C. THE REDUCTION OF GLUTATHIONE IN MAMMALIAN ERYTHROCYTES.

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GLUTATHIONE is found in most mammalian tissues [Hopkins, 1921; Blanchetière and Mélon, 1927]. Though it exists there in the reduced state as glutaminylcysteinylglycine [Pirie and Pinhey, 1929; Nicolet, 1930], little is known of the reducing mechanisms. Hopkins and Dixon [1922] studied a thermostable reducing system in muscle. They showed that an equilibrium existed between an insoluble factor in muscle called by them "fixed —SH" and soluble glutathione, according to the equation

where —SH stands for sulphydryl and —S—S— for disulphide; T for tissue and G— for the glutathione residue. It is, however, clear that this affords but a partial explanation for the presence of reduced glutathione. The system is reversible, which means that the glutathione may reduce tissue disulphide, but it gives no explanation of the ultimate presence of sulphydryl. Some system, or systems, other than muscle protein must be able to reduce pentapeptide glutathione to the tripeptide form.

Hopkins and Dixon [1922] were unable to find any dehydrogenase system which, in presence of its substrate, reduced disulphide to sulphydryl, and the claim of Wieland and Bergel [1924] that succinate acted as a reductant in liver could not be substantiated by Elliott [1928]. Hopkins and Elliott [1931] found that liver systems could reduce added —S—S— to —SH and that the systems were thermolabile. They failed, however, to ascribe the reduction to any definite reducing system. Recently Mann [1932] has found that the glucose dehydrogenase of Harrison [1931] can reduce glutathione in the presence of glucose. This may be one of the systems responsible for the reduction observed by Hopkins and Elliott.

It has now become clear that glutathione behaves differently in different tissues. Hopkins and Elliott [1931] found that it disappeared rapidly from chopped liver (and also kidney) when the reducing metabolites had been exhausted by aeration. In marked contrast to their observations stand those of Meldrum [1930] who found no evidence for the participation of glutathione in the normal respiratory mechanism of yeast. As will be found in the following protocols, glutathione is remarkably stable to oxidation in erythrocytes.

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Though glutathione is found reduced in almost every cell system in which it exists, several factors militate against the identification of what reduces it. In yeast, for example, it does not become oxidised and yeast cannot absorb added $G-S-S-G$, which remains unaffected by the cell suspension. Treatment with acetone, as in making zymin, results in an apparent loss of reducing power. Washed chopped muscle appears to be unable to reduce disulphide glutathione in the presence of added glucose. When glutathione is not reduced the absence of effect may be due to (1) the impermeability of the cell-wall preventing access; or (2) the reducing mechanism having been destroyed by the artificiality of the conditions, or method of preparation.

Now Hopkins and Elliott [1931] found liver preparations to reduce added pentapeptide glutathione to sulphydryl, but were unable to find any familiar metabolite, which, when added to the mixture, accelerated or increased the reduction. In such a case the factor which prevents analysis is the complexity of the reacting mixture. The author has endeavoured to satisfy the conditions

(1) that the element of artificiality should be a minimum,

(2) that the reduction should be controlled by the presence or absence of some definite substance.

The mammalian erythrocyte has been found to satisfy all these conditions. When washed with saline and suspended in a salt solution of the appropriate composition it approximates in state to the natural conditions, and it is improbable that its metabolism differs much from that in the blood-stream. The red blood corpuscle contains reduced glutathione even when fully oxygenated, and (as will be shown later) the process of oxidation is comparatively slow and may be neglected in comparison with the observed rates of reduction in presence of hexoses.

No attempt has been made to attach functional significance to glutathione in corpuscles. Holden [1925] suggested that glutathione was concerned in erythrocytes with the reduction of oxyhaemoglobin and in exceptional circumstances with that of methaemoglobin (formed *in vivo* perhaps as the result of the presence of nitrites and other poisons). The results of Holden's experiments were due to a combination of two circumstances:

(a) that he used impure (*i.e.* non-crystalline) glutathione;

(b) that he used systems contaminated with iron or copper.

Turner [1929] has stated that the amount of reduced glutathione is 10–15 % greater in venous blood than in arterial, and his observations were to some extent confirmed by Gabbe [1928], who has, however, in his latest communication stated that his experiments were erroneous. It seems quite clear that glutathione cannot act as an oxygen carrier to any large extent in erythrocytes.

EXPERIMENTAL.

Section I. The interaction of cysteine and glutathione with oxyhaemoglobin and methaemoglobin was studied in a series of observations made as a preliminary step to the succeeding researches. Cysteine or glutathione was

allowed to react with protein in clean test-tubes and the system studied spectroscopically. For this purpose the following solutions were made:

(1) ox-oxyhaemoglobin was dialysed for about 8 hours in the apparatus of Stadie and Ross [1926] and diluted to a concentration of about 0.5 % with metal-free water;

(2) 10 % potassium ferricyanide was added to 5 % oxyhaemoglobin and the whole electro dialysed till free from ferricyanide in Stadie and Ross's apparatus;

(3) a solution of pure cysteine hydrochloride was brought to a p_H of 7.6 in phosphate buffer, the concentration being 4 mg. per cc.;

(4) crystalline glutathione was made up at p_H 7.6 in phosphate buffer, there being 8 mg. per cc.

1 cc. protein solution was added to 2 cc. phosphate buffer p_H 7.6 in a test-tube and 2 cc. of cysteine or glutathione solution. The reduction was followed spectroscopically. The results are shown in Table I. By a "rapid reduction" is meant a reduction in about 3 mins. or less. This is shown by a + sign. A - sign indicates no reduction.

Table I.

Reducing agent	Methaemoglobin	Oxyhaemoglobin
Cysteine	+	-
Cysteine + 10^{-2} mg. Cu ...	+	+
Glutathione	-	-
Glutathione + 10^{-2} mg. Cu	-	-

These observations are in accordance with those of Krebs [1929] on cysteine oxidation by haematin derivatives. It is of interest to note that the interaction between methaemoglobin and cysteine is direct and stoichiometric, whereas that between cysteine and oxyhaemoglobin in the presence of Cu depends on the dissociation of the oxyhaemoglobin when the dissolved oxygen has been used by the cysteine. There is no direct interaction between cysteine and oxyhaemoglobin.

The results of these observations show that *in vitro* glutathione and pure cysteine are unable to react rapidly with methaemoglobin and oxyhaemoglobin, and indicate with some probability that no such interaction takes place *in vivo* in the erythrocyte. It is thus improbable that the state of the haemoglobin system affects that of the glutathione *in vivo*.

Section II. The method of estimation of glutathione was based on that of Tunnicliffe [1925]. The corpuscles were lysed with an equal volume of water and a concentrated solution of trichloroacetic acid was added, the final concentration being 10 %. The protein was filtered, ground up with 10 % trichloroacetic acid, in volume equal to the initial amount of corpuscles, and filtered. The combined filtrates were titrated with $N/100$ I in the presence of 2.5 cc. of a 25 % solution of KI per 100 cc. filtrate, starch being used as indicator. The method has been much criticised because substances other than

glutathione, which absorb iodine, are present in tissue extracts and because the total iodine uptake of glutathione depends on the concentration and acidity of the solution and on the amount of KI present. The suggested changes appear to offer little real improvement, and the simple procedure has accordingly been followed. Table II shows the iodine uptake of crystalline glutathione prepared according to the method of Hopkins [1929] as modified by Pirie [1930] when titrated as in the experiments¹.

Table II.

Wt. of glutathione mg.	Observed titre cc. 0.01040 <i>N</i> I	Calculated titre* cc. 0.01040 <i>N</i> I
10	3.13	3.14
10	2.95	3.14

* Calculated on the assumption that the reaction is $2\text{GSH} + \text{I}_2 = \text{GSSG} + 2\text{HI}$.

When starch is used as indicator the end-point of the titration is somewhat difficult. Duplicate titres were however found to agree excellently. Table III shows the agreement found.

Table III.

No. of exp.	Vol. of corpuscle suspension (cc.)	Sample No. 1 titre cc. 0.01040 <i>N</i> I	Sample No. 2 titre cc. 0.01040 <i>N</i> I
1	25	1.38	1.36
2	50	3.25	3.18
3	50	3.26	3.20
4	50	2.18	2.05
5	50	3.47	3.18
6	50	2.85	2.79

These examples were chosen at random. It is clear that the method involves an error of not more than 10%, and, if experiment No. 5 be neglected, of 5%. Any increase of the iodine titre greater than 10% of the total must be taken as indicative of a real change.

Ergothioneine (the trimethylbetaine of thiolhistidine) is present in blood-filtrates [Hunter and Eagles, 1925; Benedict, Newton and Behre, 1926], and titrates with iodine under the experimental conditions. Though the amount of ergothioneine in blood is dependent on dietary factors [Eagles and Vars, 1928] and varies much from species to species, the content of ox-blood appears to be consistently < 2 mg. per 100 cc. corpuscles [Hunter, 1928]. Ox-blood is peculiarly suitable for the investigations here described. Table IV shows the iodine uptake of ergothioneine when titrated with iodine under experimental conditions.

Table IV.

Wt. of ergothioneine mg.	Titre cc. 0.01040 <i>N</i> I	Estimated titre of ox-blood containing 20 mg. ergothioneine
20	3.8	220

The error introduced by the presence of ergothioneine is thus about 1.4% and may be neglected.

¹ The iodine was standardised with other samples of crystalline glutathione.

Section III. The following experiments on the oxidation and reduction of glutathione in erythrocytes were carried out using corpuscles washed three times on the centrifuge with isotonic saline and suspended in isotonic sodium chloride. They were stored at 2° in an ice-chest. Such corpuscles contain much glutathione in the reduced state. This can be demonstrated by the intensely positive test for sulphhydryl (ergothioneine does not react with sodium nitroprusside) and by the magnitude of the iodine titre. When such corpuscles are aerated at 25° or 37° a slow diminution occurs in the iodine titre and in the intensity of the nitroprusside test. Table V illustrates this effect.

Table V.

Exp. No.	Vol. of corpuscles cc.	Time of incubation hours	Titre cc. 0.01040 N I	Decrease %
A	50	0	2.67	0
			2.67	
	50	8.16	2.05	23
		8.16	2.18	
B	50	0	3.21	0
	50	6	2.1	34

These experiments were carried out with different samples of washed ox-corpuscles. The decreases in titre are much beyond the observed error. We have to regard glutathione in the erythrocyte as undergoing, certainly *in vitro* and probably also *in vivo*, a slow oxidation to the disulphide form. The steady state reached by the glutathione system in the red blood corpuscle depends on the relative rates of the two processes of oxidation and reduction.

Now the reduced form predominates in blood and the problem here studied is what reduces it. The reducing substances present in erythrocytes appear to consist (apart from glutathione) mainly of carbohydrates, and it seemed not improbable that they reduced glutathione, perhaps under the influence of some catalytic mechanism.

In order to test this possibility the following experiment was carried out.

Table VI.

No. of exp.	Conc. of glucose mg./100 cc.	Vol. of corpuscle suspension cc.	Time of aeration hours	Titre of experiment with glucose	Titre of experiment without glucose
1	150	50	0	1.63	1.63
			1	1.54	1.13
			15.5	2.39	0.64
2	300	50	16	3.57	0.75
3	100	25	0	1.37	—
			17	2.44	0.92
4	300	50	0	—	2.66
			2	3.00	—
			8.4	4.13	2.12
5	200	50	0	2.66	—
			3.9	3.00	—
			5.5	3.67	—
			8.25	4.13	2.05

Temp. of aeration was 37°.

Washed, aerated corpuscles were incubated with glucose at physiological concentration, and compared with similar corpuscles incubated in the absence of glucose. In each case the titre was found to be much greater with corpuscles incubated with glucose, and when most of the glutathione had originally been in the oxidised state the increase was as much as 300–400 %. The nitroprusside test was found to increase in intensity roughly in proportion to the iodine titre. Thus the disulphide glutathione had been reduced in the presence of glucose (Table VI).

Control experiments were carried out as follows. A 0.5 % solution of oxidised glutathione was made up in Ringer phosphate of p_H 7.6; 2 % glucose was made up in water, and one part boiled. The reaction took place in Thunberg tubes, evacuated and incubated at 37° for 12 hours. Experiments were done in duplicate. The protocols show the systems studied.

Table VII.

No. of Thunberg tube	...	...	1	2	3
Vol. of GSSG solution (cc.)	...	...	1	1	1
Glucose solution boiled (cc.)	...	...	0	0	2
Glucose solution unboiled (cc.)	...	...	0	2	0
Water (cc.)	...	...	2	0	0

No nitroprusside reaction could be observed in any tube after incubation overnight.

Under experimental conditions glucose alone cannot reduce oxidised glutathione.

From the data of Table VI it is clear that the reduction of oxidised glutathione existing in erythrocytes can be caused by the presence of glucose. When the corpuscles are lysed, by adding water to their suspension, or by the action of saponin, incubation with glucose is without effect on the amount of reduced glutathione. To reduce disulphide glutathione in the presence of glucose is a function of the intact corpuscle. This is shown in the following protocols.

Table VIII.

Time of aeration hours	Vol. of corpuscle suspension cc.	Amount of glucose mg.	Titre of intact corpuscles cc. $N/100$ I	Titre of lysed corpuscles
0	25	100	1.72	—
14	25	100	2.44	1.2

Lysis was caused by adding an equal volume of water to the undiluted corpuscle suspension.

When lysis was effected by adding 20 mg. saponin a similar result was obtained.

Table IX.

Time of aeration hours	Vol. of corpuscle suspension cc.	Wt. of glucose mg.	Titre of lysed corpuscles
9	25	100	1.6
9	25	0	1.5

These experiments were carried out on systems containing the glutathione which they normally possess. The lysed corpuscle system was further investigated with regard to its reducing action towards added glutathione.

25 cc. corpuscles were lysed with 20 cc. water in each of four 75 cc. Thunberg tubes. Oxidised glutathione was added and the whole incubated anaerobically at 37° with, and without, glucose for 6 hours. Had the added glutathione

Table X.

No. of Thunberg tube ...	...	1	2	3	4
Vol. of lysed corpuscles (cc.) ...	...	45	45	45	45
Vol. of 10 % glucose added (cc.)	...	1.25	1.25	0	0
GSSG solution (cc.) ...	...	0	2	0	2
Titre in cc. <i>N</i> /100 I ...	...	2.1	2.2	?	2.2

been reduced the extra titre in tubes 2 and 4 would have been 3.91 cc. The fact that the iodine titre was no greater in tube 4 than in 1 shows that the amount of the "fixed —SH" of Hopkins and Dixon [1922] in the intracellular proteins of the erythrocyte is inappreciable.

Warburg and Christian [1931] found that lysis of erythrocytes destroyed their respiration in the presence of glucose, but that it could be restored by adding Robison's hexosemonophosphate. No effect on the reduction of glutathione could be observed when hexosemonophosphate¹ was added to the lysed corpuscle suspension. This is shown in the following protocols.

Table XI.

No. of flask ...	...	1	2	3
Vol. of lysed suspension (cc.)	...	50	50	50
Glucose (mg.) ...	...	0	50	0
Hexosemonophosphate (mg.)	...	0	0	50
Titre in cc. 0.01040 <i>N</i> I ...	...	2.0	1.4	1.3

The corpuscles were incubated aerobically at 37° for 6 hours. The reducing effect of glucose on disulphide glutathione contained in the intact erythrocyte is likewise possessed by some other reducing sugars. This remarkable fact has been verified many times, invariably with positive results. On the other hand, pentoses were found to be almost devoid of reducing action, as were non-reducing sugars such as sucrose and raffinose, and the hexahydric alcohols sorbitol, mannitol and dulcitol. At first it seemed probable that the reducing action might be due to an impurity present in all the hexoses, perhaps glucose itself. As it is exceedingly hard to apply any simple test for the purity of a sugar the experiment was tried of measuring reduction as a function of the concentration of added sugar. When this was done it was found that the aldohexoses possessed the same reducing power towards glutathione whatever their chemical nature. Fructose possesses less reducing power than glucose. This seems effectively to dispose of the view that reduction with, say, galactose is due to the presence of an impurity common to it and all the other hexoses. It is

¹ A sample of hexosemonophosphate was very generously given me by Dr R. Robison.

scarcely credible that an unknown substance should be present to the same extent in glucose, galactose and mannose from widely different sources, and this view seems less likely than that the hexoses do actually reduce glutathione in the erythrocyte. The experiments with other sugars were carried out precisely as those with glucose. The suspensions of washed corpuscles were aerated at 37°, or exposed in a thin layer to clean air. The results with galactose are illustrated in Table XII, while the data obtained are summarised in Table XIII. In the experiments with galactose the reduction was studied by the use of the nitroprusside reagent. As with glucose the increase in iodine titre was found to follow the increase in comparative intensity of the nitroprusside test.

Table XII.

Vol. of corpuscle suspension cc.	Time of incubation hours	Amount of glucose mg.	Amount of galactose mg.	Titre in cc. N/100 I
25	0	0	0	1.37
25	16	0	0	0.92
25	16	50	0	2.44
25	16	0	50	2.51

It is evident that as regards net result the actions of glucose and galactose are similar.

Table XIII.

Substances which exert marked reduction	Substances which do not exert marked reduction
Glucose	Arabinose
Mannose	Xylose
Fructose	Sucrose
Galactose	Raffinose
Maltose	Mannitol
	Sorbitol
	Dulcitol

It was at first thought that the pentoses definitely exerted a slight reduction. This is always observed when the pentoses are used in concentrations of about 100 mg./100 cc. but tends to disappear when the concentration is about 20 mg./100 cc. It may thus be due to the presence of some impurity, and as the reduction was slight it is unlikely to be of biological importance, so the matter was not further investigated.

In order to study the reduction as a function of carbohydrate concentration a series of experiments was carried out, in which the cell suspension was incubated for an arbitrary suitable time with varying amounts of glucose. It was found that the reduction is independent of the amount of aldohexose within wide limits. *In vivo* this low limit can never be reached, except in hypoglycaemic convulsions of an extreme nature. The data obtained with glucose are set out in Table XIV and are plotted in Fig. 1.

In each vessel were 25 cc. corpuscle suspension, containing varying amounts of glucose. The suspensions were aerated for 9 hours at 37°.

Table XIV.

No. of vessel	1	2	3	4	5
Amount of glucose (mg.) ...	0	5	10	20	45
Titre of blood sample A ...	1.50	2.19	2.48	2.55	2.57
% increase in titre for sample A	0	46	66	73	73
Titre of blood sample B ...	1.26	1.98	—	2.26	2.24
% increase in titre for sample B	0	57	—	79	79

Fig. 1. A, Curve for blood A.
 B, Curve for blood B.
 C, % increase for blood A.
 D, % increase for blood B.

Fig. 2.

+ Glucose. ○ Galactose. ▲ Fructose.

The agreement is as good as can be expected between two different samples of blood.

Fig. 2 shows the comparative reductions shown by different carbohydrates plotted as a function of their concentration. The curves are not particularly favourable specimens, but all the experiments were carried out at the same time on one specimen of blood and for that reason are shown here. The work involved in obtaining such curves is very considerable and not many com-

parisons were made on such a scale. On the other hand many experiments were carried out using isolated points and the agreement was found to be close between the reducing powers of different hexoses.

In each vessel were 25 cc. corpuscle suspension, containing varying amounts of hexose. The suspensions were aerated for 9 hours at 37°.

Table XV.

Amount of hexose mg.	Titre with glucose cc. 0.01040 <i>N</i> I	Titre with galactose cc. 0.01040 <i>N</i> I	Titre with fructose cc. 0.01040 <i>N</i> I
0	1.31	1.32	—
5	2.02	2.08	1.55
10	2.33	—	—
15	—	2.09	—
20	—	2.26	—
100	2.25	2.28	2.23

The percentage increases in these experiments are smaller than those obtainable from the figures in Table VI. The discrepancy is more apparent than real: in the later experiments the corpuscles were used immediately after washing without preliminary aeration, so that less of the glutathione had become oxidised initially.

A considerable number of experiments were carried out in an attempt to link the reduction of glutathione with the glycolytic mechanisms of blood corpuscles described by Jost [1927]. It was found, however, that apart from lysis none of the factors such as fluorides which inhibit glycolysis exerts any marked influence on the reduction of glutathione, and no evidence could be obtained that it is in any way connected with these systems. The fact that hexoses other than glucose and fructose appear to reduce glutathione also militates against this view. Negative results were also obtained with cyanides and with phenylurethane. Neither of these appeared to influence the reduction.

DISCUSSION.

It is extremely difficult to place the data obtained in the above experiments in any general scheme. The soluble muscle enzymes of Meyerhof [1926] appear to produce lactic acid from glucose, fructose, mannose and maltose, but not from galactose. The author is aware of no system in which glucose, mannose, galactose, fructose and maltose are attacked equally, other than that described above. The question as to whether there is any general biological function attached to the system described is at present quite obscure.

One point must be emphasised. The reduction of glutathione is a function of the intact corpuscle. Now, in many cases, though glutathione is everywhere found reduced, treatment of the system preliminary to its study seems to destroy the reducing system. Thus zymine did not reduce added glutathione when incubated under apparently favourable circumstances. Chopping brain tissue seems likewise to destroy its reducing activity: and so on. It may be

that the reduction of glutathione in these systems, as in erythrocytes, is intimately bound up with the intactness of the cell membranes.

Mann [1932] has recently described a water-soluble enzyme system from liver which reduces disulphide glutathione in the presence of added glucose. The system differs from the red blood corpuscle in that other hexoses appear to be inert, and there is no evidence that it can be extracted from other tissues. It is further questionable what fraction of the reducing power is exerted by this system in liver.

The observations here described offer but a very partial solution of the problem of the reduction of glutathione and it is impossible to do more than speculate on their general significance.

SUMMARY.

1. In the intact erythrocyte the glutathione present undergoes a slow oxidation and a much more rapid reduction.

2. *In vitro* the reduction can be brought about by adding glucose to the washed suspension, and the increase can reach 300–400 %.

3. This reduction in the presence of glucose is shown only by intact corpuscles: lysis, with water or with saponin, destroys the reducing power.

4. Besides glucose other hexoses possess the power of reduction, as does maltose. Non-reducing sugars, and hexahydric alcohols derived from hexoses, cause no reduction, and in this way resemble the pentoses which reduce very feebly.

5. Curves are shown for the reduction as a function of carbohydrate concentration.

6. No evidence could be obtained that the system is in any simple way connected with the known glycolytic mechanisms of the erythrocyte.

I have very great pleasure in thanking Sir F. G. Hopkins for his interest in this work.

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