X. THE OXIDATION OF ACETOACETIC ACID AND ESTER BY HYDROGEN PEROXIDE AND ITS BIOCHEMICAL SIGNIFICANCE.

BY PERCIVAL WALTER CLUTTERBUCK
AND HENRY STANLEY RAPER.

From the Department of Physiology, The University, Manchester.

(Received December 12th, 1925.)

It is commonly believed at the present time that acetoacetic acid, when it appears as an intermediate product of animal metabolism, is produced by the incomplete oxidation of fatty acids or certain of the amino-acids. Further, it is only produced under conditions in which the oxidation of carbohydrates is in abeyance. For this reason it has been held that the complete oxidation of acetoacetic acid can only occur when carbohydrate is being simultaneously oxidised and that at this stage some form of combination between acetoacetic acid (or its reduction product, β -hydroxybutyric acid) and a carbohydrate is essential [Geelmuyden, 1904; Ringer, 1914; Woodyatt, 1916]. Shaffer [1921] has produced an *in vitro* analogy to this biological process. He finds that acetoacetic acid is more rapidly oxidised by hydrogen peroxide in the presence of glucose and other carbohydrates than in their absence, but, so far as we are aware, no explanation of the mechanism of this reaction has been given. The same facilitation of the oxidation of acetoacetic acid is produced however by the presence of other substances, not carbohydrates [Ernst and Forster, 1924], so that as an *in vitro* analogy the reaction is less satisfactory than Shaffer at first believed. Owing to the lack of knowledge as to the products produced when acetoacetic acid and other β -keto-acids undergo oxidation, we have thought it advisable to study the oxidation of acetoacetic acid by hydrogen peroxide in the hope of finding some indication of the mechanism of the reaction and thus, possibly, of the method by which it may be oxidised in the body.

We are well aware that results of experiments of this kind can only be applied to the living animal with considerable reserve, but they may often lead to a more fruitful investigation of biological processes of oxidation than would be possible without them.

There is little previous work on the subject. In an unpublished observation referred to in his book, Dakin [1924] states that sodium acetoacetate when oxidised with hydrogen peroxide yields acetic, glyoxylic and formic acids and carbon dioxide. Engfeldt [1921], oxidising sodium acetoacetate

with potassium permanganate, states that the products are probably acetic and glyoxylic acids, but that most of the glyoxylic acid is further oxidised to oxalic acid. The possibility that intermediate oxidation products might be formed other than those just mentioned was realised in our earliest experiments. It was found that an aqueous solution of the sodium compound of acetoacetic ester, or of the acid, is readily oxidised by hydrogen peroxide in the cold, the reaction in fact causing a considerable rise of temperature. When the reaction is over a substance is present which reduces Fehling's solution strongly in the cold and is therefore not glyoxylic acid. If the products of the reaction are distilled under reduced pressure, the aqueous distillate also reduces Fehling's solution in the cold and gives an osazone which indicates that the reducing substance found in the distillate is either methylglyoxal or monohydroxyacetone (acetol). Evidence collected subsequently proved that this substance is monohydroxyacetone. Since the mechanism of its production was not clear (either α - or γ -oxidation would account for its presence, thus:

or

a careful examination by various methods of the solution resulting from the oxidation was undertaken. In this way a method was gradually evolved by which a considerable number of substances could be separated from the oxidation mixture, and this is described in the Experimental Section. The oxidations were carried out in a freezing mixture. The rapid rise of temperature and disappearance of the added peroxide ceased after the addition of about two-thirds of a molecule of peroxide per molecule of acetoacetic ester. When the sodium salt of the acid is used instead of the ester the oxidation is not so rapid but appears to pursue the same course. Almost the whole of the work has been done with the sodium compound of the ester because of certain advantages which it offered in the subsequent separation of the products. The substances isolated, or separated, in the form of derivatives from the solution after oxidation by not more than one molecular proportion of peroxide in the cold are: a little unchanged acetoacetic ester and acid, ethyl acetate, ethyl isocarbopyrotritartrate, acetonylacetoacetic acid and ester, acetonylacetone, acetol and a small quantity of acetic, glyoxylic and oxalic acids. In addition, a strongly reducing but very unstable acid is present which could not be isolated in a pure state owing to its instability. It may be regarded as either α -hydroxyacetoacetic acid or $\alpha\beta$ -dihydroxycrotonic acid. These substances, together with carbon dioxide and a certain amount of acetone and alcohol produced by hydrolysis of the acetoacetic ester, account practically quantitatively, so far as can be determined by isolation methods, for the whole of the products of the reaction. The primary oxidation product appears to be the unstable reducing acid. This

probably arises by the addition of hydrogen peroxide or oxygen to the enolic form of acetoacetic acid at the double bond,

the compound produced subsequently decomposing to give acetol and carbon dioxide;

That this oxidation product is $\alpha\beta$ -dihydroxycrotonic acid or one of its isomeric forms, *e.g.* α -hydroxyacetoacetic acid, appears to be confirmed by the fact that oxidation of $\alpha\beta$ -dihydroxybutyric acid by means of Fenton's reagent gives a similar acid which reduces Fehling's solution rapidly in the cold and gives immediately the dinitrophenylhydrazone of methylglyoxal on treatment with nitrophenylhydrazine.

Of the remaining products, acetylacetoacetic ester and acid are found in considerable amounts. These may have arisen in two ways. Either a secondary oxidation occurs involving two molecules of acetoacetic ester, yielding diacetylsuccinic ester which after hydrolysis loses carbon dioxide to give acetylacetoacetic acid (I),

or one of the oxidation products shown above, viz. α -hydroxyacetoacetic acid, $\alpha\beta$ -dihydroxycrotonic acid or its peroxide form, unites with a further molecule of acetoacetic ester, *e.g.* the peroxide form and acetoacetic ester might condense, and the product chiefly losing carbon dioxide yields acetonyl-acetoacetic ester and to a small extent (0.2 g. from 13 g. acetoacetic ester oxidised) by loss of water, ethyl isocarbopyrotritartrate (II),

The very small amounts of acetic, glyoxylic and oxalic acids detected in the oxidation mixture probably arise from a subsidiary reaction involving a splitting of the enol form of acetoacetic acid at the double bond,

It appears therefore that the chief oxidation products of acetoacetic acid are α -hydroxyacetoacetic acid, and either acetylacetoacetic acid or diacetylsuccinic acid. In experiments *in vitro*, the first substance very gradually loses carbon dioxide passing to acetol. If the oxidation of acetoacetic acid *in vivo* produces α -hydroxyacetoacetic acid, it is unlikely that the decomposition into acetol occurs, since this substance although relatively non-toxic, even in moderate doses in phlorrhizinated dogs, causes haematuria and haemoglobinuria [Greer, Witzemann and Woodyatt, 1914]. It seems more probable that the reducing acid would be further oxidised to diketobutyric acid.

Two lines of further change of the diketobutyric acid suggest themselves; (1) hydrolysis with the formation of acetic and glyoxylic acids, or (2) loss of CO_2 with the formation of methylglyoxal:

The first of these reactions is in all probability that which occurs when higher members of the series of normal fatty acids undergo β -oxidation. It would result in the loss of two carbon atoms at a time and the process would be in conformity with the β -oxidation hypothesis of Knoop. The second reaction is however not without interest. It also would lead to a shortening of the fatty acid chain by two carbon atoms because the glyoxal derivatives produced by the reaction under the influence of glyoxalase could be converted into α -hydroxy-acids, which in turn by oxidation would lose one carbon atom and yield a fatty acid with an even number of carbon atoms.

In the case of diketobutyric acid itself the second reaction has added significance because it would give rise to methylglyoxal and by the intervention of glyoxalase, lactic acid. This in turn is capable of conversion into glucose. A mechanism is thus provided by which one product of the oxidation of fats may be converted into carbohydrates.

Recently, Knoop and Jost [1924] have shown that following the administration of β -hydroxybutyric acid to dogs, not inconsiderable amounts of lactic acid are found in the urine. They are not inclined to suggest that the lactic acid has arisen through oxidation of the acid administered, partly because it would involve an oxidation in the α -position. The suggestion outlined above would however explain how lactic acid might arise from the oxidation of either butyric, β -hydroxybutyric or acetoacetic acid without any sacrifice of the β -position as the site of the primary change.

One other suggestion of biological interest may be made as a result of this work. Diacetylsuccinic acid and acetylacetoacetic acid, which are products of the oxidation of acetoacetic acid, react readily with ammonia to form substituted pyrroles, *e.g.*

This reaction is possibly of importance in the production of the pyrrole nucleus of blood pigments and chlorophyll.

EXPERIMENTAL.

Oxidation of acetoacetic ester.

Acetoacetic ester (13 g.) was placed in a small conical flask in a freezing mixture. Sodium hydroxide (40 cc. 10 %) was added and the whole stirred and allowed to stand, when the sodium compound separated. When the temperature reached 0° a few drops of peroxide (30 %) were added and the liquid stirred. The temperature rose immediately and the number of drops of peroxide added was regulated so that the temperature did not rise above 10°. This was repeated until about 9 cc. of peroxide had been added. If a few drops of the solution were withdrawn at any time immediately after the addition of a little peroxide, it was found that Fehling's solution was readily reduced in the cold but that the chromate test for peroxide was negative. After about two-thirds of a molecule of peroxide (per molecule of ester) had been added, the vigorous reaction ceased. If the solution was allowed to stand it became deep red and the first few processes about to be described had to be carried out as rapidly as possible. The solution, after all the peroxide had been added, was saturated with carbon dioxide and extracted three times with ether. The ether was removed from the ethereal extracts by means of a current of air and an oil resulted which, after drying over sodium sulphate and distilling *in vacuo*, was shown to consist of unchanged acetoacetic ester with smaller amounts of ethyl acetate and a yellow oil, B.P. 110°/15 mm. (not identified), together with traces of the reducing substance and acetonyl-acetone. The aqueous solution remaining after ether extraction was then acidified with dilute hydrochloric acid. As the acid was added an oily substance appeared to separate and this on standing formed a bulky but light crystalline precipitate. This was filtered off. The substance (0.2 g. from 13 g. ester) was insoluble in cold water but soluble in alcohol and in sodium hydroxide, with ferric chloride gave a blue colour, had the equivalent 215, melted at 110°, and corresponded therefore with ethylisocarbopyrotritartrate. The acid solution after filtration was then extracted seven times with ether, and the ether removed from the combined dried ether extracts by means of a current of air. A considerable amount of yellow oil resulted. If this oil, which is strongly reducing, is allowed to stand in a test-tube at the ordinary temperature, a very gradual development of carbon dioxide takes place over a period of about 20 hours, due to the gradual decomposition of the reducing acid, α -hydroxyacetoacetic acid, into acetol and carbon dioxide. When the oil was converted into semicarbazones by addition of a little water, sodium acetate and semicarbazide hydrochloride, it was noticed that a thick precipitate formed which partially decomposed, giving carbon dioxide and a product relatively very soluble in water. The precipitate was warmed with

water and the more insoluble portion filtered off, and divided by recrystallisation from boiling water into a more soluble portion and a portion only slightly soluble in boiling water. The former semicarbazone after purification by recrystallisation from boiling water and from dilute alcohol melted at 208° and was shown to be acetonylacetoacetic acid semicarbazone, whilst the latter relatively more insoluble substance after recrystallisation from boiling water melted at 230° and corresponded with acetonylacetoacetic ester semicarbazone, being identical with the ethyl 2 : 5-dimethyl- $\Delta^{2:5}$ -dihydropyridazine-1-carbonamide-3-carboxylate of Borsche and Spannagel [1904].

Analysis:

Acetonylacetoacetic acid semicarbazone: found; N = 21.2 %; calculated for $C_8H_{11}O_3N_3$; N = 21.2 %.

Acetonylacetoacetic ester semicarbazone: found; N = 18.8 %; calculated for $C_{10}H_{15}O_3N_3$; N = 18.7 %.

Acetonylacetoacetic ester semicarbazone is also much more soluble in acetone than the corresponding compound of the acid and can therefore be extracted from the mixtures by its means.

If, on the other hand, a little of the yellow oil was dropped into cooled ammonia (conc.) and the product allowed to stand, a crystalline precipitate was formed which after filtering off was dissolved in alcohol and precipitated by the addition of water. This substance was ethyl 2.5-dimethylpyrrole-4-carboxylate, m.p. 117° , and corresponded with that of Knorr [1885] obtained by the action of ammonia on diacetylsuccinic ester followed by gentle heating to drive off one molecule of carbon dioxide.

Analysis: found; N = 8.33 %; calculated for $C_9H_{13}O_2N$; N = 8.38 %.

When a further quantity of the oil was rapidly converted in the presence of only a little water into the semicarbazones and the solids filtered off and then treated with water, a decomposition of part of the semicarbazone rapidly took place giving a new semicarbazone which went into solution. Such a solution on standing for about 24 hours deposited crystals which after recrystallisation were shown to be identical by m.p. 187° , mixed melting point with synthetic material, and by analysis, with acetone semicarbazone.

Analysis: found; N = 36.4 %; calculated for $C_4H_9ON_3$; N = 36.5 %.

Since free acetone had been removed from the acids by the method of preparation, this must have arisen by decomposition of an insoluble semicarbazone of acetoacetic acid. This acid was therefore prepared by allowing the ester to stand in contact with the theoretical amount of sodium hydroxide overnight, acidifying and extracting with ether. The acid on treating with sodium acetate and semicarbazide hydrochloride gave a semicarbazone rapidly decomposing and passing into solution from which, on standing overnight, acetone semicarbazone crystallised out. Acetoacetic acid gives also a smaller amount of another product with a characteristic odour which was not identified.

Finally, when some of the oil was boiled for a long time under reflux with

water, or for a short time with potassium carbonate, or when the oil was distilled, a substance was obtained which gave a very slightly soluble semicarbazone, crystallisable from boiling water, which was shown to be identical with acetonylacetone disemicarbazone by m.p. 224° [cf. Posner, 1901], mixed melting point with the synthetic product and by analysis.

Analysis: found; N = 36.6 %; calculated for $C_8H_{16}O_2N_6$; N = 36.8 %.

The disemicarbazone was obtained synthetically for comparison in the following way: diacetylsuccinic ester was prepared by the action of iodine on ethyl sodioacetoacetate in ether solution by the method of Rugheimer [1874] and converted into acetonylacetone by boiling with 20 % aqueous potassium carbonate for an hour [cf. Knorr, 1900]. This was then treated with sodium acetate and semicarbazide hydrochloride, the semicarbazone filtered off and recrystallised from boiling water. The diphenylhydrazone m.p. 120° , and the dinitrophenylhydrazone m.p. 212° , were also prepared and used for comparison.

Analysis:

Diphenylhydrazone: found; N = 19.2 %; calculated for $C_{18}H_{22}N_4$; N = 19.0 %.

Dinitrophenylhydrazone: found; N = 22.0 %; calculated for $C_{18}H_{20}O_4N_6$; N = 21.9 %.

Whenever the yellow oil was allowed to stand for some time, crystals of oxalic acid separated, but complete separation by means of lead and calcium salt methods showed that the amount was small.

Distillation of the oil *in vacuo* always gave a first portion containing acetic acid and having the characteristic strawberry smell of acetol, strongly reducing and readily giving methylglyoxal osazone m.p. 145° .

From the above experiments it appears that ether extraction of the acidified liquid extracts considerable amounts of (1) a very unstable reducing acid, probably α -hydroxyacetoacetic acid, which readily gives acetol, (2) acetonyl-acetoacetic acid and ester, and (3) acetoacetic acid together with small amounts of oxalic and acetic acids.

The aqueous residue after ether extraction still contains considerable amounts of the reducing acid. It was therefore neutralised to litmus and evaporated *in vacuo* from a water-bath at 50° , the distillate being removed from time to time. The first two-thirds of the distillate contained traces of both acetol and acetonylacetone, but the major part of the reducing body present as the sodium salt was concentrated in the distilling flask and a drop removed therefrom had exceedingly powerful reducing properties in the cold. The receiver was therefore finally changed and the temperature of the bath raised to about 80° and the whole distilled to dryness *in vacuo*. The residue in the distillation flask smelled strongly of acetol. The aqueous distillate reduced Fehling's solution powerfully and was treated with sodium acetate and semicarbazide hydrochloride. An almost immediate precipitation occurred of acetonylacetone disemicarbazone and, after filtering this off, the

solution was allowed to stand for 2 days. A considerable amount of practically pure acetol semicarbazone crystallised out, m.p. 197°.

Analysis: found; N = 32.2 %; calculated for $C_4H_9O_2N_3$; N = 32.06 %.

Acetol was therefore prepared by boiling bromoacetone with anhydrous potassium formate in methyl alcoholic solution under reflux [Nef, 1904] and gave with sodium acetate and semicarbazide hydrochloride a semicarbazone, m.p. 197°, identical with that obtained from the oxidation product and showing no depression of melting point when mixed with it. The presence of acetol in the distillate was confirmed by condensation with *o*-aminobenzaldehyde, when a blue fluorescing substance was obtained [cf. Baudisch and Deuel, 1922] and by preparing therefrom the osazone (m.p. 145°) and the dinitrophenylhydrazone (m.p. 302°, gives a blue colour with alcoholic sodium hydroxide which gradually becomes purple, violet, dull brown [cf. Dakin and Dudley, 1913]).

If the neutralised concentrated solution, instead of being distilled to dryness, was acidified and extracted with ether, a mixture of reducing acid and acetylacetone and a little oxalic and acetic acids was obtained and the residual solution on continuous ether extraction for 24 hours gave a further quantity of reducing acid and acetic and oxalic acids.

Methylglyoxal was prepared from a sample of ethyl $\gamma\gamma$ -diethoxy- α -methylacetoacetate (kindly supplied by Dr Dudley) by the method of Dakin and Dudley [1914], and from dihydroxyacetone both by treatment with dilute sulphuric acid [Pinkus, 1898] and by distilling *in vacuo* with phosphorus pentoxide [Fischer and Taube, 1924]. Methylglyoxal gives a disemicarbazone, m.p. 257°, both when treated with an aqueous solution of semicarbazide hydrochloride and when this solution is buffered either by bicarbonate or by sodium acetate.

Analysis: found; N = 45.3 %; calculated for $C_5H_{10}O_2N_2$; N = 45.2 %.

Ethylglyoxal on the other hand appeared to give a monosemicarbazone, m.p. 235°, on treatment with an aqueous solution of semicarbazide hydrochloride [Dakin and Dudley, 1914].

A point of great interest was the ease with which dihydroxyacetone passed to methylglyoxal. It was found that an aqueous solution of dihydroxyacetone, when mixed with a similar solution of semicarbazide hydrochloride with or without addition of sodium acetate, slowly deposited on standing methylglyoxal disemicarbazone. The passage of the primary alcohol group into a methyl group is interesting but the mechanism does not seem clear. It seems likely that some such reaction may be involved in the production of lactic acid from glucose.

An attempt was made very roughly to follow the oxidation processes quantitatively. Using 26 g. of ester (which corresponds to 20.4 g. acetoacetic acid) and 18 cc. hydrogen peroxide (100 vols.), the following were the weights of the extracts obtained:

(1) after passing carbon dioxide, 3 ether extractions	4.9 g.
(2) after acidifying with hydrochloric acid, precipitated	0.4
(3) " " " " 7 ether extractions	9.5
(4) after evaporation to $\frac{1}{2}$ bulk of the neutralised solution, acidification and 7 ether extractions	2.3
(5) after ether extraction for 24 hours	3.1
	<hr/> 20.2 g.

Extract (1) contained chiefly acetoacetic ester together with ethyl acetate, traces of acetol and a yellow oil.

(2) contained ethyl isocarbopyrotartrate.

„ (3) contained chiefly reducing acid, acetylacetoacetic acid and ester together with acetoacetic acid, acetol and acetylacetone, acetic, glyoxylic and oxalic acids.

„ (4) contained same as (3) but the amounts of acetylacetone and acetic acid were relatively larger.

„ (5) contained reducing acid, oxalic acid, acetylacetone.

During the processes of evaporation, carbon dioxide, ethyl alcohol, traces of acetol and acetylacetone were lost.

The main fraction (3) was shaken with 150 cc. water and on conversion into the semicarbazones yielded 6.0 g. dry product. This consisted almost entirely of the semicarbazones of acetylacetone, acetylacetoacetic acid and ester, the semicarbazones of acetoacetic acid (as acetone semicarbazone after decomposition) and of acetol remaining in solution. This weight of semicarbazone represents the presence of about 4.8 g. acetylacetoacetic acid. Acetylacetoacetic acid, acetylacetoacetic ester and acetylacetone are present in the mixture in decreasing amounts. Diacetylsuccinic ester was prepared for comparison and gave a semicarbazone m.p. 203° [Bülow, Riess and Sautermeister, 1905].

$\alpha\beta$ -Dihydroxybutyric acid was synthesised by oxidation in dilute solution of crotonic acid by means of barium permanganate [Fittig and Kochs, 1892]. 2.9 g. of this acid was dissolved in 50 cc. water and boiled with 0.14 g. reduced iron and the solution filtered. 14 cc. of 5 % hydrogen peroxide were added to the solution in an ice-bath. No rise of temperature occurred and the solution was therefore allowed to stand at the ordinary temperature. The temperature after about half an hour had risen to 20°. The liquid was cooled to 12° and allowed to stand about 4 hours. Samples removed from the liquid a quarter of an hour after adding the peroxide, on making alkaline, gave no reduction with Fehling's solution in the cold but after standing for a longer time the liquid readily caused reduction in the cold. The aqueous solution was powerfully reducing after standing 24 hours, and on treatment with *p*-nitrophenylhydrazine in acetic acid solution immediately gave a dinitrophenylhydrazone which on precipitation from hot nitrobenzene solution with toluene melted at 304°, gave a blue colour with alcoholic sodium hydroxide and corresponded with methylglyoxal dinitrophenylhydrazone.

SUMMARY.

(1) The oxidation of the sodium compound of acetoacetic ester or acid in the cold by means of hydrogen peroxide yields, as primary product, α -hydroxyacetoacetic acid (or $\alpha\beta$ -dihydroxycrotonic acid). This readily loses carbon dioxide, forming acetol.

(2) In addition, by subsidiary reactions, the following substances are produced; diacetylsuccinic acid, acetylacetoacetic acid, acetylacetone, acetic acid, glyoxylic acid and oxalic acid. Of these, acetylacetoacetic acid is formed in considerable amount.

(3) The mechanism of the reaction is discussed in relation to the later stages of the β -oxidation process, the possible derivation of carbohydrate from acetoacetic acid and a mode of origin of substituted pyrroles in living organisms.

We desire to express our thanks to the Government Grant Committee of the Royal Society for a grant in aid of the expenses of this investigation.

REFERENCES.

- Baudisch and Deuel (1922). *J. Amer. Chem. Soc.* **44**, 1585.
Borsche and Spannagel (1904). *Annalen*, **331**, 298.
Bülow, Riess and Sautermeister (1905). *Ber. deutsch. chem. Ges.* **38**, 2366.
Dakin (1908, 1, 2, 3). *J. Biol. Chem.* **4**, 77, 221, 227.
— (1910). *Amer. Chem. J.* **44**, 41.
— (1924). *Oxidations and Reductions in the Animal Body*. (Longmans.)
Dakin and Dudley (1913). *J. Biol. Chem.* **15**, 127.
— (1914). *J. Chem. Soc.* **106**, 2453.
Engfeldt (1921). *Z. physiol. Chem.* **112**, 176.
Ernst and Forster (1924). *Chem. Soc. Abstr.* ii, **128**, 684.
Fischer and Taube (1924). *Ber. deutsch. chem. Ges.* **57** (B), 1502.
Fittig and Kochs (1892). *Annalen*, **268**, 8.
Geelmuyden (1904). *Z. physiol. Chem.* **41**, 135.
Greer, Witzemann and Woodyatt (1914). *J. Biol. Chem.* **16**, 455.
Knoop and Jost (1924). *Z. physiol. Chem.* **141**, 55.
Knorr (1885). *Ber. deutsch. chem. Ges.* **18**, 299, 1558.
— (1900). *Ber. deutsch. chem. Ges.* **33**, 1219.
Nef (1904). *Annalen*, **335**, 191.
Pinkus (1898). *Ber. deutsch. chem. Ges.* **31**, 36.
Posner (1901). *Ber. deutsch. chem. Ges.* **34**, 3973.
Ringer (1914). *J. Biol. Chem.* **17**, 107.
Rugheimer (1874). *Ber. deutsch. chem. Ges.* **7**, 892.
Shaffer (1921). *J. Biol. Chem.* **47**, 433.
Woodyatt (1916). *J. Amer. Med. Assoc.* **66**, 1910.