CLV. THE BEHAVIOUR OF GLUTATHIONE IN YEAST.

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Introduction.

Glutathione is found in the cell largely in the reduced condition: yet knowledge of what reduces it remains much as it was when Hopkins [1921] showed that the disulphide form of glutathione was reduced to the sulphydryl form when it was incubated with kidney, liver, or skeletal muscle. This reduction is due, at least in part, to the presence of a fixed non-autoxidisable —SH group in the protein [Hopkins and Dixon, 1922] which, in reducing soluble disulphide glutathione to the sulphydryl form, becomes itself oxidised to disulphide. Bernheim and Dixon [1928] report that washed liver containing very little fixed —SH reduces much oxidised glutathione.

Many unsuccessful attempts have been made to reduce glutathione by means of a system consisting of a specific dehydrogenase, such as succinoxidase, and its substrate [Hopkins and Dixon, 1922; Elliott, 1928]. Neither Elliott nor Wieland (private communication to K. A. C. Elliott), could repeat the positive results of Wieland and Bergel [1924].

That impure glutathione oxidises spontaneously in alkaline solution was first observed by Hopkins [1921]. Following on the work of Warburg and Sakuma [1923] upon cysteine, Harrison [1924] extended their results to glutathione, and proved that traces of iron or of copper were needed for its autoxidation. Harrison added haematin to the list of catalysts, and Krebs [1929] used haemochromogens to accelerate the oxidation of cysteine. When impure glutathione is shaken with skeletal muscle (in the presence of traces of iron or of copper), an oxygen uptake results which is far in excess of that needed to oxidise the total —SH groups in the system.

Hopkins [1929] succeeded in crystallising glutathione, and found it to be a tripeptide of glycine, cysteine, and glutamic acid, while Pirie and Pinhey [1929] obtained evidence that it had the structure of glutamyl-cysteinyl-glycine. Kendall, Mackenzie, and Mason [1929] also crystallised glutathione by a different method.

Meldrum and Dixon [1930] found indications of the existence of an unknown non-metallic substance, which was present to a larger extent in the impure preparations of glutathione than in the crystalline compound. This unknown factor could be replaced by cysteine or by thiolglycollic acid, but

Meldrum and Dixon were unable to decide its precise nature. The autoxidation of glutathione in aqueous solution and the catalytic effect of the tripeptide on the oxidation of proteins appeared from their experiments to depend on the combined presence of traces of this unknown substance and of iron (or copper).

When Hopkins found that the older (impure) preparations of glutathione could oxidise proteins, fats, and fatty acids, he was studying the first thermostable oxidation system isolated from the living cell. The results were of great interest, even though the system is, perhaps, to be regarded as a model oxidation system, rather than as a representation of the actual respiratory process *in vivo*. There yet lacks a crucial experiment to decide the relationship between glutathione and respiration in the normal cell.

Two classes of substances inhibit cell respiration. At very low concentration cyanides poison the cell, apparently by preventing indophenol oxidase from oxidising cytochrome [Keilin, 1929]. In contradistinction to cyanides, narcotics act only at high concentration and inhibit respiration in proportion to their power of lowering surface tension [Warburg, 1921]. According to Keilin [1929] narcotics act by inhibiting cell dehydrogenases; thus cyanides prevent oxidation of cytochrome, and narcotics reduction of cytochrome. The object of the work here described was to study the effect on the glutathione of the cell when the respiration was controlled by various methods. If glutathione be continually reduced by the reducing mechanisms of the cell, and oxidised by the oxidising mechanisms, then those factors which inhibit the one set of reactions rather than the other should produce some change in the content of reduced glutathione. The above-mentioned contrasting effects of narcotics (and of exposure to cold and starvation) on the one hand, and of cyanides on the other, were therefore employed to bring about the desired partial inhibition of the oxidation-reduction processes in the yeast cell, comparisons of the glutathione content of yeast cells being made under the following conditions: (1) saturated[1] cells, under aerobic and anaerobic conditions, (2) saturated cells (and also zymin), with and without cyanide, (3) saturated cells (and zymin), with and without urethane, (4) saturated cells and starved[1] cells, (5) starved cells with and without urethane, (6) cells at 37° and cells at 0°.

Experimental.

5 g. of fresh baker's yeast were suspended in phosphate buffer at p_H 7·3 or 7·6. In some experiments glucose was present in 1 % concentration. The suspension was aerated in gas wash-bottles maintained at 37° in a water-bath. The results were not affected by the rate of aeration, which was always rapid when glucose was present. All experiments were done in duplicate unless otherwise stated. After aeration the suspensions of yeast were poured on to solid trichloroacetic acid, which dissolved to give a 10 % solution. The sus-

[1] "Saturated" cells are cells living in excess of 1 % glucose solution. "Starved" cells are those kept in suspension and aerated for some hours without food, till their respiration rate has fallen below 2000 mm.^{3} O_2/g. hour.

pension was rapidly brought to the boil and boiling was continued for 1·5–2 min. The mixture was cooled, brought to a known volume and centrifuged. The clear liquid was then titrated with $M/100$ iodine in potassium iodide. Starch was used as indicator in most cases; it gave slightly higher titres than did nitroprusside, but the end-points were sharp and the titration of duplicates agreed very well. It was found best to prepare fresh indicator before each set of titrations. In order to correct for the effect of cyanide and of the urethane upon the titration it was necessary to multiply the figures obtained in presence of these substances by 0·945 and 1·05 respectively. The results of these experiments are shown in Table I.

Table I.

Exp. No.	Period of aeration	p_H	Titre of control	Titre in $M/100$ KCN	Titre in 5 % ethyl-urethane	Titre from cooled suspensions	Remarks
1	0	7·6	0·710	0·703	0·728	—	In 1% glucose. Yeast warmed 25 min. at 37° then KCN and urethane added immediately and 2 g. CCl_3COOH
2	½ hr.	7·6	0·65	0·64	0·66	—	—
3	1½ hr.	7·6	1·03	1·11	1·19	—	—
4	3½ hr.	7·6	0·89	0·75	0·73	—	1 % glucose. 0·05 % phenylurethane in place of 5 % ethylurethane
5	4 hr.	7·6	0·56	0·61	0·48	—	—
6	4 hr.	7·6	0·78	0·91	0·74	—	—
7	—	7·6	0·66	0·67	0·72	—	—
8	½ hr.	7·3	0·69	0·62	—	0·60	1 % glucose
9	1¾ hr.	7·3	0·74	0·76	0·76	0·63	1 % glucose
10	40 min.	7·3	0·57 0·62*	—	—	—	
11	3 hr.	7·3	0·73	0·69	0·79	—	1·5 g. zymin in place of yeast
12	2½ hr.	7·6	0·61	0·58	0·61	—	1·2 g. zymin and 0·25 mg. cysteine hydrochloride = 0·08 cc. I_2 solution
13	1½ hr.	—	0·67	0·64	0·70	—	1·2 g. zymin in 20 cc. water

* Anaerobic control.

Exps. 1, 5, 6 and 8 were not done in duplicate. In all other cases the figures are the means of two determinations on trichloroacetic acid extract from separate portions of yeast suspension in different aeration flasks. These titrations agree within the limits of experimental error, which is about 10 %. In Exps. 11, 12 and 13 zymin was treated just as was the yeast in Exps. 1–10. In the experiments on the effect of cooling, the suspensions were first aerated at 37° and at 0° for about half an hour, by which time the temperatures were constant. Then urethane or potassium cyanide was added as desired. The anaerobic experiments were carried out in large vacuum tubes, evacuated and filled with nitrogen. No particular care was taken to remove traces of oxygen from the nitrogen, as it was felt that the yeast would rapidly use the small amount of oxygen which was present as impurity.

The figures in Table II indicate the degree of inhibition of the respiration of the yeast which was brought about by the urethane and the potassium cyanide.

Table II. *Effect of cyanides and of urethane on rate of respiration of yeast.*

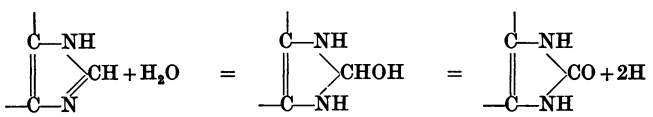
Control mm.^{3} O_2/g. hr. wet wt. yeast	KCN M/100	Urethane	% inhibition by KCN	% inhibition by urethane	Remarks
10,730	1130	0	90	100	In 1 % glucose and 15 % urethane
8,100	1704	800	77·9	90	10 % urethane
6,540	1630	2450	75	62·5	5 % urethane
7,020	3000	3120	57·1	55·5	5 % urethane
41,140	—	5650	—	86·4	In 1 % glucose and 5 % urethane
1 315	—	—	—	—	After 3½ hr. aeration in buffer

The yeast was always aerated in phosphate buffer at p_H 7·6. The true respiration rate was measured, and the rate of shaking and CO_2 output had no effect on the apparent uptake of oxygen; the temperature was 37°. The variation in the percentage inhibition by potassium cyanide is considerable, and may be due to variation in the amount of reducing substances in the yeast.

In order to test the activity of the indophenol oxidase use was made of the "Nadi" reagent. After aeration some of the yeast was diluted ten times, 5 cc. of "Nadi" reagent were added, and aeration was continued at 37°. Strongly positive reactions were obtained in all suspensions containing urethane, even in those aerated for 4 hours. No suspension containing potassium cyanide gave a positive reaction. Control suspensions aerated in the absence of glucose for 4 hours gave a weakly positive test on dilution alone. In the presence of glucose no reaction could be obtained even on diluting after aeration for 4 hours, until the yeast was warmed at 52° for 1½ hours, as described by Keilin [1929]. It seems clear that the treatment of the yeast did not destroy the indophenol oxidase.

The relationship of glutathione to the cyanide-stable respiration of yeast.

From the above results it is clear that the respiration of yeast may vary in amount within wide limits without the amount of reduced glutathione being affected. When however yeast or any other cell is poisoned by cyanide, there still exists a small residual respiration, probably due to enzymes such as xanthine oxidase. When xanthine oxidase acts on its substrate xanthine, hydrogen peroxide seems to be formed according to the equations:



$$2H + O_2 = H_2O_2$$

It is possible that glutathione is oxidised by hydrogen peroxide produced in some such way, and that this is one of the normal modes of oxidation. The disappearance of glutathione when liver is allowed to autolyse in air may be due to a similar oxidation by hydrogen peroxide. To test this hypothesis the following experiments were devised. Thermostable muscle powder was put in each cup of a Barcroft differential manometer and subjected to the action of hydrogen peroxide produced by xanthine oxidase and hypoxanthine, in the presence and in the absence of glutathione. Crystalline glutathione in such circumstances becomes stabilised to the action of oxygen [Meldrum and Dixon, 1930]. The results are shown in Table III, and demonstrate clearly that hydrogen peroxide produced during the action of xanthine oxidase on hypoxanthine can oxidise glutathione which is normally stable towards oxygen. Such hydrogen peroxide also reacts with fixed —SH in tissue, but less rapidly than with glutathione. No attempt was made to analyse the kinetics of the reaction.

Table III.

No. of Barcroft cup ...	1		2		3	
	L.	R.	L.	R.	L.	R.
Amount of GSH	0	5 g.	0	5 g.	0	0
Enzyme	0	0	0·15 g.	0·15 g.	0	0·15 g.
Skeletal muscle	0·2 g.	0·2 g.	0·2 g.	0·2 g.	0·2 g.	0·2 g.
Nitroprusside reaction:						
In muscle	+ + +	+ + +	+	?	+ + +	+
In fluid	− −	+ +	− −	?	− −	− −

Harrison [1924] showed that secondary oxidations went on in the presence of sulphydryl compounds which, perhaps, do not react so readily with hydrogen peroxide as does catalase. At any rate, experiments with methylene blue show the xanthine oxidase to be destroyed to the same extent when glutathione is present as when it is absent. Glutathione does not seem to possess the protective action of catalase [Dixon, 1925]. It seems unlikely that glutathione can be oxidised by hydrogen peroxide in any cell containing active catalase.

Discussion.

The almost universal distribution of glutathione shows that it must be of vital importance to the cell. Yet the glutathione content of any one tissue has not so far been correlated with the predominance or comparative absence of any one type of metabolism, and there seems little evidence for the statement [Mitchell and Hamilton, 1929] that greater content of glutathione goes hand in hand with greater rate of respiration. It is unfortunate that no one has yet succeeded in changing the content of reduced glutathione of any tissue respiring normally *in vivo*. Szent-Györgyi [1925] noticed this inertness of reduced glutathione: he aerated liver for 15 min. after which the amount of reduced glutathione had scarcely decreased. His work was criticised by Hopkins on the grounds that he did not aerate sufficiently long (aeration

for about 8 hours would remove all the reduced glutathione) and that he did not determine the p_H. Elliott (unpublished work) found no change in the reduced glutathione of frog's muscle after the muscle had been stimulated. Uyei [1926] starved dogs, rabbits, and guinea-pigs without affecting the amount of reduced glutathione in their tissues.

Now the aqueous extracts of most tissues show a nitroprusside reaction for the —SH group. This nitroprusside reaction is not intensified by preliminary treatment of the extract with cyanide which reduces the —S—S— to —SH. Glutathione is kept mainly reduced in the cell, presumably by several reducing factors. Fixed —SH groups in muscle form one such factor, but the work here described shows that dehydrogenase systems can play no part in this process. We know that muscle and liver proteins reduce glutathione only slowly; if then fixed —SH in proteins be the only group reducing glutathione, since the rate of oxidation is even slower, the oxygen uptake for which glutathione is responsible must be small indeed. It must be realised that this in no way affects the importance of glutathione even as an oxidising agent; for it may be that glutathione catalyses oxidations, which, though small in extent, are yet necessary to the cell for its existence, just as vitamins, of negligible calorific value, are essential constituents of food.

The interaction of glutathione with cytochrome C *in vitro* takes place very slowly indeed, and it seems evident from the work here reported that glutathione reduces cytochrome *in vivo* only to a negligible extent. Even when the cytochrome-indophenol oxidase system gives a very strong positive reaction with the "Nadi" reagents, the content of glutathione is unchanged, whether the inhibition of the dehydrogenases be produced by urethane or by cooling, or whether starvation of the yeast be carried to such an extent that the dehydrogenase systems no longer saturate the cytochrome-indophenol oxidase system, and the yeast becomes able to give the "Nadi" reaction merely on dilution.

It is clear that another problem remains untouched, namely, whether the fact that living cells always contain some soluble sulphydryl substance is due to the slow oxidation of the sulphydryl group or to the rapid reduction of the disulphide group. In either case the superficial result will be a great excess of —SH over —S—S—, but the oxygen uptake will differ widely in the two cases. The problem of the reduction of glutathione dates from 1922 and it seems little nearer solution now. The question further arises as to what oxidises glutathione, which itself scarcely reacts with oxygen. Glutathione does not reduce cytochrome readily. Is it oxidised by a specific oxidation system? The system mentioned above in which glutathione is oxidised by hydrogen peroxide, itself produced by enzymic oxidations, is admittedly a model system. Under the conditions of the experiment not only was glutathione oxidised but also the fixed —SH, though not to the same extent as if glutathione had been present. Yet this is the only mechanism found so far which will cause —SH in tripeptide glutathione to disappear with reasonable speed

under conditions which approximate, though but remotely, to those in the cell.

The part played by glutathione in the life of the cell is still obscure in the extreme, and it is possible that it is connected with protein metabolism, as has been suggested [Waldschmidt-Leitz, Purr and Balls, 1930], rather than with cell oxidations.

SUMMARY.

1. The respiration of yeast may be reduced by 60 % to 90 % (*a*) by narcotics, which inhibit the reducing systems, (*b*) by cyanide, which prevents the indophenol oxidase from oxidising cytochrome, without the amount of reduced glutathione being affected. Cooling has likewise no effect, though this is known to retard the dehydrogenases more than the indophenol oxidase.

2. Glutathione does not reduce cytochrome in yeast.

3. No evidence could be found that dehydrogenases reduce glutathione with simultaneous oxidation of their substrates, and it seems unlikely that glutathione is in any way concerned with the cytochrome-indophenol oxidase system.

4. Yeast may be starved till its rate of respiration falls to about 5 % of that in glucose solution without affecting the thiol content.

5. No evidence has been obtained that glutathione is directly concerned in carbohydrate oxidation by yeast.

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REFERENCES.

Bernheim and Dixon (1928). *Biochem. J.* **22**, 125.
Dixon (1925). *Biochem. J.* **19**, 507.
Elliott (1928). *Biochem. J.* **22**, 1410.
Harrison (1924). *Biochem. J.* **18**, 1009.
Hopkins (1921). *Biochem. J.* **15**, 286.
—— (1929). *J. Biol. Chem.* **84**, 269.
Hopkins and Dixon (1922). *J. Biol. Chem.* **54**, 527.
Keilin (1929). *Proc. Roy. Soc.* B **104**, 206.
Kendall, Mackenzie and Mason (1929). *J. Biol. Chem.* **84**, 657.
Krebs (1929). *Biochem. Z.* **204**, 322.
Meldrum and Dixon (1930). *Biochem. J.* **24**, 472.
Mitchell and Hamilton (1929). Amino-acids, p. 399. (Chem. Catalog Co., New York.)
Pirie and Pinhey (1929). *J. Biol. Chem.* **84**, 321.
Szent-Györgyi (1925). *Biochem. Z.* **157**, 50.
Uyei (1926). *J. Inf. Dis.* **39**, 73.
Waldschmidt-Leitz, Purr and Balls (1930). *Naturwiss.* 644.
Warburg (1921). *Biochem. Z.* **109**, 134.
Warburg and Sakuma (1923). *Pflüger's Arch.* **200**, 203.
Wieland and Bergel (1924). *Liebig's Ann.* **439**, 209.