XXVIII. THE SECONDARY OXIDATION OF SOME SUBSTANCES OF PHYSIOLOGICAL INTEREST.

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In a recent paper by Thurlow [1925] evidence was given for the formation of hydrogen peroxide during certain enzymic and non-enzymic oxidations and it was shown that in the presence of peroxidase this hydrogen peroxide could bring about the oxidation of nitrites. Many important oxidations of the cell may possibly be brought about by means of a similar mechanism and the present paper describes work carried out to study the effect of replacing nitrite by other substances of physiological importance.

In various experiments with the xanthine oxidase of milk, we had observed that in certain cases, when milk peroxidase was added to the xanthine oxidase with hypoxanthine, the total oxygen uptake of the system was considerably in excess of that theoretically required for the oxidation of the hypoxanthine to uric acid. This observation suggested that some substance other than hypoxanthine might be undergoing oxidation, possibly in a manner comparable to the oxidation of nitrite in the systems mentioned above. The xanthine oxidase preparations had been made according to the method described by Dixon and Thurlow [1924], involving precipitation of the enzyme together with the caseinogen from milk by half saturation with ammonium sulphate. Part of the fat had been subsequently removed by extraction of the partially dried precipitate with ether. It was thought that the substance undergoing oxidation might be fat which had escaped removal during the ether extraction. In order to investigate this possibility experiments were carried out to compare the oxygen uptake of a system containing unextracted enzyme preparation with one containing the same enzyme preparation from which fat had been removed by prolonged extraction with ether.

A xanthine oxidase preparation was made by half saturating milk with ammonium sulphate. The precipitate, after being dried on filter paper, was shaken once with twice its own volume of ether. This process results in a removal of most of the colouring matter of milk but leaves behind a large quantity of the fat. The precipitate was then dried *in vacuo* and powdered. This will be referred to as the "unextracted preparation."

In order to obtain a fat-free preparation, a portion of this was shaken again with twice its volume of ether, dried *in vacuo* and finely powdered. This second extraction with ether facilitates the grinding of the dried product to a

fine powder. The powder was then extracted continuously with ether in a Soxhlet apparatus for about 1 hour, the excess ether absorbed with filter paper, and the product dried *in vacuo*. This will be called the "extracted preparation." A solution of the enzyme was made by adding the powdered preparation to phosphate buffer of p_H 7·6 and allowing it to stand in a water-bath at 45° for about half an hour. The resulting solution, which is acid, is brought back to p_H 7·6 by addition of the requisite amount of 10 % sodium hydroxide solution. The actual concentration of enzyme solution used varied according to the activity of the particular enzyme preparation, the usual concentration being 3 g. of the dry preparation in 10 cc. of solution.

The peroxidase preparation was made from milk according to the method described by Thurlow [1925], namely, by saturating with ammonium sulphate the filtrate which remained from the milk which had previously been half saturated with ammonium sulphate. The peroxidase is brought down on the lactalbumin and this precipitate was filtered off and dissolved in a small amount of phosphate buffer of p_H 7·6, the necessary amount of sodium hydroxide being added to restore the p_H to 7·6.

The hypoxanthine, or xanthine, used was finely powdered, suspended in water and brought into solution by addition of the minimum amount of sodium hydroxide. The usual concentration employed was 10 mg. per cc.

The oxygen uptake was measured by means of the Barcroft micro-respirometer and unless otherwise stated the experiments were carried out at 37°. The final volumes quoted have been corrected to N.T.P. (allowing for the V.P. of water).

The right-hand flask of each apparatus contained 1·5 cc. of xanthine oxidase preparation, 1 cc. of either peroxidase solution or phosphate buffer of p_H 7·6, and the amount of hypoxanthine indicated in the table. The volume was in each case made up to 3 cc. with buffer. The control flasks of the apparatus contained the same amount of enzyme preparation and peroxidase solution as was placed in the experimental flask, the final volume being made up to 3 cc. with buffer. Since the only difference in the contents of the control flask and the experimental flask was, in each case, the presence of hypoxanthine in the latter, any oxygen uptake observed must necessarily be due to the oxidation of hypoxanthine itself or to some secondary oxidation induced by the oxidation of the hypoxanthine.

The apparatus was mechanically shaken in the bath for five minutes before closing the taps in order to establish temperature equilibrium. The initial rates of oxygen uptake in these experiments were found to be almost linear and, hence, in order to allow for the oxidation taking place during this time, the oxygen uptake during the five minutes immediately following the closing of the taps was noted and an equal amount added to the final uptake observed. Four of the instruments could be shaken side by side in the same bath.

All the experiments described were carried out in duplicate, and in nearly every instance the figures quoted are typical of a considerable number of

results. Table I shows the effect of milk peroxidase on the oxygen uptakes of unextracted and extracted xanthine oxidase preparations in presence of hypoxanthine (Hx).

Table I.

No. of Exp.	Contents of flask	Theoretical O_2 uptake for Hx added	Observed uptake	Observed uptake with peroxidase
		mm.³	mm.³	mm.³
1	Unextracted preparation + Hx (1 mg.)	165	164	193
	Extracted preparation + Hx (1 mg.)	165	162	168
2	Unextracted preparation + Hx (1 mg.)	165	174	252
	Extracted preparation + Hx (0·91 mg.)	150	151	159
3	Unextracted preparation + Hx (1 mg.)	165	166	215
	Extracted preparation + Hx (1 mg.)	165	176	184

It can be seen from these results that when the extracted enzyme preparation was used, both with and without peroxidase, the oxygen uptake observed was close to the theoretical uptake for the amount of hypoxanthine added. When the unextracted preparation was used without peroxidase, the oxygen uptake observed was again practically theoretical; but when peroxidase was added to the unextracted enzyme preparation plus hypoxanthine, the oxygen uptake was in every instance considerably in excess of the theoretical uptake for the amount of hypoxanthine used. It seems evident from these results that there is present in the unextracted preparation some substance which is oxidised when hypoxanthine is oxidised in the presence of peroxidase, but is not oxidised to any appreciable extent when peroxidase is absent. The fact that the peroxidase is necessary for this oxidation would seem to indicate that the oxidising agent is hydrogen peroxide, the peroxidase serving to activate this. The oxidisable substance appears to be largely removed from the enzyme preparation by thorough extraction with ether, since, when the extracted enzyme preparation is used, the oxygen uptake, either with or without peroxidase, is close to the theoretical for the amount of hypoxanthine used.

In a few exceptional cases we have obtained enzyme preparations which give with hypoxanthine, in the absence of peroxidase, an oxygen uptake somewhat in excess of theoretical, both before and after extraction with ether. The reason for this we have not so far been able to explain.

In experiments, described later in the paper, we found that the oxidation of lactic acid and β-hydroxybutyric acid could be induced if milk peroxidase were replaced by a ferrous salt. Accordingly experiments were carried out to ascertain whether ferrous iron could replace natural peroxidase in the system unextracted enzyme preparation plus hypoxanthine. The results of these experiments are given in Table II, Exps. 1–3, and show that ferrous iron can act like milk peroxidase in bringing about a similar high uptake with the unextracted preparation and that this uptake is reduced after the extraction of the enzyme with ether. Exactly equal quantities of iron as ferrous ammonium sulphate were added, together with xanthine oxidase, to the control flasks of

the apparatus, to eliminate any additional oxygen uptake due to the oxidation of the ferrous salt.

Preliminary experiments showed that in phosphate buffer solution at p_H 7·6, ferrous ammonium sulphate is oxidised very rapidly, and that the greater part is oxidised while the apparatus is attaining temperature equilibrium before the taps are closed. Further, the amount of iron added was equivalent only to a relatively very small volume of oxygen, so that any increased uptake observed in the presence of ferrous iron could not be due merely to the oxidation of the iron.

Table II.

No. of Exp.	Contents of flask	Theoretical O_2 uptake for Hx added mm.³	Iron added	Observed O_2 uptake mm.³
1	Unextracted preparation + Hx (1 mg.)	165	—	160
	" " + " (")	165	+0·14 mg. Fe as ferrous iron	236
2	" " + " (")	165	—	176
	" " + " (")	165	+0·14 " "	214
3	Extracted " + " (")	165	—	155
	" " + " (")	165	+0·14 " "	173
4	Unextracted " + " (")	165	—	175
	" " + " (")	165	+0·03 " as haemoglobin	246
	Extracted " + " (")	165	—	170
	" " + " (")	165	+0·03 " "	174
	" " + " (")	165	+0·06 " "	171
5	Unextracted " + " (")	165	—	163
	" " + " (")	165	+0·03 " "	220
	Extracted " + " (")	165	—	150
	" " + " (")	165	+0·03 " "	175
	" " + " (")	165	+0·06 " "	166
6	Unextracted " + " (")	165	—	157
	" " + " (")	165	+0·22 " as haematin	253
	Extracted " + " (")	165	—	153
	" " + " (")	165	+0·22 " "	216
7	Unextracted " + " (")	165	—	184
	" " + " (")	165	+0·22 " "	249
	Extracted " + " (")	165	—	150
	" " + " (")	165	+0·22 " "	194

Exps. 4–7 in Table II show that both haemoglobin and haematin can act very efficiently as peroxidases in bringing about the secondary oxidation of ether-soluble substances in the unextracted preparation. After the extraction of the preparation with ether, the uptake with haemoglobin in presence of hypoxanthine is reduced practically to theoretical, but with haematin, though the uptake is reduced by extraction, it is still considerably in excess of that given by the extracted preparation plus hypoxanthine alone. It would seem therefore that the oxidation of some ether-insoluble substance or substances occurs in the extracted preparation in presence of haematin. Possibly, under the influence of a strong peroxidase like haematin, an oxidation of uric acid might be taking place. We hope to carry out further investigation on this point. In the case of haemoglobin, the haemoglobin in the flask containing hypoxanthine is very rapidly oxidised to methaemoglobin,

while the haemoglobin in the control flask remains practically unchanged throughout the experiment. This change to methaemoglobin takes place almost completely during the time that the apparatus is attaining equilibrium in the bath before the taps are closed; further, any gaseous exchange involved in the conversion of the small amount of oxyhaemoglobin employed into methaemoglobin would be negligible compared with the uptakes being measured, so that this change cannot account for the increased uptake. That the extra uptake cannot be due to oxidative breakdown of the haemoglobin molecule was shown by taking equal volumes of the solutions in the control and experimental flasks at the end of an experiment, making alkaline with Na_2CO_3 and reducing to haemoglobin with sodium hydrosulphite. After shaking with air, both solutions when viewed spectroscopically gave the bands of oxyhaemoglobin with approximately equal intensity, showing that no appreciable permanent destruction of the haemoglobin molecule had taken place.

Incidentally, this oxidation of haemoglobin to methaemoglobin, which takes place only in the presence of the complete primary system hypoxanthine plus enzyme, affords another example of a secondary oxidation brought about during the oxidation of the primary system. This oxidation, as is well known, is readily brought about by hydrogen peroxide and would therefore be expected in this system.

A similar conversion of oxyhaemoglobin to methaemoglobin by peroxide in sterile extracts of pneumococci is described by Neill and Avery [1924].

The haemoglobin used in our experiments was a 20 % aqueous solution. It was prepared by treating blood corpuscles successively with ether and salt as described by Adair [1925]. The haematin solution was made by dissolving 0·25 g. of pure haematin in three equivalents of 10 % NaOH and making the solution up to 10 cc. with water[1].

During the winter months we have been unable to obtain the additional oxygen uptakes with the unextracted enzyme in presence of milk peroxidase or ferrous iron. It seems possible that this may be due to a change in the food of the cows especially as it is known that the fats found in the milk show a close parallelism with those of the diet.

The additional oxygen uptake obtained in presence of haemoglobin and haematin however has remained unimpaired.

Attempts were made to restore the fat extracted from the enzyme preparation and so produce an increased oxygen uptake with a peroxidase. The greater part of the ether from the extract in the Soxhlet apparatus was boiled off, the concentrated solution of fat then being poured over the extracted preparation and the remaining ether removed *in vacuo*. The resulting preparation gave no increased uptake with ferrous iron or haemoglobin however. Probably the readily oxidisable substances in the fat had already been oxidised by the ether peroxide formed during the extraction.

[1] We wish to thank Mr G. S. Adair for the haemoglobin and Mr R. Hill for the haematin used in these experiments.

Several experiments were carried out using inorganic ferric iron instead of haematin. The iron, in the form of ferric chloride, was added to each flask in an amount equivalent to 0·35 mg. of iron and in no case was any extra uptake of oxygen observed. This inability of free ferric iron to act as a peroxidase might well be expected, since it is incapable of giving the ordinary peroxidase reactions. But it would appear somewhat difficult to explain the peroxidase activity of ferrous iron since the latter, to a large extent at least, is, under the conditions of the experiment, very rapidly oxidised to ferric iron. Possibly during the reaction a small concentration of iron is maintained in the ferrous state by the formation of a complex which can act as a peroxidase in the system.

While it is felt to be unsatisfactory that we have not as yet been able to determine definitely whether these increased uptakes in presence of a peroxidase are actually due to the oxidation of fat, we have nevertheless given the results which we have obtained up to the present, as they appear to have a definite bearing on secondary oxidations.

Oxidation of Lactic Acid.

It has been known for some time that hydrogen peroxide is capable of bringing about the oxidation of lactic acid, especially in the presence of a ferrous salt. Fenton and Jones [1900] showed that, at low temperatures, free lactic acid is oxidised by hydrogen peroxide to pyruvic acid, and Dakin [1908] showed that by distilling ammonium lactate with hydrogen peroxide under reduced pressure acetaldehyde is formed. Since the oxidation of lactic acid plays so important a rôle in physiological processes, it would be of great interest if the oxidation of lactic acid could be correlated with such oxidising systems as the xanthine oxidase plus one of its substrates, or with a non-enzymic oxidation such as that of the sulphydryl compounds.

Experiments were therefore carried out to determine whether the oxidation of lactic acid could be induced during the oxidation of hypoxanthine or xanthine in the presence of xanthine oxidase. The results of some of these experiments are given in Table III.

Table III.

No. of Exp.	Contents of flask	Theoretical O_2 uptake for Hx or xanthine used mm.³	Observed uptake mm.³	Increase due to oxidation of lactic acid mm.³
1	X. oxidase + Hx (1 mg.) + Fe^{++} (0·14 mg.)	165	183	—
	X. oxidase + Hx (1 mg.) + Fe^{++} (0·14 mg.) + 0·4 cc. Na lactate	165	213	30
2	X. oxidase + Hx (1 mg.) + Fe^{++} (0·14 mg.)	165	173	—
	X. oxidase + Hx (1 mg.) + Fe^{++} (0·14 mg.) + 0·4 cc. Na lactate	165	219	46
3	X. oxidase + xanthine (2 mg.) + Fe^{++} (0·14 mg.)	165	165	—
	X. oxidase + xanthine (2 mg.) + Fe^{++} (0·14 mg.) + 0·4 cc. Na lactate	165	206	41
4	X. oxidase + xanthine (2 mg.) + Fe^{++} (0·14 mg.)	165	155	—
	X. oxidase + xanthine (2 mg.) + Fe^{++} (0·14 mg.) + 0·4 cc. Na lactate	165	212	57

The iron was added as ferrous ammonium sulphate, and extracted enzyme preparation was used in these experiments. A number of different samples of lactic acid were used, and all gave similar results. The free syrupy acid was neutralised with 40 % sodium hydroxide, being cooled under the tap during the addition of the alkali. The oxidation of lactic acid was not induced to any appreciable extent by milk peroxidase, but it is evident from Table III that it can be brought about by substituting a small quantity of a ferrous salt for the milk peroxidase. Xanthine appears to bring about the oxidation as readily as hypoxanthine. An equal quantity of ferrous iron was added to the control flask together with lactic acid and, as explained above, the increased uptake observed with lactic acid cannot be due to the oxidation of the iron. It was found that no appreciable oxidation of lactic acid took place unless the latter were present in fairly high concentration. This is understandable, for the hydrogen peroxide formed during the oxidation of hypoxanthine can also serve to bring about the oxidation of further hypoxanthine in the presence of its enzyme, so that we may expect the hydrogen peroxide to be shared between the hypoxanthine and lactic acid (or other secondary substrate), the amount of the oxidation of the latter depending both on its concentration and on the relative ease with which it is oxidised by hydrogen peroxide in the presence of a peroxidase. Thus, though the oxygen uptake due to the oxidation of lactic acid is relatively small, and represents the oxidation of only a small proportion of the lactic acid present, this would nevertheless be expected from the nature of the system as will be further pointed out later on. The relatively high concentration of lactic acid required does not necessarily mean that such an oxidation is unphysiological, for localised high concentrations of lactic acid are certainly present in the living body.

It was shown by Thurlow [1925] that the oxidation of nitrites could also be brought about (in the presence of peroxidase), if the sulphydryl compounds cysteine or glutathione were substituted for the systems xanthine oxidase plus hypoxanthine or xanthine. In their oxidation these two pairs of enzymic and non-enzymic systems appear to be in many ways equivalent. Accordingly experiments were made to find whether the oxidation of lactic acid could be induced during the oxidation of cysteine. The results of these experiments are summarised in Table IV.

Table IV.

No. of Exp.	p_H	Theoretical O_2 uptake for cysteine used mm.³	Observed uptake for cysteine alone mm.³	Lactic acid added cc.	Observed uptake for cysteine + lactic acid mm.³	Increase due to oxidation of lactic acid mm.³
1	6·0	250	260	0·4	313	53
2	6·0	178	144	0·4	232	88
3	6·0	178	145	0·4	272	127
4	6·2	178	163	0·4	267	104
5	6·2	250	254	0·2	319	65
6	6·2	250	252	0·4	349	97
7	7·6	178	186	0·4	236	50
8	7·6	214	209	0·4	270	61
9	7·6	178	179	0·2	239	60
10	7·6	178	180	0·4	274	94

Addition of peroxidase gave no appreciable increase in oxidation of lactic acid, and the oxidation was not much affected by changes in p_H from 6·0 to 7·6.

It is evident from these results that a definite oxidation of lactic acid is brought about during the oxidation of cysteine. Further, this oxidation can take place without the addition of a peroxidase. It has been shown by Warburg and Sakuma [1923] and Harrison [1924] that the oxidation of the sulphydryl compounds cysteine and glutathione is a catalysis by iron, and that preparations of these compounds usually contain iron. It is believed that the iron catalyses the reaction between oxygen and the sulphydryl group by forming a complex with the latter. The presence of a labile ferrous compound is maintained in solution as long as any unoxidised sulphydryl compound remains, and this ferrous compound might well be expected to act as an efficient peroxidase in the oxidation of lactic acid induced by the oxidation of cysteine.

An attempt was made to find what substance or substances are produced by the oxidation of lactic acid.

1 cc. of cysteine solution containing 100 mg., brought to p_H 7·6 with sodium hydroxide, was added to 0·4 cc. of sodium lactate and 8·6 cc. p_H 7·6 phosphate buffer. The solution was shaken in presence of air in a closed flat-bottomed flask at 37° in a water-bath. Two control flasks containing 1 cc. of cysteine solution plus 9 cc. buffer, and 0·4 cc. sodium lactate plus 9·6 cc. buffer respectively, were shaken at 37° for the same length of time. When all the cysteine was oxidised (about 4½ hours) the flasks were cooled and 8 cc. of solution was pipetted from each and distilled into 1 cc. of ice-cold water, the distillation being continued until about 3 cc. had distilled over. Rimini's test for acetaldehyde was then applied by adding two drops of a saturated solution of sodium nitroprusside and two drops of piperidine. The solution which had contained cysteine and sodium lactate gave a positive reaction, whilst the two controls were completely negative. The remainder of each of the solutions was tested for pyruvic acid by the nitroprusside test and the guaiacol test [Quastel, 1924], negative results being in every case obtained. It is thus evident that the product of this secondary oxidation of lactic acid is acetaldehyde. It seems worthy of note that if a similar oxidation of lactic acid occurs when hypoxanthine plus the enzyme preparation from milk replaces cysteine as the primary system, it would be expected that the acetaldehyde formed would be still further oxidised, since the enzyme preparation contains the Schardinger enzyme.

Oxidation of β-hydroxybutyric acid.

Preliminary experiments showed that the oxidation of β-hydroxybutyric acid by hydrogen peroxide in neutral solution at 37° can be detected by applying the nitroprusside test for acetoacetic acid and acetone, and that the oxidation is accelerated by addition of ferrous iron or milk peroxidase.

Experiments were then carried out to find whether the oxidation of β-hydroxybutyric acid could be induced during the oxidation of hypoxanthine plus xanthine oxidase, and during the oxidation of cysteine. The β-hydroxybutyric

acid used in these experiments was a sample from Kahlbaum, and contained no appreciable quantity of acetone or acetoacetic acid. The solution employed was obtained by neutralising the syrupy acid with 40 % sodium hydroxide, cooling under the tap during the addition of the alkali.

The experiments given in the following table were carried out using hypoxanthine plus xanthine oxidase as the primary oxidising system. The solutions were brought to a volume of 3·9 cc. with p_H 7·6 buffer, the iron being added as ferrous ammonium sulphate. After aerating slowly for 3½ hours at 20°, the solutions were saturated with ammonium sulphate, filtered, treated with a few drops of ammonia plus sodium nitroprusside and allowed to stand for about 15 mins. for development of colour.

No. of tube	Contents of tube	Nitroprusside test
1	2 cc. X. oxidase + 0·2 cc. β-hydroxybutyric acid + 1·5 cc. milk peroxidase	–
2	2 cc. X. oxidase + 0·2 cc. β-hydroxybutyric acid + 1·5 cc. milk peroxidase + 2 mg. Hx	–
3	2 cc. X. oxidase + 0·2 cc. β-hydroxybutyric acid + 2 mg. Hx	–
4	2 cc. X. oxidase + 0·2 cc. β-hydroxybutyric acid + 0·14 mg. $Fe^{\cdot\cdot}$	–
5	2 cc. X. oxidase + 0·2 cc. β-hydroxybutyric acid + 0·14 mg. $Fe^{\cdot\cdot}$ + 2 mg. Hx	+ +

It is evident from these results that hypoxanthine plus enzyme can bring about the oxidation of β-hydroxybutyric acid in the presence of ferrous iron, but not to any appreciable extent in the presence of milk peroxidase.

Similar experiments were then carried out using cysteine instead of hypoxanthine plus xanthine oxidase. The tubes were aerated slowly until the oxidation of the cysteine was complete and a drop of the solution gave no immediate colour with sodium nitroprusside + ammonia + ammonium sulphate. The solutions were saturated with ammonium sulphate, then filtered, treated with a few drops of ammonia and sodium nitroprusside solution and allowed to stand for about 15 minutes for development of colour.

No. of tube	Contents of tube	Nitroprusside test
1	0·05 cc. β-hydroxybutyric acid + 2·4 cc. milk peroxidase	–
2	2·4 cc. buffer + 20 mg. cysteine	–
3	0·05 cc. β-hydroxybutyric acid + 2·4 cc. buffer + 20 mg. cysteine	+
4	0·05 cc. β-hydroxybutyric acid + 2·4 cc. milk peroxidase + 20 mg. cysteine	+ +
5	0·025 cc. β-hydroxybutyric acid + 2·4 cc. milk peroxidase + 20 mg. cysteine	+

The experiments were carried out at 30° and buffer was added where necessary to make the volumes of the solution equal to 3 cc.

(The nitroprusside test is given immediately by cysteine even in very dilute solutions, whilst in the case of acetoacetic acid and acetone, at these low concentrations the colour takes about 15 minutes to develop. There is no risk therefore of confusing the reaction for acetone bodies with that given by cysteine.) Controls in which 20 mg. of cysteine in 3 cc. of 7·6 buffer were first allowed to oxidise completely, 0·025 cc. and 0·05 cc. of β-hydroxybutyric acid being subsequently added, gave no appreciable nitroprusside test after shaking at 30° for the same length of time.

After aeration a small quantity of the solution from tube No. 4 gave Arnold's test with *p*-aminoacetophenone for acetoacetic acid.

It is evident that cysteine is capable of bringing about the oxidation of β-hydroxybutyric acid without the addition of peroxidase. A similar result was obtained with lactic acid and, as pointed out above, the presence of ferrous iron in the cysteine solution is probably a necessary factor in these oxidations. It can be seen that the oxidation of β-hydroxybutyric acid by cysteine is increased by the addition of milk peroxidase.

An attempt was then made to estimate the acetoacetic acid produced in this oxidation of β-hydroxybutyric acid. Three closed flasks containing (1) cysteine + β-hydroxybutyric acid + milk peroxidase, (2) cysteine + β-hydroxybutyric acid, (3) β-hydroxybutyric acid + peroxidase, the volumes of each solution being made up to 12 cc. with phosphate buffer of p_H 7·6, were shaken in a bath at 37° until the cysteine was completely oxidised (about 5 hours). The flasks were then cooled and the acetone bodies estimated by the method described by Lublin [1922]. 5 cc. of solution were withdrawn from each flask, diluted with 45 cc. water, 2 cc. of 20 % acetic acid added and the solution distilled for 10 minutes into $N/100$ iodine in alkaline solution. The difficulty in the distillation owing to frothing was overcome by adding 20 g. of ammonium sulphate before distilling. It has been shown by Goldblatt [1925] and others that the presence of proteins does not affect the results in this distillation. The excess of iodine was liberated with sulphuric acid and titrated with $N/50$ sodium thiosulphate (factor = 0·94). The distillations were carried out in duplicate and these agreed well together.

The results are given in the following table:

Contents of flask	Thiosulphate required (for 5 cc. of original solution) cc.		Total aceto-acetic acid formed mg.	Nitro-prusside test
	I	II		
1·0 cc. β-hydroxybutyric acid + 5 cc. peroxidase	13·8	13·7	—	–
1·0 cc. β-hydroxybutyric acid + 5 cc. buffer + 100 mg. cysteine	12·6	12·7	0·35	+
1·0 cc. β-hydroxybutyric acid + 5 cc. peroxidase + 100 mg. cysteine	12·4	12·4	0·43	+ +

Attempts were made to find whether the oxidation of the acetoacetic acid can be induced by cysteine. A quantity of acetoacetic acid sufficient to give a faint but definite nitroprusside test was aerated with 20 mg. of cysteine in 2·5 cc. of milk peroxidase. No appreciable diminution in the intensity of the nitroprusside test could be observed however.

Discussion.

From the work of Thurlow [1925] it was established that a peroxide is formed during the oxidation of hypoxanthine and xanthine (in presence of the xanthine oxidase), and since such systems as oxidising cysteine and oxidising

glutathione were shown to behave in the same way in bringing about the oxidation of nitrite when peroxidase was added, it was pointed out that a peroxide is probably formed in these systems also. Strong evidence was given for the belief that in the cases of hypoxanthine and xanthine the peroxide formed was hydrogen peroxide rather than an organic peroxide. In the case of oxidising cysteine and oxidising glutathione no definite assertion was made as to the nature of the peroxide formed, though it was pointed out that by assuming the formation of hydrogen peroxide rather than an organic peroxide, the mechanism of the oxidation of sulphydryl compounds might be explained in a manner more nearly comparable to that of the other oxidising systems. It may be mentioned that Engler and Broniatowski [1904] obtained, under favourable conditions, a yellow colour with titanium sulphate during the oxidation of thiophenol and therefore believe that hydrogen peroxide is formed during the oxidation of the sulphydryl group.

Recently it has been shown by Dixon [1925] that the destruction of xanthine oxidase which occurs during the oxidation of hypoxanthine can be inhibited by the addition of catalase; and, since the enzyme is destroyed by hydrogen peroxide and since catalase acts apparently only on hydrogen peroxide and not on organic peroxides, this is further evidence for the formation of hydrogen peroxide in the hypoxanthine system.

In the present paper it has been shown that the secondary oxidation of lactic acid and β-hydroxybutyric acid brought about during the oxidation of hypoxanthine occurs only in presence of a peroxidase. This again supports the view that hydrogen peroxide is formed, since organic peroxides are capable of bringing about secondary oxidations without the addition of a peroxidase. For example, it is well known that organic peroxides are capable of blueing guaiacum and benzidine directly and Dixon (unpublished experiments) has found that benzoylhydrogen peroxide can oxidise nitrite without peroxidase.

It would have been of interest to ascertain whether the secondary oxidations brought about by cysteine require the presence of a peroxidase. Unfortunately this is difficult to prove, for it has been shown by Warburg and Sakuma [1923] and by Harrison [1924] that the oxidation of sulphydryl compounds is dependent on the presence of traces of iron which these compounds normally contain. This iron might well be expected to act as an efficient peroxidase. If the iron be removed however by very careful purification, or inactivated by cyanide, the oxidation of the sulphydryl group is very greatly inhibited and consequently it would be very difficult to detect any secondary oxidation should it occur.

The work of Dakin has given strong support to the view of the formation of peroxides during biological oxidations by showing the very close similarity which exists between the oxidations which can be brought about *in vitro* by hydrogen peroxide and those actually occurring in the body as determined by metabolism experiments. On the other hand, there appears to be no such parallelism in the oxidations brought about by the dehydrase type of mech-

anism; for example, the oxidation of succinic acid to fumaric acid, and of hypoxanthine to uric acid are typical dehydrase oxidations and are not brought about by hydrogen peroxide, whilst the oxidation of, say, the higher fatty acids is readily brought about by hydrogen peroxide and not by dehydrases. This appears to support the view that oxidations which Dakin has shown can be imitated by hydrogen peroxide *in vitro* do not take place directly by a dehydrase mechanism, but by secondary oxidation by means of peroxide on the lines described in the present paper.

The wide distribution of catalase in the living organism cannot necessarily be regarded as evidence against the occurrence of oxidations brought about by hydrogen peroxide in presence of peroxidase, for it has been shown by Chodat and Pasmanik [1907] that a partition of hydrogen peroxide between catalase and peroxidase occurs when both enzymes are present. Further, Thurlow [1925] has shown that catalase only partially inhibits the secondary oxidation of nitrites.

From the results given in Tables I–IV it can be seen that the increased oxygen uptake due to secondary oxidation is never as great as the uptake of the primary system alone, though in some cases it approaches this value. The total uptake in presence of both the primary and the secondary system is never greater than double that of the primary system alone. This is a result which would be expected if the secondary oxidation is brought about by a peroxide on the lines indicated earlier in the paper. A molecule of oxygen taken up during the oxidation of two molecules of, say, cysteine, would form one molecule of peroxide, and one atom of oxygen in this peroxide would then be available for bringing about secondary oxidation. Thus under the most favourable conditions the oxygen absorbed would be divided equally between the primary and secondary systems. On the other hand, if the secondary system were absent, the one molecule of peroxide would bring about the oxidation of a further two molecules of cysteine, the primary oxidisable substance. In any case, even in presence of the secondary system we should expect some of the peroxide oxygen to be used by the primary system and consequently the oxygen uptake due to the secondary oxidation would always be less than that due to the primary oxidation, a result which agrees with our experiments.

Hopkins [1925] has obtained a similar result in studying the induced oxidation of unsaturated fatty acids by glutathione in neutral solution[1]. Here again the oxygen uptake ceases when the total uptake is nearly double that of the sulphydryl compound alone. It would seem probable that this induced oxidation of the fatty acids is being brought about on the lines suggested above, in a similar way to the induced oxidation of lactic acid.

The induced oxidation of proteins by sulphydryl compounds recently described by Hopkins [1925] presents a difficulty, for in this case the oxygen

[1] At a decidedly acid p_H of about 3·5 the oxidation of the fatty acids appears to be induced by an entirely different mechanism, glutathione here acting catalytically as an oxygen carrier apparently in much the same way as iron acts.

uptake in presence of protein may amount to as much as ten times the oxygen uptake of the sulphydryl compound alone. A parallel case would seem to be that of the induced oxidation of linseed oil by glutathione, where the total oxygen uptake is many times greater than that of the sulphydryl compound alone. It is possible of course that the induced oxidation of proteins and linseed oil takes place by means of a different mechanism from that of the reactions which we have been considering. An alternative possibility, however, would seem to be that, in the case of protein and linseed oil, secondary oxidation is brought about by peroxide during the oxidation of the sulphydryl group in the manner described above, and that the oxidation products of the protein and linseed oil then spontaneously take up more oxygen in a reaction or series of reactions involving their more complete oxidation. The fact that, as shown by Dakin, the individual amino-acids are readily oxidised by hydrogen peroxide would make it not improbable that the secondary oxidation of proteins might be brought about on such lines.

The work of Hopkins has established a link between the oxidation of fats and the oxidation of proteins. The proteins containing a sulphydryl group reduce oxidised glutathione and the reduced glutathione then brings about the oxidation of fats. The reducing factor in the protein regulates the supply of the soluble sulphydryl compound in the tissues and is thus the controlling factor in this oxidation of fats. In the present paper we have shown that the oxidation of lactic acid and β-hydroxybutyric acid is brought about at a physiological p_H during the oxidation of the sulphydryl group, and consequently these oxidations will also be controlled by the sulphydryl groups of the proteins in the tissues. Since lactic acid is one of the most important substances concerned in the intermediary metabolism of carbohydrates, this establishes a link between the oxidation of proteins and the oxidation of carbohydrates in the living cell.

The mechanism of secondary oxidation described in this paper would form an alternative means for the oxidation in the living cell of substances such as lactic acid which are also oxidised by loss of hydrogen in presence of their specific enzymes or "hydrogen transportases" to use Thunberg's terminology. On the other hand, it might be a means of oxidation of substances such as the sugars for which no hydrogen transportase has been found in the tissues. Such a peroxide mechanism would probably be much less specific than the dehydrase oxidations, the peroxide oxygen being shared among the various oxidising substances in proportion to their concentrations, the relative ease with which they are oxidised and the speed with which their oxidation products are removed.

At the present time we have only studied the secondary oxidation of a few substances, and there seems little reason to doubt that the oxidation of many other substances may be brought about in a similar way. In using the Barcroft technique it is not possible to detect secondary oxidations unless they occur to a considerable extent, for small differences in oxygen uptake have to be

neglected since the experimental error is considerable. In the body the conditions may very possibly be more favourable, and other factors such as surface may well play a part. Further, it seems likely that a number of different substances undergoing oxidation together may mutually assist one another. For example, it has been shown by Shaffer [1921] that the oxidation of acetoacetic acid by hydrogen peroxide is much accelerated in the presence of glucose.

It is very unlikely that these secondary oxidations would be prevented owing to lack of a suitable peroxidase, for, apart from the widely distributed animal peroxidases, it has been shown that haemoglobin can act as a very efficient peroxidase in these reactions.

In this connection the respiratory pigment cytochrome, recently described by Keilin [1925] and shown to give the ordinary peroxidase reactions, may well be an important factor.

In the tissues there are present very many "primary" oxidising systems besides those we have studied. Thurlow [1925] has shown that on adding peroxidase, the secondary oxidation of nitrites can be induced during the oxidation of succinic acid in presence of succinoxidase. Doubtless many other oxidising systems are capable of inducing secondary oxidations to a greater or less extent in a similar manner, and the combined action of these various primary systems might well be expected to bring about a very considerable amount of secondary oxidation in the living organism.

Summary.

1. During the aerobic oxidation of hypoxanthine in presence of xanthine oxidase, the oxidation of some substance present in milk, possibly a fat, can be induced if a suitable peroxidase is added.

2. Milk peroxidase, ferrous iron, haemoglobin and haematin can act as peroxidases in this oxidation.

3. The oxidation of lactic acid to acetaldehyde and of β-hydroxybutyric acid to acetoacetic acid can be induced during the aerobic oxidation of cysteine.

4. The aerobic oxidation of hypoxanthine in presence of xanthine oxidase can induce the oxidation of lactic acid and β-hydroxybutyric acid if ferrous iron be added.

5. The bearing of these results on the mechanism of biological oxidations is discussed.

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