XV. THE REDUCTION OF GLUTATHIONE BY THE WARBURG-CHRISTIAN SYSTEM.

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HOPKINS [1921] suggested that the physiological significance of glutathione might lie in its ability to function as transporter of hydrogen from oxidisable molecules in the cell to molecular oxygen. His observation that certain animal tissues were able to reduce the oxidised form of the peptide, and that the reduced form was autoxidisable, lent support to this view. Furthermore he recognised that glutathione apparently always occurs in the reduced form in animal tissues.

Hopkins and Dixon [1922] discovered that "succinoxidase" was quite unable to reduce glutathione. Elliott [1928] confirmed this observation and showed moreover that the "xanthine oxidase" system would not reduce the oxidised peptide. Hopkins and Elliott [1931] studied the behaviour of glutathione in the freshly excised, chopped livers of various animals and found that livers from well-fed animals reduced glutathione more strongly than did those from starved animals. That certain definite "dehydrogenase" systems were involved in the reduction of glutathione in this tissue was strongly suggested by the fact that much of the reducing power was destroyed by heating liver for an hour at 52°. Their results proved definitely that glutathione could function as oxygen carrier in living cells. The exact nature of the system or systems which reduced glutathione in this tissue was not ascertained.

Shortly afterwards Mann [1932] discovered that the glucose dehydrogenase of liver reduced glutathione under anaerobic conditions, and almost simultaneously Meldrum [1932] observed that intact mammalian erythrocytes possessed some system capable of reducing the oxidised peptide in the presence of glucose. Apparently no other systems in living cells capable of reducing glutathione have been discovered, though it is known that certain bacteria reduce the peptide by virtue of their natural metabolites [Tarr, 1933]. The present work has shown that the isolated dehydrogenase system of Warburg and Christian strongly reduces glutathione.

EXPERIMENTAL.

The barium hexosemonophosphate "natural" ester used in these experiments was obtained from the British Drug Houses. The barium hexosemonophosphate "synthetic" ester was prepared by the method of Levene and Raymond [1931], and the corresponding barium phosphohexonic acid by oxidation of the synthetic ester with bromine [Robison and King, 1931]. Barium diphosphoglycerate was made by the method of Jost [1927] and barium fructose-

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diphosphate was kindly given to us by Prof. Keilin. All these compounds gave analyses for barium and phosphorus in good agreement with theory. The specific rotations and "aldose" and "fructose" titres corresponded closely with the values recorded in the literature except in the case of the "synthetic" ester which had a low specific rotation and gave a definite "Selivanoff test" for fructose [Macleod and Robison, 1929]. However, since the "synthetic" ester proved almost as active as the "natural" ester, it was employed in many of the following experiments.

Crude co-enzyme powder was prepared from horse erythrocytes, and from this the solution was made [Warburg and Christian, 1931]. In the Tables "mg. of co-enzyme" signifies that an amount of solution corresponding to the given weight of co-enzyme was used. The enzyme of the reducing system was prepared from rat blood [Warburg and Christian, 1931] and also from top brewery yeast and from baker's yeast [Warburg and Christian, 1932]. Experience showed that crude co-enzyme prepared as above frequently, though not always, contained fairly large amounts of a substance which, on reduction, gave a strong nitroprusside test (probably glutathione), and for this reason fairly high "iodine titres" were frequently obtained in control experiments¹.

Oxidised glutathione was prepared according to Pirie [1930; 1931], solutions being adjusted to p_H 7.4 with N NaOH before use. The amount of reduced glutathione present in experimental solutions was estimated by titrating with $N/100$ iodine after sufficient potassium iodide (25 %) had been added to bring the final concentration of this salt to 1 %. Starch was employed as indicator.

The reduction of glutathione by Warburg and Christian's reducing system in the presence of various substrates.

1. *Synthetic hexosemonophosphate, fructosediphosphate and glucose as substrates.* The substances noted in Table I were added to 75 ml. Thunberg tubes, the final volume of fluid in each tube being adjusted to 20 ml. with distilled

Table I.

Reagent	Thunberg tube No.						
	1	2	3	4	5*	6	7
$M/5$ phosphate buffer, p_H 7.4 (ml.)	5	5	5	5	5	5	5
Oxidised glutathione (mg.)	20	20	20	20	20	20	20
Synthetic barium hexosemonophosphate (mg.)	40	0	40	40	40	0	0
Fructosediphosphate (used as neutral sodium salt) (mg.)	0	0	0	0	0	0	40
Glucose (mg.)	0	0	0	0	0	40	0
Enzyme (from rat blood) (ml.)	2	2	0	2	2	2	2
Co-enzyme (mg.)	100	100	100	0	100	100	100

* Absolute control, heated 5 minutes in a boiling water-bath.

water. 0.05 ml. of caprylic alcohol was added to each tube before evacuation at a water-pump, this substance having the dual virtue of functioning as a germicide and foam preventer. The evacuated tubes were incubated for 6 hours in a water-thermostat at 37°, and then a nitroprusside test (1 ml. used) and iodine titration were made on the contents in each instance. In Table II the results of this experiment are recorded. It is apparent that reduction of glutathione is not effected by the enzyme or co-enzyme alone in the absence of hexosemonophosphate, or by the complete system when heated. Rapid and

¹ Warburg and Christian [1934] have recently stated that Nagelein has discovered that their "co-ferment II" is merely a mixture of "co-ferment I" and glutathione.

Table II.

	Tube No.						
	1	2	3	4	5	6	7
Nitroprusside test	+	+	+	+	-	-	+
ml. of <i>N</i> /100 iodine required	6.7	0.5	0.2	0.5	0.4	0.5	0.9
*Reduced glutathione in mg.	19.0	-	-	-	-	-	1.9

* Calculated from the iodine titre after subtracting the control.

practically quantitative reduction of the glutathione occurred in the presence of the complete system and hexosemonophosphate. Fructosediphosphate acted only as a very feeble donator of hydrogen in this system and glucose was not utilised at all. Unfortunately a pure sample of Neuberg's fructosemonophosphate was not obtained; crude samples obtained by the hydrolysis of fructose-diphosphate (candiolin) were found to be only slightly more active than fructose-diphosphate itself.

2. *Natural hexosemonophosphate, synthetic hexosemonophosphate, phosphohexonic acid and diphosphoglyceric acid as substrates.* The relative values of the above compounds as donators of hydrogen in the reducing system under survey were next determined. The technique was generally identical with that used in the previous experiment. Each Thunberg tube contained 2 ml. of *M*/5 phosphate buffer p_H 7.4, 50 mg. of co-enzyme, 1 ml. of enzyme (from rat blood), 50 mg. of oxidised glutathione and, with the exception of the controls, 10 mg. of the substrate under investigation. All substances were tested in duplicate, and titrations were made at the end of 12 and 24 hours at 37°. The results, which are given in Table III, show that, while both natural and synthetic

Table III.

Substrate	Time in hours			
	12		24	
	ml. <i>N</i> /100 iodine	mg. of GSH*	ml. <i>N</i> /100 iodine	mg. of GSH*
Controls (no substrate added)	0.6	0	0.6	0
Natural barium hexosemonophosphate	9.4	26.6	10.0	28.5
Synthetic barium hexosemonophosphate	6.9	19.1	7.2	20.0
Barium phosphohexonate	4.1	10.6	4.1	10.6
Barium diphosphoglycerate	0.6	0	0.6	0

* The reduced glutathione was calculated from the iodine titre after the controls were subtracted.

hexosemonophosphoric acids are readily activated, the corresponding phosphohexonic acid is less readily attacked, and diphosphoglyceric acid is not activated at all. The reduction was almost always complete after 12 hours: whether this was due to a complete utilisation of all available substrate, or to exhaustion of enzyme was not determined.

Glutathione as oxygen carrier in the Warburg-Christian reducing system.

The oxidation of hexosemonophosphoric acid by the Warburg-Christian reducing system with various concentrations of glutathione as oxygen carrier was studied employing Barcroft differential manometers. In all manometric experiments the temperature was 37° and the gas space was filled with air. Four manometers were used, and the amounts of reagents employed in each are recorded in Table IV. The final volume of liquid in each manometer cup was

Table IV.

Reagent	Manometer No.							
	I		II		III		IV	
	*L	*R	L	R	L	R	L	R
Enzyme (from rat blood) (ml.)	0	1	0	1	0	1	0	1
Co-enzyme (mg.)	0	50	0	50	0	50	0	50
Oxidised glutathione (mg.)	0	0	0	10	0	20	0	40
Barium hexosemonophosphate (mg.)	0	40	0	40	0	40	0	40
(synthetic) (mg.)								
M/5 phosphate buffer, p_H 7.4 (ml.)	1	1	1	1	1	1	1	1

* L and R refer to right- and left-hand manometer cups.

made up to 5 ml. with distilled water and 0.05 ml. of caprylic alcohol added as germicide. In the centre tubes filter-papers moistened with 6 % KOH were employed to absorb CO_2 [Dixon and Elliott, 1930]. The manometers were equilibrated for 10 minutes before the taps were closed, and readings were then taken over 10 hours at this temperature. The results are given in Fig. 1. It is evident

Fig. 1. The effect of glutathione on the oxidation of hexosemonophosphate by the Warburg-Christian reducing system.

1, Control (no added glutathione). 2, 10 mg. of GSSG added. 3, 20 mg. of GSSG added.
4, 40 mg. of GSSG added.

from this diagram that the addition of glutathione caused a considerable increase in oxygen uptake, the increase in oxidation velocity being approximately proportional to the amount of glutathione added. In no case was the oxygen uptake very rapid, and in no case comparable with that obtained by Warburg and Christian [1931; 1932; 1933] with the same reducing system when either methylene blue or yellow pigment was employed as carrier of oxygen. Perhaps a greater activity of the enzyme preparations employed by these workers accounted in part for this increased velocity. It is possible that the presence of a trace of cysteine would have accelerated the uptake in the case of glutathione experiments [Meldrum and Dixon, 1930]. At the conclusion of the experiment the fluid in each right-hand manometer cup was titrated with $N/100$ iodine solution. The results given in Table V show that the reducing system was powerful enough to maintain practically the whole of the glutathione present in the reduced form even under the strongly aerobic conditions of the experiment. Perhaps the

Table V.

Manometer No.	ml. of N/100 iodine	mg. of GSH*	mg. of GSSG originally added
I	1.5†	0	0
II	4.7	9.7	10.0
III	7.6	18.5	20.0
IV	11.6	30.0	40.0

* Calculated from iodine titre after control subtracted.

† High figure due to —SH in control (from co-ferment).

presence of reducing systems such as this in yeast may account for the results obtained during some studies of the glutathione content of yeast under various conditions [Meldrum, 1930], in which it was found that practically the whole of the glutathione present remained in the reduced form even when aerated and in the presence of cyanide or narcotics.

Cysteine as oxygen carrier in the Warburg-Christian reducing system.

This experiment was run upon similar lines to the previous one, the manometric set up being identical with the exception that cysteine (added as the neutralised hydrochloride) was used in place of glutathione. In this experiment

Fig. 2. The effect of cysteine on the oxidation of hexosemonophosphate by the Warburg-Christian reducing system.

1, Control (no added cysteine). 2, 10 mg. of cysteine added. 3, 20 mg. of cysteine added. 4, 40 mg. of cysteine added.

ordinary cysteine, and not cysteine freed from traces of iron, was used. The course of oxidation of the hexosemonophosphate at 37° was followed and the results are plotted graphically (Fig. 2). During the first hour and a half of the experiment in the case of the manometers with added cysteine the oxygen

uptake was extremely rapid, this undoubtedly being primarily due to the oxidation of cysteine to cystine. Observation showed that cystine precipitated in flasks to which cysteine had been added. Subsequently the velocity of oxidation became fairly steady and was practically identical in the three experiments in which cysteine was added. However the velocity of oxidation in all three cysteine experiments was considerably greater than in the control without cysteine. At the conclusion of the experiment the amount of —SH present was determined as in the previous experiment, the results being recorded in Table VI. It is

Table VI.

Manometer No.	Nitroprusside test	ml. of <i>N</i> /100 iodine required
I	+++	1.5
II	—	2.5
III	—	2.6
IV	—	2.7

evident that practically all the cysteine had been oxidised, and had remained in this form, and it appears therefore that the factor limiting the velocity of oxidation in this instance was the velocity of reduction of the cystine, and not, as in the case of glutathione, the velocity of re-oxidation of the reduced form. The fact that no nitroprusside test was obtained in the cystine-containing manometers suggests that the cystine causes the oxidation of the small amount of glutathione present in the co-ferment.

The velocity of the anaerobic reduction of glutathione.

75 ml. Thunberg tubes were employed as usual. Eight tubes each containing 1 ml. enzyme solution, 50 mg. of co-enzyme, 10 mg. of barium hexosemonophosphate, 1 ml. of *M*/5 phosphate buffer *p*_H 7.4 and 30 mg. of oxidised gluta-

Fig. 3. The velocity of the anaerobic reduction of glutathione by the Warburg-Christian system.

thione were prepared, and two identical tubes without glutathione as controls. The final volume of liquid in each tube was made up to 6 ml. with distilled water, and each was evacuated, incubated and the amount of —SH present determined in the usual manner, estimations being made at intervals in order to obtain an

idea of the velocity of reduction. At the end of 12 hours controls without added glutathione gave an average iodine titre of 1.7 ml. of *N*/100 solution, this being due to the —SH present in the co-ferment. In Fig. 3 the absolute —SH production (calculated as glutathione) in the series of eight tubes over a 12-hour period is shown. The reduction was rapid at first and then fell off, and at the end of the time only about 60 % of the glutathione was reduced. It was not determined whether this was due to exhaustion of enzyme or to exhaustion of substrate.

The reduction of glutathione employing yeast enzyme.

In this case the enzyme of the reducing system prepared both from brewer's top yeast and from baker's yeast was employed in place of enzyme made from rat's blood. The method of studying the reduction of glutathione was identical with that described in previous experiments, the final volume of fluid in each tube being 10 ml. The set up of the experiment is shown in Table VII. The results given in Table VIII show that an active enzyme is obtainable from top yeast and that it is inactive with glucose and fructose in place of hexosemonophosphoric acid as substrate.

Table VII.

Reagent	Tube No.					
	1	2	3	4	5	6
Oxidised glutathione (mg.)	20	20	20	20	20	20
Enzyme (from brewer's top yeast) (ml.)	2	0	2	2	2	2
Co-enzyme (mg.)	50	50	0	50	50	50
Barium hexosemonophosphate synthetic (mg.)	40	40	40	0	0	0
Glucose (mg.)	0	0	0	0	40	0
Fructose	0	0	0	0	0	40
<i>M</i> /5 phosphate buffer, <i>p</i> _H 7.4 (ml.)	1	1	1	1	1	1

Table VIII.

Tube No.	Nitroprusside test	ml. of <i>N</i> /100 iodine required	Estimated GSH in mg.
1	+++	6.3	18.8
2	—	0.1	0
3	—	0.1	0
4	—	0.1	0
5	—	0.1	0
6	—	0.1	0

An exactly similar experiment was run using enzyme from baker's yeast, only the experiments with fructose and glucose were omitted. Practically identical results were obtained, the iodine titre in the presence of hexosemonophosphate being 5.3 ml. of *N*/100 solution, which corresponds to 16 mg. of reduced glutathione.

DISCUSSION.

The results of these experiments are of some interest in that they suggest that the hexosemonophosphate system may account for a considerable fraction of the reducing power of cells toward glutathione. Unpublished observations by one of us (H. T.) have shown that Harrison's glucose dehydrogenase preparation [Harrison, 1933] is able to reduce glutathione in the presence of hexosemonophosphoric acid as substrate, and this suggests that a similar system exists in liver. It is possible that such a system was responsible for some of the reduction of glutathione by liver noted by Hopkins and Elliott [1931]. It remains to be found whether or not muscle and other mammalian tissues possess this system. Up to the present time it seems as if only those enzymes which

are intimately connected with the oxidation of carbohydrates in the cell are involved in the reduction of glutathione. Whether this fact is of any significance remains to be determined.

SUMMARY.

1. Glutathione is reduced both aerobically and anaerobically by the Warburg-Christian enzyme-co-enzyme system in the presence of hexosemonophosphoric acid.

2. Phosphohexonic acid and fructosediphosphoric acid also function as donors of hydrogen in this system, the former being much less readily utilised than hexosemonophosphoric acid, and the latter being only feebly activated. Diphosphoglyceric acid, glucose and fructose are not utilised.

3. Glutathione functions as oxygen carrier when hexosemonophosphoric acid is oxidised by the Warburg-Christian system in the presence of molecular oxygen, the velocity of oxidation being roughly proportional to the amount of glutathione added. In this case the limiting factor in the oxidation velocity appears to be the rate of re-oxidation of the reduced glutathione.

4. Cystine will also function as oxygen carrier in this system, but in this case the oxidation velocity is practically independent of the cystine concentration, because the limiting factor is the rate of reduction of the cystine.

5. The oxygen uptake in the presence of glutathione is in no instance as great as that obtained by Warburg and Christian when either methylene blue or yellow pigment was employed as oxygen carrier.

6. Enzyme obtained from brewer's top yeast or from baker's yeast can be substituted for enzyme obtained from rat's blood.

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