CXVII. THE INFLUENCE OF GLUTATHIONE ON THE OXIDATION OF FATS AND FATTY ACIDS.

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HOPKINS [1925] has investigated the influence of glutathione on the oxidation of fats and proteins. The question of the oxidation of the fats and fatty acids is not so straightforward as previously pictured, and so far the problem of the actual mechanism of the reaction has not been reached. It seems, however, desirable to record some of the results which have been obtained.

The work dealt with in the present communication concerns the oxidation of linseed oil and linolenic acid.

The principal results of Hopkins' previous work, in so far as they bear on the results here described, may be briefly summarised.

The oxidation of linseed oil and of linolenic acid or of a mixture of fatty acids was studied at neutrality and at about p_H 3·5. The behaviour of the fat or fatty acid was very different at the two reactions studied.

With linolenic acid or mixed fatty acids at neutrality, the oxygen uptake of the system fatty acid + glutathione (GSH) was about twice that required to oxidise the —SH present. At p_H 3·5, however, where the —SH group is comparatively stable, there was a prolonged uptake to many times the value required by the —SH present.

The behaviour of the oil was again different. At p_H 7·6, after a rapid uptake to approximately the value required to oxidise the —SH present, there was a prolonged rectilinear uptake to a total of many times this value. At p_H 3·5, there was an induction period of several hours, during which the uptake was very small, followed by a period of much greater activity.

The results described in this paper are not in entire agreement with the above: on the other hand, it was found that different samples of oil showed varying behaviour, and similar results to the above could only be obtained with certain conditions of the oil.

EXPERIMENTAL.

The oxygen uptakes were measured in a Barcroft differential manometer, and unless otherwise mentioned 20 mg. oil or fatty-acid in the form of an emulsion in water or buffer were shaken with varying amounts of glutathione.

Linseed oil. Three different samples of linseed oil were used in this work, and of these only one showed the behaviour previously described, of giving at p_H 7·5 a prolonged rectilinear uptake after the initial rapid uptake due to the oxidation of the glutathione had ceased.

The "active" oil. This sample of oil showed a prolonged rectilinear uptake, after the glutathione was nearly all oxidised, of about 30 mm.^{3} per hour, and the amount of this uptake, in repeated experiments, was found to be independent of the amount of GSH or of G_2S_2 + muscle residue employed. It depended merely on the quantity of oil used. (It should be noted that this uptake is much smaller than that shown in a typical curve in Hopkins' paper.) In water alone, however, as previously found, no uptake was observed in the absence of GSH. In some experiments which were carried out in Clark and Lubs' phosphate buffer, p_H 7·5, a linear uptake at a slightly greater rate, about 40 mm.^{3} per hour, was observed, even in the absence of any thiol compound.

The behaviour of this oil changed considerably on being kept for some time in a corked bottle in a cupboard. The above results were obtained from September to November 1925, but by April 1926 the oil had become much more active, and now showed an uptake of considerable amount even in water alone. The effect of adding glutathione to the emulsion in water was now simply to increase the total uptake of the system by an amount approximately equal to that of the —SH present. The rate of uptake was not changed by this addition (Fig. 1).

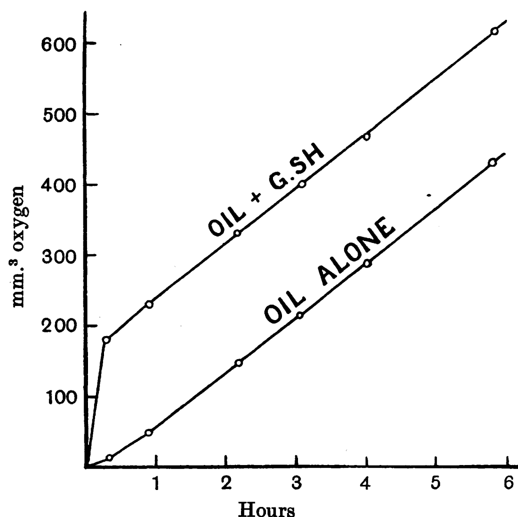

Fig. 1. "Active" linseed oil in water p_H 7·5.

At an intermediate stage (January 1926) during the maturing of this oil, the behaviour was reminiscent of Hopkins' results with the fatty acids. The uptake of a suspension of oil (in buffer) was twice that of the GSH present,

but at the end of this double uptake, a rectilinear uptake continued at the same rate (40 mm^3. per hour) as has already been observed. In water, at this time, the initial uptake was considerably greater than that of the GSH present, but it never rose to double this value.

In the later stages (April 1926) after the oil had become active, the effect of GSH in buffer solutions was peculiar. Instead of helping, or at any rate not inhibiting the oxidation of the oil, it seemed that the oxidation was actually slowed down after the GSH had itself become oxidised (Fig. 2). A control experiment with G_2S_2 showed this inhibition from the beginning. This is very like the result found for linolenic acid (see below).

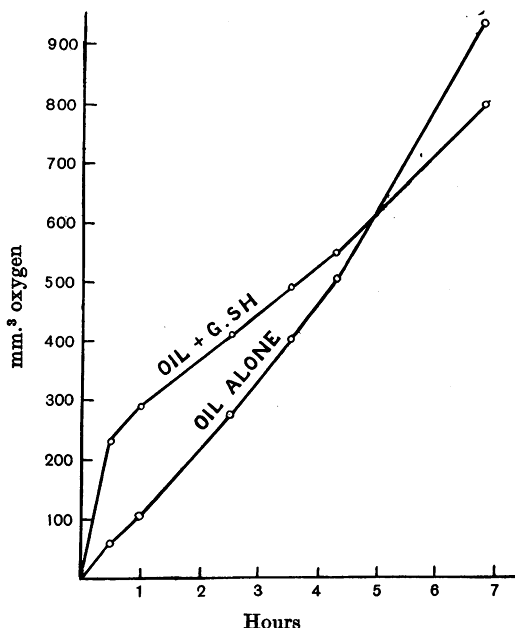

Fig. 2. "Active" linseed oil in phosphate buffer p_H 7·5.

The "inactive" oil. One of the samples of oil which previously had shown no rectilinear uptake was made to do so by aeration for $2\frac{3}{4}$ hours at 100°: by this time the iodine value, originally 180, had fallen to 165. Aeration for 2 hours, which caused no appreciable change in the iodine value, was also ineffective in bringing about an appreciable rectilinear uptake. This is significant in view of Hjort and Lund's work [1925] which is discussed later. At the same time it was noticed that the oil which at the beginning was distinctly yellow in colour, had become much paler: the oil which was "active" when the work commenced was much paler in colour than the "inactive" oil.

Hopkins [1925] says "The longer the induction period displayed by a sample of oil at p_H 3 to 4, the higher the concentration of —SH required to establish an active system at p_H 7·6."

When examined in water suspension at p_H 3·5 no appreciable uptake was observed with this "inactive" oil before aeration, either with or without glutathione. Harrison [1924] has shown the importance of iron in the oxidation of glutathione. Recent samples of glutathione have been much more iron-free than previous specimens, and the experiment was made of adding 0·02 mg. iron (in the form of ferrous ammonium sulphate) to the mixture of fatty emulsion and glutathione. A curve was obtained similar to those previously obtained by Hopkins at p_H 3·5, showing an induction period of $2\frac{3}{4}$ hours. Control experiments, using iron and oil, or iron and glutathione alone at this p_H showed a negligible uptake.

Nevertheless, even with the addition of 0·02 mg. iron, no rectilinear period was obtained with this oil at p_H 7·6 with an amount of glutathione corresponding to 250 $mm.^3$ oxygen. The uptake of the system was increased, it is true, by the addition of oil to 364 $mm.^3$ but ceased quite definitely after this. (Another inactive sample with the addition of iron at p_H 7·6 showed exactly double the uptake due to —SH present, though without iron the uptake did not reach this value.) After aeration this sample showed the typical curve, with induction period, when examined in water at p_H 3·5 in presence of glutathione.

It is of interest to note that though both the "active" oil and the "inactive" oil after aeration showed behaviour of the same general form as that previously described, the uptake after the induction period was very much smaller than that obtained by Hopkins, unless iron was also added to the system.

Fatty acids. Linolenic acid was prepared on two occasions by the reduction of hexabromostearic acid, obtained by the bromination of the mixture of fatty acids from the saponification of linseed oil, as described by Rollett [1909].

The first sample, immediately after preparation, when tested in water alone at p_H 7·5 showed a very large oxygen uptake. Unfortunately, all attempts to repeat this curve, with the same or different samples of acid were unsuccessful, the acid subsequently showing only a comparatively small uptake in water alone, though an appreciably larger one in buffer.

The results of adding GSH were very similar to those recorded above for the "active" sample of linseed oil. In water at p_H 7·5 the difference in uptake was approximately equal to that of the —SH added: in buffer there appeared, as before, an inhibition after the initial rapid uptake. Again, the addition of G_2S_2 caused inhibition from the start.

It was thought that the increased uptake of the fatty acid or oil in buffer as compared with that in water might be due to the presence of iron in the buffer. The effect of potassium cyanide was therefore tried. In 5 hours 20 mg. of a given sample of linolenic acid took up the following amounts of oxygen.

	$mm.^3$
In phosphate buffer alone	508
„ „ „ +20 mg. G_2S_2	280
„ „ „ +6 mg. NaCl	396
„ „ „ +KCN (to $M/250$)	138

The value found in presence of KCN was only slightly greater than that found in water alone.

At p_H 3·5, however, glutathione caused a definite increase in the uptake of the substance both in water and in phthalate buffer.

A sample of freshly made mixed fatty acids showed a similar behaviour. Nothing approaching the "double uptake" previously described was observed. After the mixed acids had been heated *in vacuo* at 100° from time to time during several days, the spontaneous activity in water was found to have practically disappeared, and now when examined in the presence of glutathione and a trace of iron (0·02 mg.) the characteristic "double uptake" was observed. Without the iron, under identical conditions, the uptake was greater than that of the glutathione by about 50 %. Control experiments showed that this effect was not due to the iron catalysing the oxidation of the mixed fatty acid.

The spontaneous activity of the linolenic acid could not be entirely removed by this means, and although, by the addition of iron to the system, an initial uptake could be obtained much greater than that of the glutathione itself, an accurate "double uptake" was never observed.

Discussion.

The results described above were unexpected, and modify some of the results previously obtained by Hopkins.

The two chief variables appear to be the condition of the oil or fatty acid and the iron content of the system.

Influence of iron. Different preparations of reduced glutathione vary much, not only in their content of GSH, but also in their iron content.

Hopkins [1925, p. 788] says "Since Warburg's discovery, the use of specially pure reagents has yielded preparations containing, at most, infinitesimal quantities of the metal. The employment of these, instead of earlier preparations, has made surprisingly little difference to the experimental results." Preparations since this was written have contained even less iron, and it would seem that the amount they contain is in some cases insufficient to produce a properly active system. The result with the "inactive" linseed oil at p_H 3·5 is perhaps the clearest example of this. Only in the presence of glutathione *and* iron is the typical curve developed; with neither of these separately is this result obtained.

Although Hopkins found that the use of buffers greatly reduced the uptake of a washed muscle preparation, it was not thought likely that the use of buffer would influence materially the oxidation of the oil and fatty acid, and with a view to regulating more exactly the p_H of otherwise quite unbuffered fluids, such as the suspension of the oils and fatty acids, buffers were employed in some of the experiments[1].

[1] It was found in some experiments in water, when the uptake was followed for a long time, say to 1500 or 2000 mm.^{3} per 20 mg., that the reaction of the suspension had definitely changed towards the acid side by the end of the experiment.

They were, however, found to have a marked influence on some of the results.

Both at p_H 3·5 and p_H 7·6 the use of buffer seems to increase the rate of uptake of an active oil or fatty acid: at p_H 7·6 the peculiar apparent inhibition by G_2S_2 must be considered. The results described in the experimental part seem to show that two factors may possibly be involved. The reaction is very markedly slowed by the addition of cyanide, which points to the presence of iron as the cause of the acceleration in buffer. The inhibition by G_2S_2 may possibly be due to the formation of some complex in which the iron is in some less active state. It was found, however, that the reaction was slightly inhibited by the presence of neutral salts, such as sodium chloride, and there is therefore the possibility that the action of glutathione in this connection may be, in part, due to "salt action."

Fats and fatty acids. The fact clearly emerges from the present work that the behaviour of a given sample of oil changes slowly on keeping. It was realised previously [Hopkins, 1925, p. 796] that the behaviour of a given sample of oil can be changed by previous treatment such as heating *in vacuo*, aeration, etc., but unfortunately, no initially "inactive" specimen of linseed oil was encountered during the course of that work.

The work of Hjort and Lund [1925] bears on this point. These authors investigated the oxygen uptake of cod-liver oil at 100°. Starting with perfectly fresh cod-liver oil, there was a long induction period of about 10 hours, during which the oil had a total uptake of only about 0·5 cc. per g. During the next hour or so the uptake rose to about 10 cc. per g. per hour. This long induction period would correspond to something of the order of a year at room temperature, assuming the rate of reaction to be doubled for 10° rise of temperature. It seems possible that the difference between the previous results of Hopkins and those described in this paper may be due to differences in the "maturity" of the oils used. The "active" oil used in the work here described may be assumed to have been near the end of Hjort and Lund's induction period at the beginning of the work, and to have reached the more active condition of those authors by the later part of the investigation. The "inactive" oil may be assumed to be somewhere on the earlier part of Hjort and Lund's curve, and not sufficiently near the end of the induction period to become active without previous aeration. The "active" oil and the "inactive" after aeration showed a marked tendency to harden whilst before aeration the "inactive" oil showed no tendency to harden on exposure to air.

Coffey [1921] has studied the oxidation of linseed oil, but used for his work thin films of the oil on filter papers. A definite induction period was found, which was much shorter than that obtained by Hjort and Lund with cod-liver oil. The work of the latter authors, who used the oil in much greater bulk, is probably more applicable to the changes likely to occur on keeping the oil in a bottle.

It seems quite clear that, given the right conditions, glutathione can induce

at p_H 7·5 a coupled oxidation of either the oil or fatty acid, causing them to take up an amount of oxygen equal to that taken up by the glutathione itself. As far as can be seen, the requisite conditions seem to be (*a*) the oil or fatty acid used must not show great spontaneous activity, (*b*) a definite amount of iron is necessary in the system. There may be other factors which are not at present realised.

How much the glutathione is responsible for the rectilinear uptake at p_H 7·5 is not so clear. The fact that the rate of uptake seems independent of the amount of —SH present, and that uptake at the same rate occurs in buffer in the absence of glutathione, would seem to rule out glutathione as a cause of this. The reason for the absence of uptake in water alone in the earlier experiments is obscure. In the later experiments, when the oil had become really active, an uptake was constantly found in water in the absence of glutathione.

As regards the fatty acids, the spontaneous activity of these is not in accord with Meyerhof's remarks [1923, p. 547].

It is true that glutathione exerts its influence most clearly at the acid reaction, and that with an already active oil or acid the influence of glutathione is not obvious at neutrality; but under certain conditions, the glutathione can undoubtedly induce a coupled oxidation of the oil or fatty acid.

Coffey [1921, p. 1160] makes reference to the common opinion that the fatty acids are less easily oxidised than the oils and states that his experience is otherwise. In neither his work nor in that of Hjort and Lund do the free fatty acids show any definite induction period. The work described above bears out the fact that the acids themselves are more easily oxidised.

SUMMARY.

The influence of glutathione on the oxidation of linseed oil and of linolenic acid has been studied.

The principal results are as follows:

(1) Not all samples of linseed oil or linolenic acid give the results described by Hopkins.

(2) The behaviour of the fatty acids was found to be much more like that of the glycerides than would appear from previous work.

(3) A given sample of oil undergoes changes merely on keeping under ordinary conditions, becoming more "active" as time goes on. The same increase in the "activity" of a sample of oil can be produced by aeration.

(4) Some doubt is thrown on the part played by glutathione in establishing a rectilinear uptake with linseed oil at p_H 7·5.

(5) The "double uptake" previously obtained with the fatty acids at p_H 7·5 seems to depend upon the lack of spontaneous activity of the fatty acid and the iron content of the system, and possibly other factors. Given the right conditions, it can also be obtained with the oils.

(6) Linolenic acid and some samples of linseed oil were found to have a definite uptake in aqueous suspension without the addition of glutathione.

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