XCV. THE REDUCTION OF GLUTATHIONE BY A LIVER SYSTEM.

By PHILIP JOCELYN GEORGE MANN.

From the Biochemical Laboratory, Cambridge.

(Received May 3rd, 1932.)

SINCE the isolation of glutathione by Hopkins [1921] it has been commonly assumed that this substance acts as a catalyst of tissue respiration. This assumption is based upon its —SH content, its wide distribution, and its behaviour when added to washed tissue. More recently objections have been raised to this view owing to the fact that glutathione occurs in the tissues almost entirely in the reduced form and seems to be relatively inert. Thus Meldrum [1930] suggests that the constancy of the concentration of reduced glutathione in yeast is due not to its maintenance by reducing processes, but to the stability of the thiol form. Objections of this nature have been answered by the work of Hopkins and Elliott [1931] on the survival respiration of liver, if one may assume that the conditions of their experiments approximated to *in vivo* conditions. They have shown that when fresh chopped liver is suspended in Ringer's solution and shaken in air reduced glutathione is gradually oxidised. Thus in the case of rabbit-liver the reduced glutathione disappears after 6 to 8 hours' aeration at room temperature, while at 37° it disappears in 3 hours or less. They have conclusively proved that during this time reduction and oxidation processes are both proceeding, though the velocity of reduction gradually falls owing to the using up of the reducing substrate in the excised liver.

While glutathione may thus well function as a carrier of oxygen to reducing systems, it appears that it can be responsible for only a fraction of the total tissue respiration, and an estimation of its importance in this respect cannot be made until the nature of the systems involved in its reduction are known. This work was started with the intention of investigating the systems in liver responsible for the reduction. The only tissue system so far discovered to be capable of reducing glutathione is the "thermostable residue" of Hopkins and Dixon [1922], but it has been shown by Hopkins and Elliott [1931] that thermolabile systems also play an important part, since the reducing power of liver tissue is largely diminished by heating for 1 hour at 50°. Of thermolabile systems, most dehydrogenases have been tried, with negative results; the method usually employed being to wash the tissue thoroughly with water

and attempt to form a reducing system by the addition to it of such metabolites as lactic, citric or succinic acid, or hypoxanthine.

The work to be described here deals with a liver system which reduces glutathione.

EXPERIMENTAL.

In the experiments to be described the tissue generally employed was ox-liver, though sheep- and rabbit-livers were also occasionally used with similar results. The experiments usually consisted in incubating the washed tissue or tissue extracts with GSSG, in phosphate buffer at p_H 7.4 at 37°. The GSSG employed was prepared by oxidation of GSH with H_2O_2 [Pirie, 1931]. The amount of GSH produced during the course of the experiment was estimated either by the method of Tunncliffe [1925] or of Kühnau [1931]. The reactions were carried out in 100 cc. flasks which were evacuated at the water-pump. In most cases toluene was added to the reaction mixtures. When it was not employed the experimental times were kept as short as possible, and the results were rejected if there was any obvious sign of bacterial growth.

Substrate in watery and alcoholic extracts.

There is no doubt that when minced liver tissue is repeatedly washed with water, its power of reducing glutathione is largely decreased, indicating that some part of the reducing system is washed out. This was the first point to be investigated.

Fresh ox-liver was minced and washed by shaking for 30 minutes with approximately twice its volume of distilled water. After centrifuging, the aqueous layer was removed, boiled, filtered, and stored *in vacuo* at 0°. The residual tissue was dried in a vacuum desiccator over sulphuric acid, and when dry was powdered, washed again with water and finally made up into a suspension with water. 10 cc. lots of this suspension were incubated overnight in phosphate buffer at p_H 7.4 with excess GSSG, with and without the addition of 10 cc. of the boiled watery extract. The amount of GSH formed was estimated by titration against $N/100$ iodine, with the following results:

	Increase in titre (cc. $N/100$ I)
Washed tissue + watery extract	3.28
Washed tissue alone	1.45

In a further experiment an extract of minced tissue was made with approximately 50 % alcohol. The filtrate was evaporated down to a small volume under reduced pressure and dried in a vacuum desiccator. The residue was taken up in a small volume of water for the purposes of the experiment. This concentrated extract was then incubated as before with a suspension of water-washed tissue. The results were as under:

	Increase in titre (cc. $N/100$ I)
Tissue + alcoholic extract ...	1.55
Tissue alone	0.31
Boiled tissue + alcoholic extract	0.20

It will be seen that while the tissue alone shows little activity, the addition of a boiled watery or 50 % alcoholic extract of tissue produces an active system. This was interpreted as indicating that the reducing substrate was soluble in water or 50 % alcohol. When the washed tissue suspension was boiled, addition of the extract failed to bring about a reduction of glutathione, showing that the system is thermolabile.

Enzyme in watery extract.

The capacity for reducing glutathione of a system produced by adding boiled watery extracts of liver to the washed tissue does not compare at all favourably with that of the unwashed tissue. A feasible explanation of this is that the enzyme responsible is itself water-soluble, and that much of it is lost in the watery extracts which are subsequently boiled. An attempt was made to prepare a cell-free preparation of the enzyme, based on the assumption that the latter was water-soluble.

Fresh ox-liver was minced, spread in thin layers in Petri dishes and rapidly dried in vacuum desiccators over sulphuric acid. The dried tissue was ground to a powder and extracted by grinding with water in a mortar for 2 to 3 hours, approximately 300 cc. of water being used for 50 g. of dried tissue. The mass was centrifuged, and the supernatant liquid was dialysed for 4 hours against a large volume of distilled water. On centrifuging, a clear red liquid was obtained, the activity of which for reducing GSSG was estimated by incubating it overnight in 10 cc. samples with oxidised glutathione, with and without the addition of a 50 % alcoholic extract of tissue. The following results were obtained:

	Increase in titre (cc. N/100 I)
Centrifugate alone	4.20
Centrifugate + alcoholic extract	4.65

The centrifugate thus showed a satisfactory degree of activity. It was further dialysed overnight and centrifuged again, and the clear liquid was saturated with ammonium sulphate. The precipitate was collected and dried. The yield from 50 g. of dried tissue was approximately 8 g. This preparation when combined with watery extracts of liver showed considerable activity in reducing GSSG, as is indicated by the following results obtained by incubating 0.25 g. of the precipitate with the extract overnight.

	Increase in titre (cc. N/100 I)
Enzyme preparation + watery extract	8.4
Enzyme preparation alone	0.3
Enzyme preparation + alcoholic extract	0.9

The alcoholic extract employed was made by extracting the dried tissue with 97 % alcohol, evaporating the extract to a small volume under reduced pressure, and finally drying in a vacuum desiccator. The residue was taken up in water for use.

It appears from the results that the reducing substrate, while being soluble in 50 % alcohol, is practically insoluble in 97 % alcohol.

The enzyme having been thus obtained in a reasonably stable cell-free form, an attempt was made to form a system capable of reducing GSSG by incubating it with metabolites likely to function as reducing substrates, such as lactic, citric, and succinic acids, glucose and hypoxanthine. A small but definite reduction was obtained with glucose, the concentration of this substance employed being 0.25 *M*; this, according to Harrison [1931], is the optimum for glucose dehydrogenase. It was at first thought that the low activity of the system might be due to the fact that when glutathione was used as the hydrogen acceptor the optimum concentration of glucose differed from that when methylene blue was used. Using glutathione as the hydrogen acceptor the concentration of glucose producing maximum velocity was found to be *M*/10, but even with this optimum concentration of glucose the rate of reduction compared very unfavourably with that obtained by the addition of boiled watery extract of liver to the enzyme. Thus, in a typical experiment of 4 hours' duration, using 0.25 g. of enzyme, the following results were obtained:

	Increase in titre (cc. <i>N</i> /100 I)
Enzyme + boiled watery extract	4.40
Enzyme + <i>M</i> /10 glucose ...	0.55

i.e. when boiled watery extract was used in place of glucose the reduction proceeded with 8 times the velocity. The almost complete absence of substrate from a 97 % alcoholic extract of liver also seemed to indicate that the glucose system could not be responsible for the reduction of glutathione by liver.

An attempt was next made to obtain some idea of the properties of the reducing substrate by a fractionation of tissue extracts. A start was made by taking advantage of the apparent insolubility of the substrate in alcohol.

50 g. of dried tissue were extracted with 300 cc. of water and the extract after boiling and filtering was evaporated under reduced pressure to a volume of approximately 50 cc. Ten times the volume of 97 % alcohol was then added, and after standing for 1 hour in the refrigerator the supernatant liquid was poured off, and the precipitate was washed with alcohol and dried. The supernatant liquid was evaporated to a small volume under reduced pressure and finally taken to dryness in a vacuum desiccator.

In view of the results previously obtained it was expected that the substrate would be found in the alcohol precipitate. However, on testing the fractions in the usual manner, the alcohol precipitate proved to have practically no activity, while the filtrate from the alcohol precipitation was appreciably active. When now both fractions were combined an activity was obtained which was much greater than would correspond to a summation of the two separate systems, and which approached that of the original watery extract.

	Increase in titre (cc. <i>N</i> /100 I)
Enzyme + alcohol precipitate + filtrate	7.05
Enzyme + filtrate	3.10
Enzyme + alcohol precipitate ...	0.55

Some substance contained in the alcohol precipitate is thus essential for the activity of the system.

The effect of this alcohol precipitate was then tried on the reduction of glutathione by glucose dehydrogenase prepared as above, and glucose. The results given below were obtained using 0.1 g. of enzyme with 0.1 g. of the alcohol precipitate in *M*/10 glucose. The duration of the experiment was 4 hours.

		Increase in titre (cc. <i>N</i> /100 I)
Enzyme + alcohol precipitate + glucose		2.80
Enzyme + glucose		0.25
Enzyme + alcohol precipitate		0.40

The alcohol precipitate is thus an activator of glucose dehydrogenase, and the complete system is capable of readily reducing glutathione.

The alcohol precipitate has a similar effect upon the reduction of methylene blue by glucose dehydrogenase. If the enzyme is prepared by half-saturation with ammonium sulphate instead of full saturation and is well dialysed before use, the enzyme and glucose alone show almost complete inability to reduce methylene blue. If however the activator is also present a rapid reduction is obtained. Thus in one experiment, using in each methylene blue tube 1 cc. *M* glucose, 1 cc. enzyme solution (4 %), 0.5 cc. phosphate buffer (p_H 7.4), 0.5 cc. 1/5000 methylene blue, with and without the addition of 1 cc. of activator (5 % solution), made up to a total volume of 4 cc., the following reduction times were obtained:

Enzyme + activator + glucose	9 mins.
Enzyme + activator	 32 mins.
Enzyme + glucose	 3 hours +

Qualitatively similar results were obtained at other p_H values.

It is thus certain that the alcohol precipitate contains a substance which is an essential part of the glucose dehydrogenase system. The nature of this factor has not yet been ascertained. The preparation also contains varying amounts of substances which are capable, in presence of the enzyme, of reducing both methylene blue and glutathione. It is necessary to fractionate the preparation and obtain the activator free from such substrates before its action can be demonstrated in an entirely satisfactory manner.

DISCUSSION.

From the preceding results it is evident that glutathione is readily reduced by the glucose dehydrogenase system. It is however difficult to assess the quantitative importance of this system in the reduction of glutathione by liver. Hopkins and Elliott [1931] showed that the falling off of the reduction processes during the aeration of chopped liver was due to the using-up of the reducing substrate. In the course of such experiments, however, one would expect an increase rather than a decrease in the concentration of glucose, owing

to glycogen breakdown; and it thus appears that there must be systems in liver which reduce glutathione more actively than does the glucose dehydrogenase system. It seemed a possible alternative explanation that glucose dehydrogenase might have been destroyed during the vigorous shaking which takes place in the aeration experiments. However, attempts to test this point made by addition of the enzyme to excised liver during survival respiration failed to yield satisfactory results. In the course of the present work indications have not been lacking that there are other systems in liver capable of reducing glutathione. For instance, the activator as prepared by alcoholic precipitation of watery extract of liver is capable, when added to the enzyme preparation, of forming a system which in some cases actively reduces glutathione.

Further work on this problem is proceeding.

SUMMARY.

1. Glutathione is reduced by glucose in presence of glucose dehydrogenase prepared from liver by a method similar to that of Harrison [1931].
2. The reaction proceeds slowly. It is greatly accelerated by an activator which can be extracted from liver. The activator is soluble in water but insoluble in alcohol.

I wish to thank Sir F. G. Hopkins and Prof. J. B. S. Haldane for advice and encouragement during the course of this research.

The expense was defrayed by a grant from the Department of Scientific and Industrial Research to Sir F. G. Hopkins.

REFERENCES.

- Harrison (1931). *Biochem. J.* **25**, 1016.
Hopkins (1921). *Biochem. J.* **15**, 286.
—— and Dixon (1922). *J. Biol. Chem.* **54**, 529.
—— and Elliott (1931). *Proc. Roy. Soc. Lond. B* **109**, 58.
Kühnau (1931). *Biochem. Z.* **230**, 353.
Meldrum (1930). *Biochem. J.* **24**, 1421.
Pirie (1931). *Biochem. J.* **25**, 619.
Tunnicliffe (1925). *Biochem. J.* **19**, 194.