black, S., Harte, E. M., Hudson, B. & Wartofsky, L. (1960). *J. biol. Chem.* **235**, 2910.
 Dohan, J. S. & Woodward, G. E. (1939). *J. biol. Chem.* **129**, 393.
 Eldjarn, L. (1950). *Scand. J. clin. Lab. Invest.* **2**, 202.
 Eldjarn, L. & Pihl, A. (1957). *J. Amer. chem. Soc.* **79**, 4589.
 Eldjarn, L. & Pihl, A. (1959). *25th Anniversary Publication from the Norwegian Radium Hospital*, p. 253. Oslo: J. Chr. Gundersen.
 Eldjarn, L., Pihl, A. & Sverdrup, A. (1956). *J. biol. Chem.* **223**, 353.

- Fischer, P. & Goutier-Pirotte, M. (1954). *Arch. int. Physiol.* **62**, 76.
- Hatch, M. D. & Turner, J. F. (1960). *Biochem. J.* **76**, 556.
- Henneman, D. H., Altschule, M. D. & Gonez, R. M. (1954). In *Glutathione*, p. 299. Ed. by Colowick, S. P. *et al.* New York: Academic Press Inc.
- Ingram, V. M. (1955). *Biochem. J.* **59**, 653.
- Kolthoff, J. M., Stricks, W. & Morren, L. (1954). *Analyt. Chem.* **26**, 366.
- Narahara, H. T. & Williams, R. H. (1959). *J. biol. Chem.* **234**, 71.
- Nickerson, W. J. & Falcone, G. (1956). *Science*, **124**, 318.
- Pihl, A. & Eldjarn, L. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **13**, 43.
- Pihl, A., Eldjarn, L. & Bremer, J. (1957). *J. biol. Chem.* **227**, 339.
- Pranker, T. A. J. (1961). *The Red Cell*, Oxford: Blackwell Scientific Publications.
- Racker, E. (1954). In *Glutathione*, p. 165. Ed. by Colowick, S. P. *et al.* New York: Academic Press Inc.
- Racker, E. (1955). *J. biol. Chem.* **217**, 867.
- White, A. & Beach, E. F. (1938). *J. biol. Chem.* **122**, 219.

Biochem. J. (1962) **82**, 197

Catabolism of γ -Globulin by the Isolated Perfused Rat Liver

By S. COHEN,* A. H. GORDON AND CHRISTINE MATTHEWS†
National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 31 July 1961)

Experiments with ^{14}C -labelled amino acids have shown that plasma protein with the electrophoretic mobility of γ -globulin is not synthesized by the normal rat liver (Miller & Bale, 1954). In the present investigation the catabolism of γ -globulin by the isolated, perfused rat liver has been investigated and directly compared with that of albumin. In these experiments, γ -globulin prepared by chromatography, labelled with radioactive iodine and screened in a living rat for 2 days before use, was catabolized at a rate equivalent to about 30% of that observed in the whole animal. On the other hand albumin, which is known to be synthesized exclusively in the liver (Miller, Bly, Watson & Bale, 1951; Gordon & Humphrey, 1960), is broken down at a significantly slower rate equivalent to less than 15% of the total catabolism of this protein *in vivo* (Cohen & Gordon, 1958). These experiments indicate that to a large extent the catabolic sites of both albumin and γ -globulin differ from their respective sites of synthesis.

In the present investigation, as in previous studies, it was observed that the concentration of plasma protein in the perfusing blood usually increased progressively during the course of the experiment, despite the fact that haemoconcentration did not occur. This phenomenon appears to be relevant to an assessment of the functional state of the perfused liver. The amount of protein discharged into the perfusing blood has therefore been measured, and plasma samples have been assayed

for the presence of solubilized liver protein; this part of the investigation has been the subject of a brief preliminary report (Gordon, 1961).

METHODS

Perfusion apparatus and technique. This was basically the same as that used by Miller *et al.* (1951) but having the modifications described by Cohen & Gordon (1958). Haemoconcentration, which readily occurs during the course of a perfusion, was not completely controlled by measures previously adopted (Cohen & Gordon, 1958); for this reason 0.9% NaCl equivalent to 1.5 times the volume of bile produced was added to the blood reservoir at intervals of about 1 hr.

Liver donors were hooded male rats that had been starved overnight. The perfusion technique used in these experiments was that described by Cohen & Gordon (1958).

Preparation of blood for perfusion. Blood donors were rats of the hooded strain maintained at the National Institute for Medical Research. For perfusions 122–128 (Table 1) 20–30 mg. of heparin (2000–3000 i.u.) was added to 65 ml. of rat blood. In the remaining experiments, the erythrocytes obtained from similar samples of fresh heparinized blood after removal of the plasma and leucocyte layer were washed in 2 vol. of 0.9% NaCl and suspended in an appropriate volume of serum which had been prepared on the previous day by the following methods: (i) In perfusions 131 and 133, plasma containing 0.167 vol. of 3.8% (w/v) sodium citrate was clotted by addition of CaCl_2 (0.015 m-equiv./ml. of plasma). (ii) In perfusions 136, 137 and 138, plasma separated by centrifuging of blood in plastic tubes was subsequently allowed to clot. Serum was stored overnight at 2° and mixed with the washed erythrocytes immediately before the experiment.

Preparation of protein fractions. γ -Globulin was prepared from a sample of pooled rat serum by chromatography on diethylaminoethylcellulose (Peterson & Sober, 1956). The

* Present address: Department of Immunology, St Mary's Hospital Medical School, London, W. 2.

† Present address: Radiotherapeutic Research Unit, Hammersmith Hospital, London, W. 12.