CXXXIX. α -HYDROXYACETOACETIC ACID

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I. PREPARATION, PROPERTIES AND ESTIMATION

α -HYDROXYACETOACETIC acid has aroused the interest of biochemists for two reasons: first, because it was assumed to be a possible intermediate in the oxidative breakdown of acetoacetic acid. Clutterbuck & Raper [1926] studied the oxidation of sodium acetoacetic ester by 1 mol. H_2O_2 in the cold and obtained a solution containing a large variety of oxidation and condensation products. Amongst these there was an acid with strong reducing properties which spontaneously decomposed into CO_2 and acetol and which they therefore considered to be α -hydroxyacetoacetic acid.

The second reason was the possible existence of the following tautomeric equilibrium between the two ketohydroxy- and the enediol-forms:

The enediol tautomeride will share with the other known enediol compounds (which have also been called "reductones") a strong tendency to be dehydrogenated to the diketo-compound. Karrer & Hershberg [1934], in an attempt to prepare these substances, reduced diketobutyric ester and obtained three fractions which they considered to be the ethyl esters of the three tautomerides.

A method is described below for the preparation of solutions containing, in addition to sodium *dl*- α -hydroxyacetoacetate, only an equimolecular quantity of sodium acetate and a little phosphate buffer, and which are therefore suitable for the investigation of the chemical and physiological properties of this substance. The solutions were obtained by hydrolysis of ethyl α -acetoxyacetoacetate [Dimroth & Schweizer, 1923]; these authors have already observed that the ester easily consumes alkali and that it is decomposed to acetol, acetic acid and CO_2 in acid solution. They have not however studied the hydrolysis in detail.

On acid hydrolysis an almost immediate evolution of CO_2 sets in, whilst on alkaline hydrolysis in air an absorption of O_2 occurs. If the hydrolysis is carried out in dilute NaOH under anaerobic conditions, however, and the excess alkali is neutralized before admission of O_2 , the solution thus obtained shows no appreciable absorption of O_2 or evolution of CO_2 . Hydrolysis is complete at 25° after about 1 hr., when 2 mol. of NaOH have been consumed (Fig. 1).

0.01 ml. or 12 mg. ethyl acetylhydroxyacetoacetate (=0.064 mMol.) used 1.28 ml. $N/10$ NaOH. Calculated (for 2 mol. NaOH per mol. ester): 1.28 ml. $N/10$ NaOH.

The hydrolysis curve was determined in a series of Warburg manometers with two side bulbs, filled with pure N_2 . At t_0 the ester (0.01 ml.) was tipped from one side bulb into 3.0 ml. $N/20$ NaOH. After the time t a measured excess of HCl was tipped from the other side bulb, the vessel quickly disconnected and at once titrated with NaOH against phenolphthalein.

Fig. 1. Hydrolysis of α -acetoxyacetoacetic ester in absence of O_2 . The broken line indicates the theoretical value for 2 mol. NaOH.

The yield of hydroxyacetoacetic acid depends greatly on the initial concentration of NaOH. If this does not exceed $N/10$, the yield is about 90 %, but it declines with higher initial concentrations, probably owing to polymerization (Table I). The final solution is freed from alcohol and concentrated by distillation *in vacuo*.

Preparation of M/10 sodium hydroxyacetoacetate: 0.2 ml. ethyl α -acetoxyacetoacetate is added to 26 ml. $N/10$ NaOH in an atmosphere of pure N_2 . After incubation for 1 hr. at 25° 0.5 ml. M NaH_2PO_4 is added in a stream of N_2 . The solution is concentrated *in vacuo* from a bath at 30° to about 10 ml.

Table I. Yield of hydroxyacetoacetic acid after hydrolysis of ethyl acetoxyacetoacetate in varying initial concentrations of NaOH. Aniline method

Initial concentration of NaOH (N)	Yield %
0.1	87
0.2	81
0.37	60
0.87	40

Whereas the ester linkage of ethyl acetoxyacetoacetate is not hydrolysed in dilute $NaHCO_3$ or only very slowly, the hydrolysis of the acetyl group is accomplished with great ease. This process can be followed manometrically and, in absence of O_2 , 1 mol. of acid is formed (Fig. 4, curve I). By anaerobic incubation in $NaHCO_3$ α -hydroxyacetoacetic ester can thus be prepared from ethyl α -acetoxyacetoacetate.

0.01 ml. or 12 mg. ethyl acetoxyacetoacetate liberated 1450 μ l. CO_2 from $N/10$ NaHCO_3 in pure N_2 . Calculated (for 1 mol. of CO_2 per mol. of ester): 1438 μ l. Warburg manometers filled with Clerici solution were used for these experiments.

Properties of the solutions obtained by hydrolysis: the solutions of sodium hydroxyacetoacetate are colourless and odourless. If the hydrolysis has been carried out in an initial concentration of $\text{NaOH} > N/10$ they have a distinct yellow colour.

Colour reactions: the solutions give a blue colour with FeCl_3 and an orange colour with nitroprusside in NH_3 -alkaline solution. Characteristic colours are obtained with phenols and conc. H_2SO_4 : with orcin a green colour, with resorcinol a purple, changing to violet and blue, with pyrogallol blue-green, with phloroglucinol dark green.

Reaction with dinitrophenylhydrazine: on addition of excess 2:4-dinitrophenylhydrazine a brown precipitate of methylglyoxal-bis-2:4-dinitrophenylhydrazone appears (M.P. 296°), insoluble in NaHCO_3 and alcohol, soluble in alcoholic KOH with intense violet-blue colour.

The solutions are free from acetoacetic acid and acetone.

In discussing the hydrolysis of ethyl α -acetoxyacetoacetate reference has been made to three reactions: decarboxylation, autoxidation and polymerization. These will now be dealt with separately.

Decarboxylation: hydroxyacetoacetic acid is fairly stable at neutral reaction and low temperature. Thus at $\text{pH } 7.4$ the times for 50% decarboxylation are: 6 hr. at 37.5° , 24 hr. at 25° and about 600 hr. at 0° . No difference was found

Fig. 2. Half-life time of hydroxyacetoacetic acid as a function of temperature.

between a bicarbonate/ CO_2 buffer and a phosphate buffer. At $\text{pH } 4.6$ the half-life time is only about two-thirds of that at $\text{pH } 7.4$ (Fig. 2).

Higher temperatures and lower pH greatly accelerate the decarboxylation. At 100° the acid is decarboxylated in a few minutes. In $N/2$ H_2SO_4 at 25° the decarboxylation is complete in 90 min. (Fig. 3).

Fig. 3. Decarboxylation of hydroxyacetoacetic acid. I, in presence of aniline; II, in $0.5 N$ H_2SO_4 .

The relative stability of the acid at pH 4.6 and 25° allows the manometric estimation of bicarbonate in presence of hydroxyacetoacetic acid by acidification with strong acetate buffer pH 4.6.

The decarboxylation of hydroxyacetoacetic acid is, like that of other β -ketonic acids, catalysed by aniline [Ljunggren, 1925; Ostern, 1933; Edson, 1935]. In absence of other β -ketonic acids this is the most convenient method of estimation. The solution is brought to pH 4.6 with 1/10 vol. of $3 M$ acetate buffer before the aniline (aniline citrate reagent according to Edson [1935]) is added. The CO_2 evolved is measured manometrically at 25° . The decarboxylation is complete in 20 min. (Fig. 3). The decarboxylated product is acetol which can be detected by the very sensitive and apparently specific fluorescence reaction of Baudisch [1918], based on the formation of 3-hydroxyquinoline by condensation with *o*-aminobenzaldehyde.

Autoxidation: (1) *Autoxidation of ethyl hydroxyacetoacetate.* Whereas in absence of O_2 only 1 mol. acid is formed from α -acetoxyacetoacetic acid in $N/10$ $NaHCO_3$, due to the hydrolysis of the acetyl group, in presence of O_2 1 mol. O_2 is absorbed and 3 mol. acid are formed (Fig. 4).

Three Warburg manometers filled with Clerici solution were used. They all contained ml. $N/10$ $NaHCO_3$ in the main part and 0.008 ml. ethyl acetoxyacetoacetate in the side bulb. Manometer I was filled with pure N_2 and served for the estimation of acid formation during

anaerobic hydrolysis. Manometers II and III contained air. 0.2 ml. 10% NaOH was placed in the centre well of manometer vessel II. O_2 absorption and CO_2 evolution during aerobic hydrolysis were calculated from the pressures observed in manometers II and III. At the end of the experiment (after 6 hr.) an aliquot was taken from the solution in vessel III and its bicarbonate content was determined manometrically. The decrease of $NaHCO_3$ corresponded closely to the CO_2 evolution which was therefore due to acid formation and not to decarboxylation.

Fig. 4. Hydrolysis and autoxidation of ethyl α -acetoxyacetoacetate in $N/10$ $NaHCO_3$.
I, CO_2 evolution in pure N_2 ; II, O_2 absorption in air; III, CO_2 evolution in air.

The liberation of 3 mol. CO_2 from $NaHCO_3$ during aerobic hydrolysis of ethyl acetoxyacetoacetate is due to the formation of 2 mol. acetic acid and 1 mol. ethyl hydrogen oxalate. These are found in the solution in theoretical quantity (the latter as oxalic acid after acid hydrolysis).

0.008 ml. (=1150 μ l.)¹ ethyl α -acetoxyacetoacetate yielded after anaerobic hydrolysis: 1120 μ l. volatile acid (estimated as described below); after aerobic hydrolysis: 2160 μ l. volatile acid. No formic acid was found.

For the estimation of oxalic acid the solution after acid hydrolysis for 5 min. at 100° was adjusted to pH 5 with dilute NH_3 , an excess of $CaCl_2$ was added and the precipitate centrifuged after 12 hr., washed with NH_3 1:50, dissolved in hot NH_2SO_4 and titrated with $N/50$ $KMnO_4$ at 70° .

Oxalic acid found after anaerobic hydrolysis: 0; after aerobic hydrolysis: 1080 μ l.

¹ Analytical figures are expressed in μ l. (1 mMol. of compound in gaseous form at N.T.P. = 22,400 μ l.) in order to facilitate the comparison with manometric experiments.

The reactions probably proceed according to the following equations:

H_2O_2 could not be detected in the solution at the end of the reaction. But its intermediate formation in autoxidations is now generally assumed. The suggested action of H_2O_2 on ethyl diketobutyrate (3) is supported by the observation of Friedemann [1927] that glyoxals are oxidized by H_2O_2 in alkaline solution according to the general scheme:

(2) *Autoxidation of hydroxyacetoacetic acid.* In marked contrast to ethyl hydroxyacetoacetate the autoxidation of the acid is slow in NaHCO_3 . In $N/6$ NaOH , however, 1 mol. O_2 is rapidly taken up and 1 mol. CO_2 is formed. No H_2O_2 is present in the solution at the end of the reaction.

0.3 ml. $M/20$ hydroxyacetoacetate which with aniline yielded 325 μl . CO_2 was tipped into 2 ml. $N/6$ NaOH and absorbed 312 μl . O_2 in 60 min. On acidification with 0.3 ml. 10% acetic acid ($p\text{H}=5$) 310 μl . CO_2 were evolved (corrected for blank).

No CO_2 was formed from a solution treated in exactly the same way in an atmosphere of pure N_2 . The decarboxylation must therefore occur after oxidation. The process might be formulated thus:

According to this scheme the consumption of 1 mol. alkali (after boiling out the CO_2) and the formation of 1 mol. acetic acid and 1 mol. formic acid would be expected. In fact however no alkali has been bound at the end of autoxidation. Further analysis, carried out under mild conditions (distillation *in vacuo*), shows that only 1 mol. volatile acid has been formed and this consists entirely of formic acid. But if the solution is heated with acid for several hours and then analysed for volatile acids, 2 mol. are found of which one is formic acid. Obviously a neutral polymerization product is first formed which quantitatively yields acetic acid on heating with acid. Its nature is discussed below.

2 ml. $M/20$ hydroxyacetate (=2580 μl . by the aniline method) and 0.3 ml. 10% NaOH were mixed and immediately titrated with $N/10$ HCl at 100° against methyl red: 7.5 ml. were used. An identical sample was titrated after 5 hr. incubation at 25° , when 2620 μl . O_2 had been absorbed. Again 7.5 ml. $N/10$ HCl were used.

A third sample of 2 ml. $M/20$ hydroxyacetoacetate + 0.3 ml. 10% NaOH was, after exhaustive autoxidation, analysed for volatile acids as follows: the solution was acidified with H_3PO_4 and

made up to about 30 ml. It was now distilled *in vacuo* until the residue was concentrated to about 5 ml.; 10 ml. water were added and the solution again concentrated to 5 ml. This process was repeated five times. A further distillate was free from acid. The combined distillates were titrated with $N/10$ $\text{Ba}(\text{OH})_2$. Found: before autoxidation: 2600 μl . volatile acid; after autoxidation: 5220 μl . volatile acid. Therefore 1 mol. volatile acid had been formed during autoxidation.

The neutralized distillates were concentrated on the water bath to about 6 ml. After cooling they were acidified with N H_2SO_4 , made up to 10 ml. with a solution of 10% MnSO_4 in 10 N H_2SO_4 and filtered. An aliquot of 2 ml. was taken from the filtrate and transferred to the main part of a manometer vessel, the side bulb of which contained 0.5 ml. 1.5 N KMnO_4 . On tipping, formic acid was oxidized, liberating 1 mol. CO_2 . The reaction was completed after 3 hr. at 25°. Formic acid found before autoxidation: 0; after autoxidation: 2540 μl .

1 ml. $M/10$ hydroxyacetoacetate (=2100 μl . by the aniline method) + 7 ml. $N/10$ NaOH were left in an open flask at room temperature for 24 hr. The solution was neutralized and made up to 12 ml. 6 ml. were analysed for volatile acids immediately, the remaining 6 ml. after heating on the water bath with 0.6 ml. 10 N H_2SO_4 .

Exp.	1	2	
Unheated	4200 μl .	4230 μl .	} Volatile acid.
Heated 1 hr.	5250 μl .	Heated 4 hr. 6440 μl .	

No oxalic acid was found after autoxidation of hydroxyacetoacetic acid.

Polymerization. It is obvious that the failure to detect acetic acid by mild analysis and its gradual appearance after heating with acid, as well as the formation of only 1 mol. acid instead of two, are due to polymerization of hydroxyacetoacetic acid or of one of its oxidation products in alkaline medium. The constitution of the product of condensation as it appears after complete autoxidation has not been further investigated, but in view of the fact that it is built up of the $\text{CH}_3\text{—CO—}$ moiety of the molecule only, that it is not acid and that it yields acetic acid quantitatively after heating with acid, the following formula of a trihydroxyparaldehyde may be tentatively suggested.

A very similar formula has been proposed by Evans & Waring [1926] for the trimeric form of hydroxypyruvic aldehyde and the paraldehyde type of condensation is also favoured by Levene & Walti [1931] for crystalline dimeric lactaldehyde. In aqueous alkaline solution lactaldehyde, according to Dworzak & Prodinger [1928], polymerizes to a trimolecular product. Hydroxypyruvic and lactic aldehydes are, like hydroxyacetoacetic acid, ketohydroxy-compounds.

The ketohydroxy-enediol tautomerism

(1) *The equilibrium.* The concentration of enediol form in a freshly prepared $N/10$ solution of hydroxyacetoacetic acid at $p\text{H}$ 6.5 and 0° has been determined by iodine titration and found to be 1.35%. It is thus considerably higher than the enol content of a solution of acetoacetic acid under similar conditions, which was found by Grossmann [1924] to be under 0.1%. The concentration of the enediol form is of course greatly increased in a more alkaline medium and, if

autoxidation has been prevented, the ketohydroxy form will be reformed on neutralization. If an equilibrium exists between the three tautomerides, one would expect the enediol form to be converted partly into the α -keto- β -hydroxy- and partly to the α -hydroxy- β -keto-compound. α -Keto- β -hydroxybutyric acid has not yet been described, but it is reasonable to assume that, as an α -ketonic acid, it will not be decarboxylated by aniline, whereas it may be attacked by carboxylase. A solution of hydroxyacetoacetate was therefore incubated in $N/10$ NaOH at 25° for 2 hr. in an atmosphere of pure N_2 and subsequently neutralized. Aliquots were then subjected to the action of aniline or carboxylase. It was found that the CO_2 evolution on addition of aniline had not decreased substantially, whilst carboxylase had no effect. A small increase of CO_2 output was due to non-enzymic catalysis of decarboxylation of the β -ketonic acid, since it occurred also with a boiled sample of carboxylase. It appears therefore that of the tautomeric equilibria:

only the left-hand half exists in aqueous solution.

A similar case is that of ascorbic acid; 2-ketogulonic acid does not form a true equilibrium with the enediol (ascorbic acid) and requires very drastic treatment for conversion into the latter. Ascorbic acid, on the other hand, forms a true equilibrium with 3-ketogulonic acid, which lies almost entirely on the side of the former [Reichstein & Grüssner, 1934].

(2) *The velocity of oxidation.* If iodine is added to a solution of hydroxyacetoacetic acid the first few drops are reduced very quickly, until all the preformed enediol component has been oxidized. A further drop is reduced only very slowly. The same observation is made if the titration is carried out with an indicator, e.g. 2:6-dichlorophenolindophenol. It follows that, in certain cases at least, the reaction of the enediol form with the oxidant is fast compared with the velocity of enolization. Therefore the velocity of oxidation is a measure of the ease with which the compound is enolized. It was of interest to compare the tendencies to enolization of ethyl hydroxyacetoacetate, hydroxyacetoacetate and acetol by measuring the velocities of oxidation under identical conditions. Quite generally the following order was found: ethyl hydroxyacetoacetate $\gg$ hydroxyacetoacetate $\gg$ acetol.

(a) The autoxidation of ethyl hydroxyacetoacetate in $N/10$ $NaHCO_3$ is complete in less than 2 hr. (Fig. 4). In other experiments it was even considerably faster. The autoxidation of hydroxyacetoacetate on the other hand is very slow in neutral or weakly alkaline solution. It varies somewhat from one experiment to another, probably owing to the amount of heavy metals accidentally present.

In Fig. 5 the autoxidations of hydroxyacetoacetate and of acetol in $N/10$ NaOH are compared; whereas the autoxidation of hydroxyacetoacetate is complete after 15 min. that of acetol has only reached about 60% after $3\frac{1}{2}$ hr. The solutions of acetol used in these experiments were prepared by boiling a solution of hydroxyacetoacetate for 5 min. under reflux. Equal quantities of the unboiled and boiled solution were taken for comparison.

(b) If carried out in a bicarbonate- CO_2 buffer many oxidation-reduction reactions can be followed manometrically by the liberation of acid. Thus the reduction of 1 mol. methylene blue is accompanied by the liberation of 1 mol. CO_2 from the $NaHCO_3$ [Reid, 1931]. Preliminary experiments with Thunberg

Fig. 5. Autoxidation of hydroxyacetoacetic acid (I) and acetol (II) in $N/10$ NaOH. The broken line indicates the quantity of CO_2 evolved from the same volume of the hydroxyacetoacetic acid solution after addition of aniline.

Fig. 6.

Fig. 7.

Fig. 6. Oxidation of ethyl hydroxyacetoacetate by methylene blue.

Warburg manometer filled with Clerici solution was used. The manometer vessel contained 3 ml. $M/40$ NaHCO_3 in the main part, 0.004 ml. of ethyl acetoxyacetoacetate in side bulb I and 0.5 ml. 3% methylene blue in side bulb II. The gas space contained purified N_2 with 5% CO_2 . The ester was tipped in at the beginning and the methylene blue after 1 hr. (arrow). 25° .

Fig. 7. Oxidation of hydroxyacetoacetic acid by methylene blue.

The main part of the manometer vessel contained 2 ml. $M/40$ NaHCO_3 and 1 ml. 1.2% methylene blue. The gas space was filled with purified $\text{N}_2/5\%$ CO_2 . 0.1 ml. $M/10$ hydroxyacetoacetate was tipped from the side bulb at the beginning of the experiment which was carried out at 25° . The broken line indicates 1 mol. as determined by the aniline method.

tubes showed that the following indicators were completely reduced by an excess of hydroxyacetoacetate: phenolindophenol, 2:6-dichlorophenolindophenol, thionine, brilliant cresyl blue, methylene blue, pyocyanine, methyl Capri blue, indigotrisulphonate, though in most cases the process is very slow and takes many hours. Phenosafranine and neutral red were not reduced. Figs. 6 and 7 show the reduction of methylene blue by ethyl hydroxyacetoacetate and by hydroxyacetoacetate in manometric experiments ($M/40$ bicarbonate- CO_2 buffer $\text{pH } 7.4$, 25°). In Fig. 6 the hydrolysis of ethyl acetoxyacetoacetate is recorded in the first period; the acid liberated indicates the exact amount of ethyl hydroxyacetoacetate present. When the hydrolysis is finished, the methylene blue is tipped in from a side bulb. 1 mol. methylene blue is reduced in about 2 hr. With hydroxyacetoacetate the reaction is much slower; 1 mol. acid is formed after 15 hr., but the reaction still continues. It has not been possible to observe the end value, but it is probable that it would have reached 2 mol., especially if it is remembered that the spontaneous decomposition of hydroxyacetoacetate is appreciable over such long periods. No reduction of methylene blue was observed with acetol under the same conditions.

Fig. 8. Oxidation of hydroxyacetoacetic acid by a copper-bicarbonate solution (I) and by a ferricyanide bicarbonate solution (II).

The oxidation of hydroxyacetoacetate by a copper-bicarbonate or a ferricyanide-bicarbonate solution can also be followed manometrically (Fig. 8). In the case of the copper reduction the reaction can be schematically written thus:

2 mol. CO_2 are actually liberated in the experiment. The reaction is rapid, being complete after 2 hr. The oxidation with ferricyanide is much slower. The underlying reaction is similar [cf. Haas, 1937]:

The greater velocity of the reaction with the copper solution than of that with the ferricyanide solution may be explained by the fact that the positive copper ions have a higher affinity to the anion of the acid than the negative ferricyanide ions. Neither the copper nor the ferricyanide solution reacted with acetol under the conditions of the experiment.

The experiments were carried out at 25° as follows: 0.1 ml. of ca. *M*/10 hydroxyacetoacetate (the exact concentration of which was previously determined by aniline analysis) and 2 ml. *M*/10 NaHCO₃ were placed in the main part of the manometer vessel. The gas space was filled with 5% CO₂ in N₂, purified over hot copper. After equilibration 0.4 ml. of the copper or ferricyanide solution was tipped from the side bulb. The copper solution had the following composition: 4 g. copper acetate, 15 g. Rochelle salt, 3.2 g. NaHCO₃ made up to 100 ml. The ferricyanide solution contained 5 g. K₃Fe(CN)₆ dissolved in 100 ml. *M*/10 NaHCO₃.

The fact that the tendency to enolization increases from acetol to hydroxy-acetoacetic acid and from hydroxyacetoacetic acid to ethyl hydroxyacetoacetate is easily understood in the light of the electronic theory. Enolization is actually a dissociation according to the scheme:

If *R* is a carboxyl or a carbethoxyl group the mobility of the dissociable proton will be greater than if *R* is a H-atom. On the other hand the negative charge at the carboxylate group has a restraining influence on the release of the proton, compared with the electrically neutral carbethoxyl group. The same reasons explain why if the enediol acid is converted into the ketohydroxy-acid the α -hydroxy- β -keto-acid only is formed. The tendency to release an electron will be stronger at the α - than at the β -position and the free proton will be oriented towards the negative forces of the carboxylate group.

The estimation of acetol by reduction methods

For tissue experiments a method was required by which the sum of hydroxy-acetoacetic acid and the acetol formed by decarboxylation could be estimated. This is possible by using any of the current methods for the estimation of glucose by reduction. If a standard solution of acetol, prepared as described below, is heated for 15 min. in a boiling water bath with the copper solution of Shaffer-Hartmann [Peters & Van Slyke, 1932, p. 466, reagent 1], 2 Cu⁺⁺ are reduced for 1 mol. acetol, corresponding to $\frac{1}{2}$ mol. O₂. 1 ml. *N*/100 thiosulphate therefore indicates 112 μ l. acetol in the final titration.

If a standard solution of acetol is heated for 30 min. in a boiling water bath with 0.05 *N* K₃Fe(CN)₆ in 7% Na₂CO₃, acidified after cooling and titrated with *N*/100 ceric sulphate, using setopaline as indicator [Miller & Van Slyke, 1936], 4 mol. ferricyanide are reduced by 1 mol. acetol, corresponding to 1 mol. O₂. 1 ml. *N*/100 ceric sulphate therefore indicates 56 μ l. acetol.

Standard solution of acetol (*M*/50): 520 mg. recrystallized bis-acetoldimethylether [Nef, 1904], m.p. 127°, were heated under reflux with 25 ml. *N* HCl on the water bath for 30 min., cooled, neutralized and made up to 250 ml.

1 ml. *M*/50 acetol + 3 ml. Shaffer-Hartmann solution used 2.60 ml. *N*/100 thiosulphate. 3 ml. Shaffer-Hartmann solution used 6.60 ml. Difference: 4.00 ml. *N*/100 thiosulphate.

1 ml. *M*/50 acetol + 3 ml. ferricyanide solution, titrated with *N*/100 ceric sulphate, used 8.00 ml.

If a freshly prepared solution of hydroxyacetoacetate is converted into acetol and CO₂ by boiling, the ferricyanide method gives values which agree closely with the amount of CO₂ liberated by aniline from the unboiled solution. The values obtained by the copper method are, however, low (70–80%) for reasons which are not clear. This is especially surprising since the oxidation by

copper gave a theoretical result in the manometric experiments referred to above.

0.2 ml. of *ca.* *M*/10 hydroxyacetoacetate yielded 552 μ l. CO_2 on addition of aniline.

0.2 ml. of this solution, after boiling + 3 ml. Shaffer-Hartmann solution, 15 min. at 100° : titrated 3.45 ml. *N*/100 thiosulphate = 386 μ l. (70%).

0.2 ml. of this solution + 3 ml. ferricyanide solution, 30 min. at 100° : titrated 9.45 ml. *N*/100 ceric sulphate = 530 μ l. (96%).

This estimation is of course not very specific. Separation from other reducing substances, especially sugars, was not possible. A blank with an equal amount of tissue but without hydroxyacetoacetate was run in every experiment and the result corrected accordingly. This blank is small with most sliced tissues, except liver.

The ferricyanide method cannot be used in presence of acetoacetic acid or acetone, since acetone reacts with alkaline ferricyanide at 100° .

Conversion of acetol into lactic acid

Denis [1907] and Evans & Hoover [1922] have shown that acetol is oxidized by KMnO_4 at room temperature in neutral or weakly acid solution yielding equimolecular amounts of acetic acid and CO_2 . The reaction can be studied manometrically, but it is so slow that it is not convenient for analytical purposes. No volatile carbonyl compounds are formed in the oxidation of acetol by KMnO_4 ; acetol does not therefore interfere with lactic acid estimation, nor does hydroxyacetoacetic acid. If, however, a solution of acetol is subjected to the copper-lime treatment, it is converted almost quantitatively into lactic acid, being first oxidized to methylglyoxal [cf. Denis, 1907] which, in alkaline solution, is converted into lactic acid by intramolecular dismutation. It is important to realize this if the formation of lactic acid is studied in presence of acetol. A freshly prepared solution of hydroxyacetoacetic acid, free from acetol, does not form any lactic acid when treated with copper-lime. The acid is apparently completely removed from the solution by the copper-lime treatment.

10 ml. *M*/300 acetol, containing 790 μ l., analysed for lactic acid by the method of Friedemann & Kendall [1929]: no lactic acid.

The same + 1 ml. 25% CuSO_4 + 1 ml. 12.5% $\text{Ca}(\text{OH})_2$. Left at room temperature for 30 min. with repeated shaking. Filtered and analysed for lactic acid: 635 μ l. lactic acid (80%).

II. METABOLISM IN ANIMAL TISSUES

Clutterbuck & Raper [1926] suggested that the physiological oxidation of acetoacetic acid follows the same path as the oxidation *in vitro* by H_2O_2 in alkaline solution. According to this scheme acetoacetic acid would be oxidized to hydroxyacetoacetic acid and further to diketobutyric acid. This substance would be decarboxylated to methylglyoxal which would be converted into lactic acid. Some support was given to this scheme by experiments of Haarmann [1935] who reported formation of lactic acid from β -hydroxybutyric acid by various tissues under anaerobic conditions.

Since the decarboxylation of diketobutyric acid can be assumed to take place spontaneously and the conversion of methylglyoxal into lactic acid is a

well-known enzymic reaction, the steps to be proved are (1) the oxidation of acetoacetic acid to hydroxyacetoacetic acid, (2) the oxidation of hydroxyacetoacetic acid to diketobutyric acid or methylglyoxal. The preparation of solutions of hydroxyacetoacetate, described in the preceding section, made it possible to test the second of these reactions with tissues. If hydroxyacetoacetic acid is oxidized by tissues, increased respiration and aerobic disappearance of the substrate are to be expected. It is known that the tissue where the oxidative breakdown of acetoacetic acid is highest is kidney. This is true for the intact perfused organ [Snapper & Grünbaum, 1927] as well as for kidney slices [Quastel & Wheatley, 1935; Edson & Leloir, 1936]. Special attention has therefore been directed to experiments with kidney.

Methods. Tissue experiments were carried out at 37.5° with the Warburg apparatus. The results are expressed in the conventional Q -units.

Respiration of rat tissues in presence of hydroxyacetoacetic acid

When measuring the respiration of tissue slices in presence of hydroxyacetoacetic acid it is necessary to correct the results for the small autoxidation of the latter. This has been done with the results collected in Table II. It is also

Table II. *Respiration of rat tissues*

Phosphate-saline. O_2 . HAA = Hydroxyacetoacetic acid

Exp.	Tissue	Substrate	Q_{O_2}		
			1st hr.	2nd hr.	3rd hr.
1	Kidney (slices)	0	- 15.3	- 10.5	- 9.3
		$M/100$ HAA	- 21.7	- 16.7	- 15.5
		$M/100$ acetic acid	- 21.5	- 16.7	- 16.8
2		0	- 19.4	- 15.6	- 12.3
		$M/100$ HAA	- 28.6	- 23.5	- 13.7
		$M/100$ acetic acid	- 25.6	- 23.6	- 22.3
3	Brain (slices)	0	- 6.5	- 3.3	- 1.2
		$M/100$ HAA	- 6.0	- 2.2	- 1.1
4	Liver (slices)	0	- 9.2	- 8.7	
		$M/100$ HAA	- 13.7	- 13.1	
5		0	- 9.5	- 8.0	
		$M/100$ HAA	- 15.3	- 14.5	
		$M/100$ acetic acid	- 13.9	- 12.6	
6	Heart (slices)	0	- 3.4	- 2.6	
		$M/100$ HAA	- 10.5	- 10.6	
7		0	- 3.1	- 2.8	
		$M/100$ HAA	- 8.5	- 6.4	
		$M/100$ acetic acid	- 6.7	- 7.1	
8	Testis	0	- 6.8	- 4.9	
		$M/100$ HAA	- 8.2	- 3.7	
9	Jensen rat sarcoma (slices)	0	- 11.5	- 9.7	
		$M/150$ HAA	- 11.1	- 8.2	
		$M/150$ acetic acid	- 9.5	- 8.4	

to be kept in mind that the solutions of hydroxyacetoacetate contain an equimolecular amount of acetate. An increase of respiration may be due to oxidation of either substrate. Only if the respiration in presence of hydroxyacetoacetic acid were considerably higher than in presence of an equimolecular amount of acetic acid could the effect be attributed with certainty to hydroxyacetoacetic acid. But the increase of respiration where it is observed is in most cases not or only slightly higher than with an equimolecular quantity of acetic acid.

Disappearance of hydroxyacetoacetic acid added to rat tissues

Hydroxyacetoacetic acid disappears very quickly if incubated with slices of rat kidney. The rate of disappearance is about the same in presence as in absence of O₂ (Table III).

Table III. *Disappearance of hydroxyacetoacetic acid in presence of slices of rat kidney*

1 hr. incubation in bicarbonate-saline. Amount of hydroxyacetoacetic acid present at start: 550 μ l. Estimation of hydroxyacetoacetic acid present at the end with aniline at 25°

Mg. dry wt.	Gas phase	μ l. HAA final	Disappearance due to tissue μ l. HAA	Q_{HAA}
0	O ₂ /5% CO ₂	470	—	—
5.61	O ₂ /5% CO ₂	226	244	-43.5
6.64	N ₂ /5% CO ₂	152	318	-47.7

These results were obtained with the aniline method which measures the disappearance of the β -ketonic acid only. This disappearance might be due to simple decarboxylation: in this case corresponding amounts of acetol and bicarbonate would be found. Or the β -ketonic acid might have been converted into another compound which is not decarboxylated by aniline, e.g. an intramolecular dismutation to α -keto- β -hydroxybutyric acid or a reduction to dihydroxybutyric acid. This possibility can be excluded, since the amount of bicarbonate formed aerobically as well as anaerobically accounts for the disappearance of β -ketonic acid. The sum of β -ketonic acid and bicarbonate remains constant.

The experiments were done with four vessels provided with two side bulbs. Unbuffered, bicarbonate-poor Krebs-Ringer solution was used as suspension fluid. The gas space was filled with O₂ or N₂. Vessel I contained neither substrate nor tissue, vessel II contained hydroxyacetoacetic acid without tissue, vessel III contained tissue without substrate and vessel IV tissue + hydroxyacetoacetic acid. After 1 hr. incubation at 37.5° the tissue was removed and 0.3 ml. 3 *M* acetate buffer pH 4.6 was placed in one side bulb and 0.4 ml. aniline solution in the other side bulb. The manometers were now shaken in a bath at 25° and bicarbonate and β -ketonic acid contents were subsequently analysed by tipping in first the acetate buffer and then the aniline solution (see Table IV).

Table IV. *Formation of bicarbonate and disappearance of hydroxyacetoacetic acid in presence of slices of rat kidney*

Mg. dry wt.	Gas phase	μ l. bicarbonate formed	μ l. HAA final	Sum
0	O ₂	58	206	264
5.38	O ₂	164.5	103	267.5
0	N ₂	42.5	218	260.5
5.94	N ₂	200	48	248

It follows that the disappearance of hydroxyacetoacetic acid is accompanied by decarboxylation. At least as far as the aerobic experiments are concerned, however, this decarboxylation could take place after oxidation of hydroxyacetoacetic acid to diketobutyric acid or, alternatively, acetol could be oxidized after the decarboxylation. In both these cases one would find a decrease of the sum of hydroxyacetoacetic acid + acetol (= "total acetol").

The disappearance of total acetol has been examined in a series of experiments using both reduction methods described (Table V).

At the end of the experiment 0.2 ml. 2*N* HCl was added. The tissue was removed and the solution neutralized with *N* NaOH. The solution was made up to about 8 ml. and heated in a boiling water bath for 5 min., after a little kieselguhr had been added. The filtered solution was analysed for acetol. Blanks without substrate were carried out in every case and the results corrected in proportion to the dry weights of tissue.

Table V. *Disappearance of total acetol in rat tissues*

2 hr. incubation in O₂ if not stated otherwise

Tissue	mg. dry wt.	μ l. total acetol final	Decrease μ l.	Remarks
Exp. 1. Total acetol present at start, 443 μ l. Ferricyanide method				
0	—	372	71	—
Brain	14.94	388	55	—
Kidney	10.85	400	43	—
Liver	18.87	295	148	—
Heart	17.02	367	76	—
Testis	49.41	440	3	—
Exp. 2. Total acetol present at start, 390 μ l. Ferricyanide method				
0	—	375	15	—
Liver	15.69	300	90	—
Heart	14.90	325	65	—
Exp. 3. Total acetol present at start, 440 μ l. Copper method				
0	—	413	27	—
Kidney	9.49	438	2	—
Testis	37.57	432.5	7.5	—
Heart	26.57	360	80	—
Exp. 4. Total acetol present at start, 475 μ l. Ferricyanide method				
0	—	377	98	—
0	—	472	3	Anaerobic exp.
Liver	16.16	440	35	—
	16.13	475	0	Boiled soln. of HAA
	15.75	472	3	Anaerobic exp.
Heart	30.23	385	90	—
	24.85	475	0	Boiled soln. of HAA
	31.23	466	9	Anaerobic exp.
Exp. 5. Total acetol present at start, 368 μ l. Ferricyanide method				
Jensen rat	0	344	24	—
sarcoma	11.61	350	18	—

The interpretation of these results which are typical of a greater number of experiments is complicated by the fact that there is sometimes considerable disappearance of total acetol even in absence of tissue, probably owing to autoxidation of hydroxyacetoacetic acid. In most cases this disappearance is not increased but rather reduced by the presence of tissues. It is impossible to say how much of the disappearance found in presence of tissues is due to enzymic processes and how much to autoxidation. Liver and heart are the only tissues with which the disappearance is sometimes definitely increased, but even this is not a constant observation. With kidney the result was always clearly negative; yet kidney is the tissue where the oxidative removal of hydroxyacetoacetic acid should be highest if this substance is an intermediary of acetoacetic acid metabolism.

Summarizing the evidence so far presented it appears that there is no effect on respiration which could with certainty be attributed to hydroxyacetoacetic acid and that a possible small oxidative disappearance is observed only with liver and heart. The only effect which is observed in presence of kidney is a rapid

decarboxylation to acetol and bicarbonate. It is of course possible that this decarboxylation is too rapid to allow the hydroxyacetoacetic acid added to the suspension fluid to penetrate to the centre of oxidation within the cell.

Formation of hydroxyacetoacetic acid by tissues

A series of experiments was undertaken to test the first step in the hypothetical sequence of reactions which lead from acetoacetic acid to lactic acid, viz. the oxidation of acetoacetic acid to hydroxyacetoacetic acid. It is quite clear from earlier work [Snapper & Grünbaum, 1927; Friedemann, 1936; Quastel & Wheatley, 1935; Edson & Leloir, 1936] that the body is able to oxidize acetoacetic acid and that this process is specially active in kidney, but we are still quite ignorant about the intermediary stages of the process.

The anaerobic disappearance of acetoacetic acid was first investigated, together with the formation of β -hydroxybutyric acid. Edson & Leloir presented figures for the anaerobic disappearance of acetoacetic acid in various tissues and under varying conditions, but they have not examined the simultaneous formation of β -hydroxybutyric acid. Quastel & Wheatley on the other hand published two experiments with guinea-pig kidney where the respiration was poisoned with HCN. They found in one experiment a formation of β -hydroxybutyric acid which accounted for 78 % of the acetoacetic acid disappearing, but in the other experiment it accounted only for 52 %.¹ I have found quite regularly that the β -hydroxybutyric acid formed accounted only for about 50 % of the acetoacetic acid disappearing (Table VI).

The estimation of acetoacetic acid and β -hydroxybutyric acid was carried out in the same solution by the method previously described [Weil-Malherbe, 1937]. A yield of 76–78 % of acetone from β -hydroxybutyric acid was obtained with great regularity if the following details were adhered to: to the solution in the distilling flask were added 6 ml. 66 vol. % H_2SO_4 and water to make a total volume of 30 ml. The solution was now heated to boiling and 10 ml. 0.1 % $\text{K}_2\text{Cr}_2\text{O}_7$ were slowly added from the dropping funnel. The flame was reduced so that the solution was just kept boiling and the rate of distillation was not higher than 1 drop in 4 sec. The distillation of the 25 ml. should be finished in not less than 30 min. Water is added from the dropping funnel to keep the volume in the flask constant.

Table VI. *Dismutation of acetoacetic acid with rat tissues*

Bicarbonate saline. $\text{N}_2/5\%$ CO_2 . $M/100$ acetoacetate		
Tissue	$Q_{\text{Acetoac.}}$	$Q_{\beta\text{-Hydroxybut.}}$
Brain	–0.67	0.33
	–0.55	0.30
Testis	–0.98	0.48
	–0.69	0.35
Kidney	–1.57	0.81
	–1.64	0.85
+ $M/100$ $l(+)$ alanine	–2.30	1.24

It is reasonable to assume that, if 50 % of the acetoacetic acid disappearing is reduced, the other 50 % is oxidized, i.e. that the anaerobic disappearance of acetoacetic acid is due to a dismutation, as is the case with other keto-acids [cf. Weil-Malherbe, 1937].

Since acetoacetic acid disappears only in proportion to the velocity with which it can be reduced, its removal is primarily an oxidative process. This is a

¹ Quastel & Wheatley's experiments in presence of glucose and pyruvate are deliberately left out of the discussion; here the reactions are complicated by a possible dismutation between pyruvic and acetoacetic acids and also by a possible formation of β -hydroxybutyric acid from pyruvic acid [Krebs & Johnson, 1937].

valuable clue since it excludes other non-oxidative reactions which have been suggested, such as hydrolytic cleavage into two molecules of acetic acid or hydration to dihydroxybutyric acid [Haarmann & Schroeder, 1938].

The anaerobic CO_2 production of tissue incubated anaerobically in bicarbonate-saline is not at all or only insignificantly greater in presence than in absence of acetoacetic acid. The dismutation of acetoacetic acid is therefore not accompanied by decarboxylation or acid formation.

All these facts could be explained if it were possible to identify the oxidative equivalent of the dismutation with hydroxyacetoacetic acid and it was these experiments which originally led to the present investigation. But all efforts to detect the formation of hydroxyacetoacetic acid from acetoacetic acid failed. If hydroxyacetoacetic acid were really formed from acetoacetic acid, its own oxidation should be sufficiently checked under anaerobic conditions to allow some accumulation. The measurement of the rate of dismutation allowed an estimate of the quantity of oxidative equivalent that could be expected and the dimensions of the experiment were so chosen that at least 100 $\mu\text{l.}$ of the oxidative equivalent were formed (about 100 mg. dry wt. of slices in a 2 hr. experiment). At the end of the experiment all enzymic processes were stopped by acidification before admission of O_2 . After removal of the tissue the solution was deproteinized by boiling with kieselguhr and filtered. The filtrate was then analysed by one of the following methods: (1) heating with Shaffer-Hartmann's copper solution did not reveal the presence of a reducing substance; (2) the filtrate was acidified and distilled and the distillate was tested with *o*-aminobenzaldehyde for acetol; no acetol could ever be detected, though the test was intensely positive if model estimations with the expected amount of acetol were performed; even a few $\mu\text{l.}$ acetol produced obvious blue fluorescence in the light of a mercury lamp; (3) the filtrate was analysed for lactic acid by the method of Friedemann & Kendall [1929]; if hydroxyacetoacetic acid had been further oxidized even under anaerobic conditions, perhaps by undergoing a dismutation itself, the product formed would be lactic acid; no formation of lactic acid was, however, observed (Table VII). The analysis was sometimes carried out with the kieselguhr filtrate without further treatment, in other experiments after treatment with copper-lime. In the latter case any acetol present would have been partly estimated as lactic acid.

If hydroxyacetoacetic acid were formed from acetoacetic acid in the body, one would expect that just as in the case of acetoacetic acid it would partly escape further oxidation by spontaneous decarboxylation. Acetol should therefore appear in the urine of persons with ketosis where the body has to deal with large amounts of acetoacetic acid. Through the courtesy of Dr F. K. Herbert I examined the urine of a patient in diabetic coma. The freshly passed urine

Table VII. *Formation of lactic acid by slices of rat kidney in presence of acetoacetic and hydroxyacetoacetic acids*

Bicarbonate-saline. 2 hr. HAA = hydroxyacetoacetic acid			
Substrate	Gas phase	Pre-treatment	Q _{Lact.}
0	$\text{O}_2/5\% \text{CO}_2$	Kieselguhr	0.31
M/50 acetoacetate	—	—	0.24
M/100 HAA	—	—	0.34
0	$\text{N}_2/5\% \text{CO}_2$	Kieselguhr	1.5
M/50 acetoacetate	—	—	1.4
M/100 HAA	—	—	1.8
0	$\text{N}_2/5\% \text{CO}_2$	Copper-lime	0.59
M/50 acetoacetate	—	—	0.53

which had a strongly positive FeCl_3 reaction was acidified and distilled, and the distillate was tested with *o*-aminobenzaldehyde; the test was negative.

Under aerobic conditions considerable quantities of a reducing substance are formed from acetoacetic acid. This substance does not give the fluorescence test with *o*-aminobenzaldehyde and is therefore not identical with acetol. Its nature is being further investigated. No formation of lactic acid was detected in aerobic experiments (Table VII).

The failure to detect hydroxyacetoacetic acid or acetol amongst the oxidation products of acetoacetic acid in tissue experiments does not exclude conclusively the possibility of their intermediate formation. It is still possible to argue that nascent hydroxyacetoacetic acid disappears too quickly or that our means of detection are too inadequate to obtain positive evidence of its intermediate formation. This objection can be effectively answered since it is possible to demonstrate a case where hydroxyacetoacetic acid is indeed formed in kidney slices, viz. by oxidation of threo-1:2-dihydroxybutyric acid. There is no reason to assume that hydroxyacetoacetic acid behaves differently whether it is formed from acetoacetic acid or from dihydroxybutyric acid; therefore if it is found in one reaction it should also be detectable in the other reaction, and if not it may be inferred that it is in fact not formed. These results confirm, in our view definitely, the conclusion that hydroxyacetoacetic acid is not an intermediary of the physiological oxidation of acetoacetic acid.

The oxidation of dl-threo-1:2-dihydroxybutyric acid by slices of rat kidney

The respiration of slices of rat kidney is increased in presence of *dl*-threo-dihydroxybutyric acid, but not of *dl*-erythro-dihydroxybutyric acid (Table VIII).

Table VIII. *Respiration of slices of rat kidney*

Substrate	Phosphate-saline. O_2			
	Q_{O_2}			
	Before tipping substrate 10 min.	1st hr.	2nd hr.	3rd hr.
0	-26.1	-19.4	-15.6	-12.3
M/50 <i>dl</i> -threo-1:2-dihydroxybutyric acid	-24.4	-27.5	-22.5	-18.2
M/50 <i>dl</i> -erythro-1:2-dihydroxybutyric acid	-25.9	-20.3	-16.2	-13.4

After aerobic incubation of rat kidney slices in presence of threodihydroxybutyric acid hydroxyacetoacetic acid or acetol can be detected in the solution by the following three reactions: (1) a positive fluorescence test with *o*-aminobenzaldehyde, (2) an evolution of CO_2 upon addition of aniline and (3) a reduction of Shaffer-Hartmann's copper solution or of an alkaline ferrieyanide solution. All three reactions are negative with pure solutions of threodihydroxybutyric acid and also with solutions in which tissue had been incubated without addition of this substrate.

Owing to the rapid decarboxylation of hydroxyacetoacetic acid by kidney slices the amount of β -ketonic acid present after a 2 hr. experiment is only about 20 % of the total acetol, as estimated by the reduction method. It is of course uncertain whether the reducing substances formed are entirely "total acetol". If, however, the fluorescence test is carried out with a quantity of acetol corresponding to the amount found by the reduction test and is compared with a test performed on a solution from a tissue experiment, the fluorescences of both solutions are of very similar intensity.

The formation of "total acetol" has been compared with the disappearance of dihydroxybutyric acid. Both analyses were done on aliquots of the same solution.

1:2-Dihydroxybutyric acid was estimated with periodic acid which oxidizes α -glycols specifically [Fleury & Lange, 1932]. The method used was very similar to the procedure described by Fleury & Fatome [1935] for the estimation of glycerol. To the deproteinized, neutralized solution (about 10 ml.) were added 2 ml. $N/10$ periodic acid and 1 ml. $N/10$ HCl and the mixture was left at room temperature for 15 min. 10 ml. saturated NaHCO_3 , 2 ml. 0.11 N NaAsO_2 in 7% NaHCO_3 , 2 ml. 10% KI and 1 ml. starch indicator were then added. After 10 min. the solution was titrated with $N/100$ I_2 . It is necessary to pipette the periodic acid and the arsenite very accurately. The difference between $N/100$ iodine used in the experimental solution and that used in the blank indicates the quantity of α -glycol present. 1 ml. $N/100$ $\text{I}_2 = 112 \mu\text{l.}$ dihydroxybutyric acid. The method gives theoretical results with pure solutions. It was not possible to separate dihydroxybutyric acid and sugars. The value obtained in the blank, corrected for the dry weight of tissue used, was therefore subtracted from the result. Acetol was removed before the estimation by evaporation of the neutral solution to dryness on a water bath.

Table IX. *Disappearance of dl-threo-1:2-dihydroxybutyric acid and formation of "total acetol" by slices of rat kidney*

Phosphate-saline. O_2 . 2 hr. incubation		
Exp.	$Q_{\text{Dihydroxybut.}}$	$Q_{\text{Acetol.}}$
1	-4.2	2.4
2	-3.6	1.5

Four large Warburg vessels of about 40 ml. volume with side bulb were used for each experiment. They each contained 10 ml. phosphate saline and about 30 mg. of kidney slices (dry wt.). Vessels II and IV contained in addition 0.3 ml. $M/10$ dihydroxybutyric acid in the side bulb, which was added to the tissue at t_0 . The reaction was interrupted by acidification in vessels I and II at t_0 , in vessels III and IV after 2 hr.

It appears from Table IX that the acetol formed, as estimated by the reduction method, accounts for about half of the dihydroxybutyric acid disappearing.

Whether this reaction is of physiological significance or whether it is merely due to a limited specificity of the β -hydroxybutyric dehydrogenase or another enzyme will not be discussed. It is important however that hydroxyacetoacetic acid or acetol, when really formed, accumulate to a considerable extent and can be detected without difficulty.

After these experiments had been completed, a paper by Haarmann & Schroeder [1938] was published. These authors found increased formation of lactic acid from β -hydroxybutyric and dihydroxybutyric acids, both aerobically and anaerobically, with minced tissues of the dog and cat. On the basis of this result they put forward the very same mechanism of acetoacetic acid oxidation which I was led to reject as a result of my experiments. Though the possibility of a conversion of dihydroxybutyric acid into lactic acid is not denied and is indeed made probable by our experiments, the experiments of Haarmann & Schroeder are in my opinion open to certain criticisms. Haarmann & Schroeder write: "Die Dioxybuttersäure stört die Bestimmung der Milchsäure nicht." This statement cannot be accepted without a detailed description of the method employed to separate these acids. I have found that with the method of Friedemann *et al.* [1927], used by Haarmann & Schroeder (see Haarmann [1932]), up to 70% is analysed as "lactic acid". This is in agreement with an observation of Friedemann [1928] who found 50% "lactic acid" formed from dihydroxybutyric acid.

The copper-lime precipitation does not remove dihydroxybutyric acid quantitatively. Analysing a copper-lime filtrate I have still found 36 % "lactic acid". With the periodic acid method it can be shown that only about 40 % of the dihydroxybutyric acid is removed by the copper-lime treatment. Moreover, if the copper-lime precipitation were used, as was described by Haarmann in earlier work [1932], the lactic acid found may have been formed from acetol in a purely chemical, non-enzymic way, and evidence has been presented in this investigation that acetol is indeed thus formed from dihydroxybutyric acid. These points need clarifying before the evidence for the formation of lactic acid from dihydroxybutyric acid by tissues can be accepted.

It has already been pointed out that Haarmann & Schroeder's suggestion that dihydroxybutyric acid may be formed from acetoacetic acid by hydration is improbable on the ground that the process by which acetoacetic acid disappears anaerobically is a dismutation, i.e. the formation of equal amounts of reductive and oxidative equivalents. Since dihydroxybutyric acid is oxidized to hydroxyacetoacetic acid, the latter should also be formed by the dismutation of acetoacetic acid if the hydration mechanism operates; it has been shown that this is not the case.

These criticisms do not of course apply to the formation of lactic acid from β -hydroxybutyric acid described by Haarmann [1935] and by Haarmann & Schroeder [1938]. But it may be emphasized that in these experiments where a large formation of acetone takes place the preliminary removal of this substance before lactic acid estimation must be very thorough. Using this precaution I could not find any formation of lactic acid from acetoacetic acid with rat kidney slices.

SUMMARY

1. A method is described for the preparation of solutions containing equivalent amounts of α -hydroxyacetoacetic and acetic acids by the anaerobic hydrolysis of ethyl α -acetoxyacetoacetate in dilute alkali.

2. The stability of hydroxyacetoacetic acid in aqueous solution at pH 7.4 and 4.6 has been determined for 0, 25 and 37.5°.

3. Ethyl hydroxyacetoacetate is oxidized in $M/10$ NaHCO_3 by molecular O_2 . 1 mol. O_2 is absorbed and 1 mol. acetic acid and 1 mol. ethyl hydrogen oxalate are formed.

4. Hydroxyacetoacetic acid is oxidized in $N/10$ NaOH by molecular O_2 . 1 mol. O_2 is absorbed and 1 mol. CO_2 , 1 mol. formic acid and 1 mol. acetic acid are formed. The latter appears as a polymerization product which is built up of the $\text{CH}_3\text{—CO—}$ moiety of hydroxyacetoacetic acid and which yields acetic acid quantitatively after heating with acid.

5. The velocity of oxidation which is in many cases a measure of the tendency to enolization is of the following order: ethyl hydroxyacetoacetate > hydroxyacetoacetic acid > acetol. A number of oxidation reactions have been studied manometrically.

6. The estimation of acetol by reduction methods is described.

7. There is no effect on the respiration of rat tissues which could with certainty be attributed to hydroxyacetoacetic acid. A possibly oxidative removal is observed only with liver and heart. The only effect observed in presence of kidney slices is rapid decarboxylation.

8. If acetoacetic acid is incubated anaerobically with rat tissues a dismutation takes place, since the β -hydroxybutyric acid formed accounts only for 50 % of the acetoacetic acid disappearing. But no evidence has been found which would

allow the identification of the oxidative equivalent with hydroxyacetoacetic acid.

9. *dl*-Threo-1:2-dihydroxybutyric acid is oxidized by slices of rat kidney to hydroxyacetoacetic acid. Its amount accounts for about 50 % of the dihydroxybutyric acid disappearing. This proves that the failure to detect hydroxyacetoacetic acid amongst the oxidation products of acetoacetic acid is not due to a too rapid disappearance of hydroxyacetoacetic acid or to inadequate technique of detection.

I wish to acknowledge with gratitude the gift of samples of *dl*-threo- and erythro-1:2-dihydroxybutyric acids by Dr J. W. E. Glattfeld of the University of Chicago. My thanks are also due to Dr F. Dickens for his interest and encouragement.

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Note added 26 May 1938. Recent experiments indicate that the reducing substance found in the suspension fluid after aerobic incubation of rat kidney slices with acetoacetic acid is glucose.