XXVIII. GLUTATHIONE.

RELATION BETWEEN THE TISSUES AND THE OXIDISED DIPEPTIDE.

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IN his original paper on glutathione Hopkins [1921] showed that the hydrogen-ion concentration of the medium had a considerable effect on the velocity of reduction of methylene blue by tissues. This was the case both with fresh tissues and with washed tissues artificially supplied with oxidised glutathione.

He noted that, whereas at reactions slightly on the alkaline side of neutrality the addition of the oxidised dipeptide markedly accelerated the reduction of the dye, alteration of the reaction to the acid side of the neutral point resulted in a retardation of the reduction on the addition of the dipeptide. The explanation was advanced that the —SH groups formed by the reduction of the disulphide form were unable to transfer their hydrogen at an acid reaction, and thus the oxidised glutathione was acting as a competitor with the methylene blue for the available labile hydrogen of the tissues.

In view of the later results of Dixon and Tunncliffe [1923], who showed that even at p_H 5.0 reduced glutathione was able to reduce methylene blue, it appeared to be of value to continue the investigation of the effect of p_H on the systems described by Hopkins, and Hopkins and Dixon [1922].

EXPERIMENTAL.

Methylene blue technique.

Details of a typical experiment are given below.

Sheep liver was minced and washed six times with distilled water. It was then ground under alcohol, dried and again washed with distilled water. To each of a series of tubes were added 0.5 g. of the tissue, 0.3 cc. of 1/5000 methylene blue solution, and the requisite quantities of oxidised glutathione and phosphate buffer solutions. In each case the total volume was kept the same. The tubes were then evacuated and placed in the water-bath at 37°. The times for decolorisation of the dye were noted and are tabulated below.

Oxidised glutathione mg.	Reduction time (minutes)	
	at p_H 7.6	at p_H 6.2
0	145	245
2	120	210
4	90	180
8	40	122

Similar results have been obtained with both heated and unheated preparations of the muscle of the rabbit, rat, dog and cat. In no case within the limits of p_H examined has there been observed a retarding effect on the addition of oxidised glutathione.

The reactions occurring in the system may be represented as follows:

where $T \begin{array}{c} \text{H} \\ \diagup \quad \diagdown \\ \text{H} \end{array}$ represents the hydrogen donator of the tissues.

MB and MBH_2 methylene blue and methylene white.

G.S.S.G and G.SH the oxidised and reduced forms of the dipeptide respectively.

The above result shows that the direct reaction (i) is slowed by increasing acidity. The combined reactions (ii) and (iii) proceed at a greater rate than the single direct reaction no matter whether the p_H be on the acid or alkaline side of the neutral point. As might be expected from the results of Dixon and Tunncliffe [1923] the whole process (i), (ii) and (iii) proceeds more rapidly in alkaline than in acid solutions. Further analysis of the results is rendered somewhat difficult owing to the complicated nature of the system. The fact that the reaction (iii) is slowed by increasing acidity is suggestive that the effect of p_H on the whole system is due to its effect on reaction (iii).

The method throws no light on what is perhaps the most interesting of the reactions—that between the hydrogen donators of the tissue and the oxidised glutathione. An attempt has therefore been made to attack the problem directly.

The method used was as follows. A known weight of the tissue preparation was suspended in a solution of oxidised glutathione at the required p_H and the tube exhausted. After being kept for the necessary time at 37° the tube was opened and the reduced glutathione estimated as described in a previous paper [Tunncliffe, 1925]. The end point can be determined either by the use of starch, or by using sodium nitroprusside as an external indicator in the usual manner. In the case of heated tissue preparations the end point with starch is fairly sharp, but in the case of the cold washed preparation it is preferable to use the external indicator. The results by the two methods agree fairly closely.

The results of a typical experiment showing the course of reduction of oxidised glutathione by a muscle preparation are given graphically in Fig. 1.

Fig. 1.

Fig. 2.

Fig. 1. Reduction of oxidised glutathione by muscle.
Thermostable preparation of cat muscle, 0.2 g.
Oxidised glutathione, 8 mg.
Phosphate buffer solution, p_H 7.6.

Fig. 2. Reduction of oxidised glutathione by muscle.

—x—x— Washed, heated.
—●—●— Washed, unheated.

Equal weights of washed unheated rabbit muscle were used for each determination in the two series. The muscle in the second (heated) series was plunged for 5 minutes into boiling water before use.

Phosphate buffer solution, p_H 7.6.

A series of tubes was used and estimations made on separate tubes at suitable intervals. It will be observed that the reduction occurs rapidly for the first three hours, and that after this time the rate of the reaction diminishes until after five hours the reaction is complete. The amount of reduced glutathione formed is considerably less than the amount of oxidised glutathione added. This point will be referred to again in a later section of the paper.

In view of the statements of Abderhalden and Wertheimer [1923] that frog muscle can be washed until it shows no power of reducing cystine, and that the reducing power can be restored by the addition of "Kochsaft," or by heating, these points were examined in some detail.

On exposure of various tissue preparations to the air it has frequently been observed that there was a perceptible diminution in the capacity of reducing the oxidised dipeptide; particularly when exposed in thin layers in the moist state. Hopkins and Dixon [1922] also noted this fact. For this reason the tissues have been washed anaerobically by shaking with water in a stoppered cylinder through which nitrogen had been bubbled.

Frog muscle was minced by a special machine to a much finer state of division than can be obtained by chopping with scissors. It was then washed anaerobically with boiled tap water until all traces of haemoglobin were removed, and finally shaken in nitrogen for 4 hours with distilled water and toluene. After drying with alcohol in the usual manner the preparation was found to reduce oxidised glutathione and cystine vigorously.

The effect of heating the tissue preparation for a short time at 100° on the course of the reduction was also examined. The results are shown in Fig. 2 (p. 201). The reaction proceeds somewhat more smoothly in the case of the heated preparation, but within the limits of experimental error the total reduction is the same in both cases. The alteration in permeability of the preparation due to the heating would in all probability account for the greater ease of reduction in the case of thermostable preparations.

As mentioned previously the amount of oxidised glutathione added to the tissue preparation was in excess of the reducing capacity of the tissue used. An experiment was therefore carried out using in each case the same weight of muscle preparation but gradually increasing the amount of oxidised glutathione added. The reaction was allowed to proceed in each case for 6 hours and the reduced glutathione then estimated. In Fig. 3 the abscissae represent the amounts of oxidised dipeptide added and the ordinates the amounts of reduced glutathione formed. The results show clearly that below a certain amount the oxidised glutathione is practically completely reduced; after this point further increase in the amount of the oxidised form leads to no further formation of reduced glutathione. This maximum amount of oxidised glutathione which a given weight of a tissue will reduce is constant within the limit of experimental error for any given preparation, and is to be regarded as a measure of that labile hydrogen of the tissue which is concerned in the reaction

Fig. 3.

Fig. 4.

Fig. 3. Relation between amount of reduction and amount of oxidised glutathione present. For each determination 2 g. of a thermostable preparation of cat muscle was used, suspended in phosphate buffer solution p_H 7.6.

Fig. 4. Effect of p_H and nature of medium on course of reduction.

- Phosphate buffer solution p_H 7.6.
- " " " p_H 8.0.
- ×— " " " p_H 6.0.
- Solution adjusted to p_H 7.6 with dilute Na_2CO_3 solution.

The effect of changes in the nature of the medium in which the tissue was suspended was next examined. The results shown in Fig. 4 may briefly be stated as follows. No significant difference in the rate or amount of reduction

could be detected by altering the p_H or the salts of the medium. Thus there was no difference between the course of reduction in phosphate buffer solution at p_H 7.6, and that in a dilute sodium carbonate solution of approximately the same p_H .

The bearing of the facts above may now be considered in relation to the complete system (Tissue + Oxidised Glutathione + Hydrogen Acceptor). Where methylene blue is the hydrogen acceptor a clear conception of the process can be obtained.

The first point of interest is that the amount of reduced glutathione formed by a given weight of a tissue preparation in contact with excess of the oxidised dipeptide corresponds extremely well with the maximum amount of methylene blue which the same weight of the preparation will reduce. Thus 1 g. of a muscle preparation was found to reduce 6.5 mg. of the dipeptide. The same amount of tissue was found by the method described by Hopkins and Dixon [1922] to reduce between 4.8 and 4.9 cc. of 1/1000 solution of methylene blue. Calculating from the equation $2G.S.H + MB = G.S.S.G + MBH_2$ this is equivalent to 6.4 mg. of reduced glutathione.

The result of alterations of hydrogen-ion concentration on the system is also clearly explained. At p_H 7.6 and 6.0, for example, the rate of the reaction $T \begin{matrix} \swarrow H \\ \searrow H \end{matrix} + G.S.S.G$, as shown above, will be the same in both cases.

The rate of the final reaction $2G.S.H + MB$ will however be considerably less at the lower p_H and consequently the time for reduction of the methylene blue will be greater in the second of the two cases.

When oxygen is the hydrogen acceptor the bearing of the results described is more interesting and at the same time more complex. Calculating, as in the case of methylene blue, the volume of oxygen equivalent to the reduced glutathione formed by 1 g. of a thermostable muscle preparation, the oxygen uptake is found to be of the order of 140–150 mm.³. This figure is of course unaffected by the nature of the medium in which the tissue is suspended, for, as has been pointed out, the amount of reduced glutathione formed is independent of changes in the medium.

The oxygen uptake actually measured in the Barcroft apparatus is in phosphate buffer solution 400 mm.³ per g. of tissue preparation. In Ringer solution or in the absence of phosphate ions the measured uptake is increased to four times this amount, *i.e.* 1600 mm.³ per g. approximately.

There seems no doubt therefore that in addition to the oxidation of those substances which anaerobically are solely connected with the reduction of oxidised glutathione, there must also occur an aerobic oxidation of some other substance associated with the thermostable tissue residue. This additional oxidation occurs in the presence of phosphate ions but to a very much larger extent in their absence. Meyerhof [1923] has recently shown that the oxidation of an unsaturated fatty acid, linolenic acid, by oxygen, is greatly accelerated by the presence of a sulphydryl group which is also undergoing oxidation at

the same time. The oxidation of the linolenic acid in presence of a sulphydryl group is greatly diminished by phosphate ions. Meyerhof used as a sulphydryl compound thioglycollic acid. He also showed that the alcohol-ether extract of thermostable muscle preparations contained an unsaturated acid associated with the lecithin fraction. Through the kindness of Professor Hopkins I am able to state that the oxidation of unsaturated fats and fatty acids, in particular linolenic acid, by oxygen is greatly increased when the reduced form of glutathione is simultaneously undergoing oxidation. Phosphate ions also inhibit this oxidation of the double bonds of linolenic acid in the presence of reduced glutathione.

There seems therefore to be no doubt that the oxidation of the unsaturated fatty acid is the cause of the discrepancy between the oxygen uptake as measured in the Barcroft apparatus, and the uptake calculated from the amount of reduced glutathione which the preparation will form anaerobically.

Meyerhof however appears to regard the unsaturated acid as being the substance responsible for the reduction of the disulphide (dithiodiglycollic acid in his experiments) to the sulphydryl form according to the equation

This view is at variance with the results which have been described above, and the following experiments were therefore carried out to determine whether there was such a possibility in the case of glutathione. The substances shown in the table below were kept in evacuated tubes in the water-bath at 37° for 20 hours.

Tube	Linolenic acid mg.	Oxidised glutathione mg.	Medium	pH	Methylene blue 1/5000 cc.
1	20	10	Borate	6.5	0.0
2	20	10	"	6.5	0.3
3	20	10	"	7.8	0.0
4	20	10	"	7.8	0.3
5	20	10	"	8.4	0.0
6	20	10	"	8.4	0.3

Tubes 1, 3 and 5 were opened and the nitroprusside test was found to be negative in each case. There was therefore no reduction of the disulphide form to the sulphydryl form. This was confirmed by the fact that the methylene blue was not reduced in tubes 2, 4 and 6, although there was a strong adsorption of the dye on the surface of the oil droplets which separated out.

It is thus clear that linolenic acid is unable to reduce oxidised glutathione; if such reduction did take place the fact (communicated to me by Professor Hopkins) that the disulphide form causes no increase in the oxidation of the linolenic acid by atmospheric oxygen, would be meaningless.

This has also been confirmed as follows: thermostable residues were extracted for 6 hours in a Soxhlet apparatus with alcohol, ether and acetone in the order described. The total extraction lasted 18 hours and should have

removed thoroughly any fatty acid which was present. Despite this violent treatment the tissue residue still possessed over 50 % of its original capacity for reducing the oxidised dipeptide.

What diminution was observed may well have been due to oxidation of the hydrogen donors of the tissue during the extraction, since ether when boiled tends to form an ether peroxide.

The picture of the aerobic oxidation process suggested previously is thus supported. The linolenic acid is oxidised concurrently with the oxidation of the sulphydryl groups of the glutathione which are established by the reduction of the disulphide form by the hydrogen donors of the tissues. So long as the supply of the donors remains unexhausted there will be a cyclic process involving the alternate reactions

Concurrently with reaction (ii) will occur the oxidation of the double bonds of the unsaturated acid. When the hydrogen donors are exhausted reaction (i) ceases, the sulphydryl groups are oxidised, and the system ceases to take up oxygen. With the disappearance of the sulphydryl groups the oxidation of the double bonds of the unsaturated fatty acid ceases.

The effect of alteration of the hydrogen-ion concentration on the aerobic process remains to be considered. At p_{H} 7.6 the oxidation of reduced glutathione proceeds very rapidly compared with the rate at which the tissue can reduce the oxidised dipeptide. A very low concentration of the sulphydryl form would therefore be expected. This is in agreement with fact, for under the conditions described the nitroprusside reaction is exceedingly faint. The amount of reduced glutathione present was insufficient for estimation by the method described. At p_{H} 6.0 the rate of oxidation of the reduced glutathione is much slower than at p_{H} 7.6. As the rate at which the oxidised dipeptide is reduced is unaltered by alteration of p_{H} a higher concentration of the —SH form is therefore to be expected at the lower p_{H} , other conditions being the same in the two cases. This is also confirmed experimentally, both by estimation and by means of the intensity of the nitroprusside reaction, particularly in the earlier stages of the aeration of the system.

In cases where the dipeptide is originally present in the reduced form, the conditions will obviously be different from the cases in which it was added in the oxidised form. At a p_{H} of 7.6 the oxidation of the —SH groups already present will proceed with considerable rapidity and will be practically complete in one hour. There will thus be an initial rapid uptake of oxygen, after which the conditions will approximate closely to those in the system commencing with the glutathione in the —S—S— form. The final total uptake of oxygen will be greater in the case where the glutathione was originally in the —SH form, by an amount of oxygen equivalent to this quantity of —SH groups.

At a more acid reaction, *e.g.* p_H 6.0, the oxidation of the —SH groups already present will be very much slower than at p_H 7.6 and there will thus be a higher concentration of the —SH form at the lower p_H .

The exact effect of the varying concentrations of —SH groups on the velocity of oxygen uptake by the system is difficult to predict; the fact that the disulphide form as shown by Dixon and Tunncliffe accelerates the oxidation of the reduced form of the dipeptide [1923], and the close relation between the oxidation of the unsaturated fatty acids and that of the —SH groups introduce factors which render such an attempt at present inadvisable. The ultimate solution will rest on a further knowledge of the kinetics of the reaction

both in the absence and presence of unsaturated fatty acid linkages.

It is hoped to continue the investigation from this point of view.

SUMMARY.

(1) A study has been made of the reaction between the thermostable tissue residue and oxidised glutathione.

(2) The bearing of the results obtained is discussed with reference to the reduction of methylene blue, and to the uptake of oxygen by tissue residues in the presence of oxidised glutathione.

(3) Linolenic acid was found not to be the substance in the thermostable residue responsible for the reduction of oxidised glutathione.

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