CCLVI. STUDIES IN FAT METABOLISM.

II. THE OXIDATION OF NORMAL SATURATED FATTY ACIDS IN THE PRESENCE OF LIVER SLICES.

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THE methods described in Part I of this series [Jowett and Quastel, 1935, 1], together with others described here, have been used to study the oxidation of the normal saturated fatty acids, containing from two to ten carbon atoms, by slices of rat and guinea-pig liver. The work has special reference to the formation of "ketone bodies" as products of oxidation and provides new and quantitative findings.

The results form a contribution to the problem of the mechanism of oxidation of fatty acids. It has been possible to estimate the approximate yields of ketone bodies from some of the fatty acids, which prove to be much higher than those hitherto obtained. The production of ketone bodies is definitely established as a major process in the oxidation; lack of evidence had hitherto led such authorities as Leathes and Raper [1925] to a sceptical view of their importance.

It has also been found that the simple β -oxidation theory of Knoop as applied by Embden to the breakdown of fatty acids in the liver, is unable to account for the facts observed. It will be remembered that according to the view of Embden and Marx [1908] decanoic acid is broken down successively to octanoic, hexanoic and butyric acids before acetoacetic acid is formed.

EXPERIMENTAL METHODS.

Details of most of the methods used will be found in Part I. Briefly, liver slices have been shaken for two hours at 37° in various media in manometric vessels of the Warburg type. After the tissue slices have been removed to be dried and weighed, the residual solutions are employed for estimation of acetoacetic acid, or for the gravimetric estimation of ketone bodies, described later.

Estimations of acid-base changes have been made in a manner generally similar to that of Negelein [1925]. In these experiments lead perchlorate solution of density about 2.09, prepared according to Krebs [1930], has been used as manometer fluid in order to measure greater outputs of carbon dioxide than can be measured with Brodie's fluid. Lead perchlorate has also been used in a number of other experiments, and it has been found that it leads to an accuracy no less than with Brodie's fluid, since although the manometer changes are halved the readings are steadier.

In estimating the changes in fixed acid, account must be taken of the fact that when the solutions are acidified to decompose bicarbonate the acetoacetic acid present will be partially decomposed. The CO₂ evolution ascribed to bicarbonate will be slightly increased by this decomposition and the subsequent

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CO₂ output in presence of aniline, ascribed to acetoacetic acid, will be lowered. A correction has been calculated on the basis of our measurement of the rate of decomposition of acetoacetic acid in solutions of p_H below 2 (Part I). A correction of 7.5% is added to the "observed" values for acetoacetic acid, when the solution has been maintained at the low p_H for 15 min. at 37° (for 20 min., 10% is added). A correction of the same absolute (not relative) amount is added algebraically to the value derived for production of fixed acid. The corrections do not essentially alter the values. The amount of acid added, usually about 0.15–0.2 ml. of $N H_2SO_4$, has always been sufficient to bring the p_H below 2 (c_H approximately 0.03), allowance being made for the amount of bicarbonate to be decomposed and for the buffering action of salts of weak acids present. The evolution of CO₂ is complete in 10–15 min. After reading the pressure changes the tissue slices are removed, and if acetoacetic acid is to be estimated the solution is brought at once to a p_H near 4.5 by addition of sodium acetate solution, the amount required being usually determined by calculation.

Gravimetric estimation of ketone bodies. The method of Van Slyke [1917] and of Peters and Van Slyke [1932] for blood has been adapted to a micro-scale. All details of the method of precipitation of the mercury compounds have been retained, such as times of refluxing and concentrations of reagents, but the size of apparatus has been reduced, and the volumes of solutions reduced from 175 ml. to 35 or 22 ml. The precipitates are washed on the centrifuge and transferred by suction to Jena sintered glass filters of type G4, which are weighed on a micro-balance.

In the accompanying table are summarised the results of control estimations. Sodium acetoacetate, freshly freed from acetone, has been standardised by the manometric technique of Part I, and the specimen of sodium β -hydroxybutyrate

Control estimations.

Acetoacetic acid.			
mg. taken cal- culated as acetone	Volume during refluxing (ml.)	mg. ppt.	Wt. ppt. Wt. acetone
0.143	22	2.15	15.1
0.286	"	5.01	17.5
0.428	"	8.53	19.9
0.571	"	11.24	19.7
0.386	35	6.52	16.9
0.445	"	8.18	18.4
0.857	"	17.68	20.6
β -Hydroxybutyric acid.			
mg. taken cal- culated as acid	Volume (ml.)	mg. ppt.	Wt. ppt. Wt. acid
0.280	35	2.69	9.6
0.560	"	5.36	9.6
0.776	"	7.52	9.7
0.839	"	8.75	10.4
1.119	"	11.09	9.9
1.552	"	16.38	10.55

used, when dried at 100°, has been taken as pure. For acetoacetic acid the factor tends to be lower for small quantities than Van Slyke's factor of 20, and an empirical correction has been applied for the deviations, although the use of data based on precipitates weighing less than 5 mg. has been almost completely avoided. For β -hydroxybutyric acid the factor 9.85 has been found and employed, which compares with Van Slyke's factor 8.45, and probably indicates a more complete oxidation to acetone than was found by Van Slyke.

Estimations show that in our experiments the quantities of acetoacetic acid and β -hydroxybutyric acid formed from fatty acids are usually of the same order, and to calculate "total ketones" we have therefore assumed that the amounts formed are equal.

The data and assumptions above lead to the following factors by which the weights of precipitates (mg.) are divided in order to convert weights of precipitate into μ l. of gas at N.T.P.: acetone + acetoacetic acid 0.052; β -hydroxybutyric acid 0.046, total ketones 0.049.

In our experiments, summarised later, in which ketone bodies are estimated by this method, the contents of the manometer vessel, after removal of the tissue slices, are made up to known volume (usually 10–18 ml.) with water and 1/10 volume of the acid mercuric sulphate reagent of Van Slyke. After filtration through paper, a known volume of filtrate is at once taken for the estimation. Where manometric estimations are also made, these are effected on the contents of another manometer vessel which has undergone simultaneous shaking in the bath with similar solutions and a roughly equal quantity of liver slices.

Manometric method for acetoacetic acid. In applying the manometric method for acetoacetic acid, described in Part I, to the determination of a product derived from higher fatty acids, account must be taken of the possibility that the higher fatty acids may yield homologues of acetoacetic acid, which will probably react with aniline in a similar manner to acetoacetic acid.

Dakin [1923] has shown by liver perfusion experiments that hexanoic acid does not give rise to butyrylacetic acid, but only to acetoacetic acid. He compared the quantities of ketone bodies obtained after distilling the products of oxidation, (a) by the iodine titration method, which estimates propyl methyl ketone as well as acetone, and (b) by the gravimetric mercuric sulphate method, which estimates only acetone, as was shown by Dakin himself. The question raised is therefore answered for hexanoic acid.

We have found that in our experiments the products obtained react with aniline at the same rate as does acetoacetic acid within the experimental error of 5%. This statement applies to the products of oxidation of hexanoic, octanoic and decanoic acids, and to the product given by rat liver without added fatty acids. In a slightly less quantitative way the same statement may be made with respect to the other fatty acids used, which give small outputs of gas. Now it is probable that higher homologues of acetoacetic acid would react with aniline at rates different from that of acetoacetic acid. This result therefore provides some evidence that acetoacetic acid is the chief β -keto-acid produced in the oxidation of the fatty acids.

Much stronger evidence is derived from a direct comparison of (a) the acetoacetic acid as determined by the manometric method, and (b) the acetoacetic acid + acetone as determined by the gravimetric methods. Figures of this kind are given later and provide convincing evidence that acetoacetic acid is the only β -keto-acid produced in significant amounts from the fatty acids.

ACETOACETIC ACID PRODUCTION AND RESPIRATION OF GUINEA-PIG LIVER IN PRESENCE OF FATTY ACIDS.

In Table I are summarised illustrative experiments which show the rates of respiration and acetoacetic acid formation by guinea-pig liver in presence of the normal straight-chain fatty acids containing from two to ten carbon atoms. Every acid raises the respiration, and most of them increase the acetoacetic acid production. Usually there is an optimum concentration for acetoacetic acid production.

Table I. *Acetoacetic acid production and respiration in presence of fatty acids.*

Exp.	Fatty acid	Guinea-pig liver. Oxygen. Glycerophosphate buffer.					
		Conc. (<i>M</i>)					
1	Acetic		0	0.005	0.01	0.02	
		Q_{O_2}	5.51	7.35	7.00	7.03	
		Q_{Ac}	0.24	0.49	0.49	0.60	
2	Propionic	Conc. (<i>M</i>)	0	0.005	0.01	0.02	
		Q_{O_2}	4.73	8.87	9.68	10.34	
		Q_{Ac}	0.15	0.09	0.12	0.20	
3	Butyric	Conc. (<i>M</i>)	0.0025	0.005	0.01	0.02	
		Q_{O_2}	8.34	9.22	10.1	10.7	
		Q_{Ac}	0.81	1.68	2.49	2.60	
4	Valeric	Conc. (<i>M</i>)	0	0.005	0.01	0.02	
		Q_{O_2}	5.04	10.76	11.44	13.14	
		Q_{Ac}	0.19	0.55	0.61	0.41	
5	Hexanoic	Conc. (<i>M</i>)	0	0.005	0.0075	0.01	
		Q_{O_2}	5.52	11.67	12.37	12.20	
		Q_{Ac}	0.28	3.26	3.40	3.00	
6	Heptanoic	Conc. (<i>M</i>)	0	0.004	0.007	0.01	
		Q_{O_2}	5.03	12.35	13.49	13.90	
		Q_{Ac}	0.28	0.83	0.91	0.84	
7	Octanoic	Conc. (<i>M</i>)	0	0.003	0.006	0.009	
		Q_{O_2}	5.05	7.97	9.39	9.23	
		Q_{Ac}	0.26	1.22	1.44	1.23	
8	Nonanoic	Conc. (<i>M</i>)	0	0.002	0.004	0.006	
		Q_{O_2}	5.84	10.09	10.71	11.28	
		Q_{Ac}	0.34	0.72	0.76	0.78	
9	Decanoic	Conc. (<i>M</i>)	0	0.0015	0.002	0.0025	0.003*
		Q_{O_2}	4.00	6.19	6.78	7.12	7.05
		Q_{Ac}	0.32	1.14	1.12	1.13	1.03

* At this concentration the solubility limit of calcium decanoate in the medium is reached.

Table II summarises the data obtained for the rate of acetoacetic acid production at concentrations of each fatty acid near the optimum. The acids with 4, 6, 8 and 10 carbon atoms produce acetoacetic acid the most rapidly, the 8- and 10-carbon acids being less active than the others. Acetic acid also produces acetoacetic acid, the rate being low but definitely above the very low rate found in its absence. Propionic acid does not produce any acetoacetic acid. Thus far the results are in accord with the findings of Embden and Marx [1908] on dog liver by the perfusion method, and of other workers.

A new result is seen on turning to valeric, heptanoic and nonanoic acids. These acids definitely produce acetoacetic acid, although at rates very much smaller than those characteristic of the neighbouring even-numbered acids. It was considered possible that, although acids of good quality were employed, the preparations of the odd-numbered acids might contain small amounts of the even-numbered acids, which might be responsible for the acetoacetic acid formation. We therefore compared, in one experiment, the rates of acetoacetic acid production using specimens of heptanoic acid obtained from three different sources (an old Kahlbaum specimen; a fresh B.D.H. specimen, and a fresh specimen from Cohen (Berlin), this last being freshly redistilled before use). All three specimens, used at a concentration 0.007 *M*, gave the same values of Q_{Ac} within the experimental error, the values observed being 1.36, 1.32 and 1.30, whilst the values for the respiration were all very similar. It is very improbable that values of this magnitude could be due to impurities, and that impurities of presumably varying amounts would bring about an equal rate. We therefore conclude that

Table II. *Acetoacetic acid production from fatty acids. Mean values.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.					
Acid	Conc. of acid (<i>M</i>)	No. of exps.	Q_{Ac} (Range)	Q_{Ac} (Mean)	Av. deviation from mean
—	—	42	0.00–0.68	0.20	±0.07
Acetic	0.01–0.02	3	0.23–0.60	0.42	±0.10
Propionic	0.01–0.02	3	0.05–0.20	0.12	±0.05
Butyric	0.01	39	1.09–4.44	2.55	±0.46
Valeric	0.01	2	0.40–0.61	0.51	—
Hexanoic	0.004–0.0075	6	2.19–3.26	2.62	±0.34
Heptanoic	0.004–0.007	4	0.31–1.33	0.88	—
Octanoic	0.005–0.006	5	1.44–2.25	1.80	±0.28
Nonanoic	0.004–0.006	3	0.48–0.92	0.72	—
Decanoic	0.002	3	1.12–1.59	1.39	—

Acid	Approx. optimum concentration for acetoacetic acid production <i>M</i>	Approx. optimum concentration for respiration <i>M</i>
Acetic	0.01 –	0.01 (?)
Butyric	0.01 –	>0.02
Valeric	0.01	>0.02
Hexanoic	0.007	>0.04
Heptanoic	0.007	>0.01
Octanoic	0.006	0.008
Nonanoic	0.006	>0.01
Decanoic	0.002	0.0025

acetoacetic acid production from fatty acids with odd numbers of carbon atoms is a real phenomenon, which must be taken into account in any theory dealing with the mechanism of oxidation of fatty acids.

No experiments have been made with acids containing more than 10 carbon atoms. With decanoic acid the low solubility of the calcium salt and the lowered rate of acetoacetic acid production were already beginning to make quantitative work difficult, and these difficulties will presumably be greater, though not insurmountable, with the higher acids.

The relation between respiration and acetoacetic acid production.

The data obtained show that there is a real relation between the increase in acetoacetic acid production and the increase in respiration brought about by fatty acids. Table III shows that, at approximately the optimum concentrations

Table III. *Acetoacetic acid oxygen quotients, i.e. $\frac{\text{Extra } Q_{Ac}}{\text{Extra } Q_{O_2}}$*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.					
Acid	Conc. (<i>M</i>)	No. of exps.	Range of quotients	Mean quotient	Mean values of extra Q_{O_2}
Acetic	0.01	3	0.03– 0.17	0.11	1.4
Acetic	0.02	2	0.14– 0.23	0.18	1.5
Propionic	0.01– 0.02	3	–0.02– +0.01	0.00	5.6
Butyric	0.01	7	0.56– 0.69	0.63	3.8
Valeric	0.01	2	0.04– 0.07	0.05	5.7
Hexanoic	0.004–0.0075	4	0.42– 0.55	0.49	4.9
Heptanoic	0.004–0.007	4	0.03– 0.14	0.08	7.9
Octanoic	0.005–0.006	4	0.27– 0.41	0.34	4.3
Nonanoic	0.005–0.006	3	0.08– 0.10	0.09	5.1
Decanoic	0.002	3	0.28– 0.44	0.37	2.8

for acetoacetic acid production, there tends to be a characteristic ratio for each fatty acid. The value of the ratio falls off as the concentration of the fatty acid rises, as can be calculated from many of the data given in Tables I and IV.

This is connected with the fact that whilst acetoacetic acid production passes through a maximum at a certain fatty acid concentration, when the acid has from 5 to 10 carbon atoms, the respiration continues to rise as the concentration is further increased. This in turn may, however, pass through a maximum at a higher fatty acid concentration (Table I, see also Quastel and Wheatley [1933]). The maximum concentrations tend to decrease as we ascend the series of fatty acids.

Table IV. *Inhibitory effect of higher concentrations of fatty acids on acetoacetic acid formation.*

Exp.	Acid	Guinea-pig liver.				
		Conc. (<i>M</i>)	0.005	0.01	0.02	0.04
1	Hexanoic	Q_{O_2}	10.18	11.74	11.82	12.25
		Q_{Ac}	2.63	2.75	2.22	2.0
		Conc. (<i>M</i>)	0	0.002	0.004*	0.006*
		Q_{O_2}	4.97	7.81	7.39	5.37
2	Decanoic	Q_{Ac}	0.31	1.45	1.20	0.82
		Conc. (<i>M</i>)	0	0.002	0.004*	0.006*
		Q_{O_2}	4.97	7.81	7.39	5.37
		Q_{Ac}	0.31	1.45	1.20	0.82

* In these concentrations calcium decanoate is precipitated in the medium. There is a rapid decrease of the respiration with time in the strongest concentration.

The ratios given in Table III show very clearly the great difference in behaviour between the acids with even numbers of carbon atoms and those with odd numbers. The difference is mainly due to the difference in acetoacetic acid production, but is exaggerated a little by the interesting fact (Table III) that the odd-numbered acids raise the respiration of liver more than do their even-numbered neighbours. This fact suggests that odd-numbered acids are oxidised more completely in the liver than are even-numbered acids.

It is seen in Table IV that raising the concentration of hexanoic acid well above its optimum value depresses the acetoacetic acid formation definitely, but that the inhibition is only partial. A similar effect was observed earlier with crotonic acid. The phenomenon, which is probably fairly general, is presumably an inhibition of a secondary type due to the high adsorption of the fatty acid anions. With decanoate, the inhibitory effect of higher concentrations may be due in part to the precipitation of calcium ion.

ACETOACETIC ACID PRODUCTION AND RESPIRATION OF RAT LIVER IN PRESENCE OF FATTY ACIDS.

The behaviour of rat liver is very similar to that of guinea-pig liver with respect to acetoacetic acid production, but there are a number of differences. Rat liver produces acetoacetic acid faster than does guinea-pig liver from the even-numbered acids (Table V). Butyric, hexanoic and octanoic acids yield acetoacetic acid at very similar rates, whilst the rate with decanoic acid is rather lower.

Rat liver without any added fatty acid yields acetoacetic acid at a definitely measurable rate, several times higher than that with guinea-pig liver. This phenomenon complicates the decision as to whether the other added acids can yield acetoacetic acid. In the case of acetic acid it is clear that acetoacetic acid is produced, and at a rate higher than with guinea-pig liver. Valeric, heptanoic and nonanoic acids do not however affect definitely the nett rate of acetoacetic

Table V. *Mean rates of acetoacetic acid production.*

Rat liver. Oxygen. Glycerophosphate buffer.					
Acid	Conc. (<i>M</i>)	No. of exps.	Range of Q_{Ac}	Mean Q_{Ac}	Av. deviation from mean
—	—	22	0.74–1.59	1.12	± 0.17
Acetic	0.01	4	2.11–2.71	2.38	± 0.20
Butyric	0.01	9	3.42–5.68	4.53	± 0.57
Valeric	0.01	1	—	1.04	—
Hexanoic	0.007	2	4.59–4.90	4.75	—
Heptanoic	0.007	1	—	1.33	—
Octanoic	0.006	3	4.24–4.68	4.40	—
Nonanoic	0.006	1	—	1.06	—
Decanoic	0.0025	3	3.02–3.99	3.36	—

acid production at the one set of concentrations at which they have been examined (Table VI).

That the odd-numbered acids are oxidised by rat liver is clear from the fact that they increase the respiration. The fatty acids generally do not however raise the respiration of rat liver as much as they do that of guinea-pig liver (Table VII), although they cause greater acetoacetic acid production. A possible explanation is that with rat liver there may be greater competition between the added fatty acids and the substrates already present. However this may be, the fact finds its expression (Table VII) in higher values for the ratio extra acetoacetic acid production/extra respiration for the even-numbered acids.

Table VI. *Acetoacetic acid production and respiration in presence of fatty acids.*

Rat liver. Oxygen. Glycerophosphate buffer.						
Exp.	Fatty acid	Conc. (<i>M</i>)	Q_{O_2}	Q_{Ac}		
1	Acetic	0	10.27	11.60		
			1.10	2.46		
2	Butyric	0	9.77	12.74		
			0.96	4.55		
3	Fatty acid	—	—	Valerate	Heptanoate	Nonanoate
	Conc. (<i>M</i>)	—	—	0.01	0.007	0.006
	Q_{O_2}	11.14	13.24	13.78	15.65	
	Q_{Ac}	1.09	1.04	1.33	1.06	

Table VII. *Acetoacetic acid oxygen quotients.*

Rat liver. Oxygen. Glycerophosphate buffer.						
Acid	Conc. (<i>M</i>)	No. of exps.	Range of quotients	Mean quotient	Av. deviation	Extra Q_{O_2}
Acetic	0.01	4	0.45–1.04	0.83	—	1.6
Butyric	0.01	8	0.91–1.33	1.08	± 0.12	3.2
Valeric	0.01	1	—	—0.02	—	2.1
Heptanoic	0.007	1	—	0.09	—	2.6
Octanoic	0.006	2	0.65–0.69	0.67	—	5.0
Nonanoic	0.006	1	—	—0.01	—	4.5
Decanoic	0.0025	1	—	0.85	—	2.3

Q_{Ac} in presence of a mixture of fatty acids.

In Table VIII an experiment shows that when butyrate and hexanoate are present together, the acetoacetic acid production is not raised above the level shown by either acid separately and is actually no higher than that given by

Table VIII. *Experiments with two added substrates.*

Oxygen. Glycerophosphate buffer.					
Exp. 1. Guinea-pig liver	Conc. butyrate (<i>M</i>)	—	0.02	—	0.02
	Conc. hexanoate (<i>M</i>)	—	—	0.006	0.006
	Q_{O_2}	4.21	9.72	8.41	10.42
	Q_{Ac}	0.14	3.10	2.32	2.18
Exp. 2. Rat liver	Conc. butyrate (<i>M</i>)	—	0.01	0.01	
	Conc. acetate (<i>M</i>)	—	—	0.01	
	Q_{O_2}	9.43	12.34	13.05	
	Q_{Ac}	1.00	4.13	5.00	

the acid showing the lower rate of production. A similar result was previously obtained for crotonic and butyric acids. Competition of this kind is probably a general phenomenon, indicating some common mode of oxidation for the different fatty acids.

Acetic acid however proves to be an exception (Table VIII), for Q_{Ac} in presence of butyrate and acetate is higher than when the acids are present alone. It would be expected that the mechanism of formation of acetoacetic acid from acetic acid would be of quite a different type from that involved in the oxidation of other fatty acids, and this result therefore accords with expectation.

The formation of β -hydroxybutyric acid and acetone.

It may appear to have been tacitly assumed in previous discussions, particularly in Part I, that acetoacetic acid is the only product of fatty acid oxidation which concerns us. This of course is not true, for acetoacetic acid is transformed in the liver in two ways.

One possible mode of breakdown of acetoacetic acid in liver is to acetone and carbon dioxide. Some evidence that acetone as well as acetoacetic acid resulted from fatty acid oxidation in the perfused dog liver was obtained by Embden and Engel [1908], but the decomposition may have been due to the perfusing blood, since according to Grégoire [1933] dog blood decomposes acetoacetic acid to acetone. A few figures we have obtained (Table IX) suggest that some acetone is formed during oxidation of fatty acids by rat liver. The rate of formation of acetoacetic acid + acetone, estimated gravimetrically, is greater than the rate of acetoacetic acid formation estimated manometrically, and probably therefore acetone is being formed from acetoacetic acid. A more definite and interesting conclusion to be drawn from the figures is that acetoacetic acid is the only β -keto-acid formed to an appreciable extent in the experiments. If a higher homologue of acetoacetic acid were formed it would probably be estimated by the manometric method but not, in view of Dakin's experiments already mentioned, by the gravimetric method.

Acetoacetic acid is also transformed in another manner in liver, namely to β -hydroxybutyric acid. We have already shown that in the oxidation of butyric and crotonic acids by guinea-pig liver β -hydroxybutyric acid is not an intermediary product in the formation of acetoacetic acid. In these cases therefore the formation of β -hydroxybutyric acid results from a reduction of acetoacetic acid, and it is likely that the same is true for other fatty acids. The figures given in Table IX show that quantitatively β -hydroxybutyric acid is an important product of fatty acid oxidation in the liver, the amount formed in most of our experiments being of the same order as that of acetoacetic acid. Formation of β -hydroxybutyric acid by the liver has been shown to take place from butyric acid [Friedmann and Maase, 1910, and others] and from hexanoic acid [Dakin,

Table IX. *Gravimetric estimations of ketone body formation.*

The symbol Q_{Ac} , hitherto used to indicate rate of acetoacetic acid production by the manometric method, is also used to indicate rate of formation of acetoacetic acid + acetone, by the gravimetric method, and is then always distinguished by the statement that the gravimetric method was used.

Q_{oxy} indicates similarly the rate of formation of β -hydroxybutyric acid, and Q_{ket} the rate of formation of total ketones, both estimated gravimetrically.

Rat liver.

Medium—Ringer's solution of initial p_H 7.4.

Either 1.5% CO_2 , 0.008 M $NaHCO_3$; or 5% CO_2 , 0.025 M $NaHCO_3$.

Exp.	Substrate	Q_{Ac} (manom.)	Q_{Ac} (grav.)	Ratio
1	Butyrate	1.74	1.96	0.89
1	Hexanoate	1.27	1.51	0.84
2	Octanoate	1.52	2.16	0.70
		Q_{Ac} (grav.)	Q_{oxy}	
3	Butyrate	2.89	1.62	
1	Butyrate	1.96	1.40	
2	Octanoate	2.16	2.58	
		Q_{Ac} (manom.)	Q_{ket}	Ratio
1	Butyrate	1.74	3.36	0.52
1	Hexanoate	1.27	2.08	0.61
4	Octanoate	1.02	1.61	0.63
2	Octanoate	1.52	4.74	0.32
4	Decanoate	1.20	2.22	0.54
2	Decanoate	1.27	3.42	0.37

Guinea-pig liver.

Ringer's solution: 1.5% CO_2 , 0.009 M $NaHCO_3$.

Exp.	Substrate	Q_{Ac} (manom.)	Q_{ket}	Ratio
5	Butyrate	2.71	4.07	0.67
6	Valerate	0.95	2.11*	0.45 (?)
7	Hexanoate	1.50	2.94	0.51
8	Heptanoate	0.80	1.30	0.61
9	Octanoate	0.73	0.79	0.92
10	Octanoate	1.22	1.37	0.89
11	Decanoate	0.81	1.55	0.52

* This value is too high, for, it has been found, in confirmation of the work of Butts *et al.* [1935], that valeric acid itself gives a precipitate in the Van Slyke estimation.

Ringer's solution: 5% CO_2 , 0.025 M $NaHCO_3$.

Exp.	Substrate	Q_{Ac} (manom.)	Q_{ket}
5	Butyrate	2.71	4.07
5	Butyrate + 0.002 M benzoate	1.53	2.25
5	% inhibition	43	45
		Q_{oxy}	
12	Butyrate 0.01 M	0.60	
12	Acetoacetate 0.01 M	1.18	

1923], but as far as we are aware it has not previously been shown to take place from other normal saturated fatty acids.

A very rough estimate of the average ratios of the substances making up the total ketone bodies produced from fatty acids by rat liver in our experiments is acetoacetic acid 0.5, acetone 0.1, β -hydroxybutyric acid 0.4. The proportions will not necessarily be the same for other experimental conditions. The breakdown of

acetoacetic acid to acetone is almost certainly irreversible, and the proportion of acetone will therefore increase with time. The ratio of acetoacetic acid to β -hydroxybutyric acid varies in our experiments, and the nett rate of transformation of one of these substances into the other will presumably depend on the supply of other metabolites to the liver cells. The cells will tend to set up a dynamic equilibrium between these two substances, the position of the equilibrium being dependent on the conditions.

Accepting, as we do, the view that acetone and β -hydroxybutyric acid are products derived from acetoacetic acid, it is important for us to know whether the nett acetoacetic acid formation in any one liver bears a constant ratio to the formation of total ketone bodies, for on this view the total ketone bodies measure the gross acetoacetic acid formation. It is, for instance, desirable for us to know whether a substance that inhibits the nett acetoacetic acid production to a certain extent inhibits also the gross acetoacetic acid production to an equal extent.

We cannot claim that this is always the case, but where the point has been tested by us it is true. An experiment recorded in Table IX shows that benzoate inhibits the nett formation of acetoacetic acid from butyric acid by guinea-pig liver to the same extent as it inhibits total ketone formation. Similar evidence of a more qualitative kind regarding the inhibiting action of benzoate is presented later.

If β -hydroxybutyric acid is derived from acetoacetic acid, and not from butyric acid more directly, it would be expected that acetoacetic acid might yield β -hydroxybutyric acid at a rate faster than butyric acid does. This expectation has been verified by an experiment with guinea-pig liver (Table IX), which therefore supports the views put forward.

The yield of ketone bodies from fatty acids.

In the experiments recorded in other sections of this work we have endeavoured to maintain the fatty acid concentrations at levels which do not fall very much with time, so that the rates of oxidation are measured under fairly definite conditions.

In this section, on the other hand, we present experiments in which the object has been to allow the tissue to remove the fatty acid completely during the experiment, the period of which has however been restricted as before to two hours. The fatty acid concentrations have been lowered and the quantity of tissue increased. Several manometer vessels have been set up in each experiment. In each vessel the volume of solution and the initial concentration of the fatty acid have been the same, but the weight of tissue has been varied. By comparing the quantities of ketone bodies formed in the presence of varying weights of tissue, it should be possible to tell whether any of the quantities of tissue has been sufficient to transform the fatty acid completely, for when this object is attained the quantity of ketone bodies formed will be expected to become constant. We appear to have been successful in this object, but two difficulties arise in the exact interpretation of the experiments.

(1) In the absence of added fatty acids, liver gives rise to some acetoacetic acid. Increasing the quantity of tissue indefinitely will therefore probably increase the quantity of ketone bodies indefinitely. We can to some extent meet the difficulty (a) by choosing a liver with relatively low "spontaneous" acetoacetic acid production, *i.e.* guinea-pig liver rather than rat liver, and (b) by attempting to correct for the "spontaneous" acetoacetic acid formation.

(2) A second source of error, which however is small in our experiments, is the necessity of correcting for the quantity of ketone bodies removed from the vessels with the tissue slices. We find that the loss in weight of the vessels when the tissue slices are removed is for guinea-pig liver about six times the dry weight of the slices found subsequently. We have calculated the correction to be applied on the basis that the ketone bodies removed are equal to the quantity contained in an equal quantity of solution. As there may not be equal partition of ketone bodies between liver slices and medium, the assumption may lead to an error of a few per cent. in our results¹.

Details of one of our experiments are recorded in Table X, which make clear the nature and magnitude of the corrections applied. The results of the experiments are shown in Table XI, where the several values obtained in each experiment, both corrected and not corrected for the "spontaneous" acetoacetic acid formation, are shown.

Table X. *Experiment showing yield of acetoacetic acid from hexanoic acid.*

O ₂ . 3.2 ml. of solution containing glycerophosphate buffer. Guinea-pig liver.						
	Vessel	...	1	2	3	4
Initial conc. hexanoate (<i>M</i>)	—		—	0.0025	0.0025	0.0025
After 117 min. shaking at 37°, tissue removed from vessels, to which were added 0.2 ml. aniline hydrochloride and 0.3 ml. <i>N</i> acetic acid. <i>v_F</i> =3.7.						
Dry wt. tissue (mg.)			27.6	48.0	39.7	21.4
<i>k</i> _{CO₂}			1.393	1.492	1.548	1.553
Δ <i>h</i> (aniline)		+ 18.1		+ 118.1	+ 107.2	+ 75.3
μl. CO ₂ (uncorrected)		25.2		176.2	165.9	116.9
μl. CO ₂ (corrected for aniline blank)		13.2		164.2	153.9	104.9
μl. CO ₂ (corrected for loss due to tissue removal)*		13.9		178.9	165.3	109.1
μl. CO ₂ (corrected for spontaneous acetoacetic acid formation)†		0		154.7	145.3	98.3
μl. hexanoate added		0		179.2	179.2	179.2
* $\frac{\text{Acetoacetic}}{\text{Hexanoic}}$ (uncorrected)		—		1.00	0.92	0.61
† $\frac{\text{Acetoacetic}}{\text{Hexanoic}}$ (corrected)		—		0.86	0.81	0.55
<i>Q</i> _{O₂} (14–117 min.)			4.59	7.82	7.80	8.13
<i>Q</i> _{Ac} (0–117 min.)			0.26	1.91	2.14	2.61

In Table XI are also shown the results of a similar series of experiments carried out in the presence of 0.04 M sodium malonate. Malonate raises the spontaneous acetoacetic acid formation in guinea-pig liver [Jowett and Quastel, 1935, 2] and it was thought that by increasing the correction for this it would be made clear whether or not this correction should be applied. It was found later, however, that malonate inhibits the breakdown of acetoacetic acid by liver, and the complete interpretation of the experiments is, therefore, difficult.

The first set of experiments may be taken to show that butyric acid yields about 0.3–0.5 mols. of acetoacetic acid, hexanoic about 0.9, octanoic 0.6, and decanoic acid 0.6.

The highest yields of acetoacetic acid previously obtained from these fatty acids were derived from the experiments of Embden and Marx [1908] with dog

¹ It may be pointed out that the correction discussed has not usually been applied to experiments in other sections of this work. For 20 mg. of guinea-pig liver in 3.2 ml. solution, the correction amounts only to 4%, and for relative results in any one experiment is much less.

Table XI. *Yields of ketone bodies from fatty acids.*

Guinea-pig liver.								
Acid	Initial conc. <i>M</i>	μ l. fatty acid	Acetoacetic acid in mols. per mol. of fatty acids. Several weights of tissue were employed in each experiment, the result for the largest weight being at the left of each column					
			Uncorrected			Corrected		
Butyric	0.004	285	0.49	0.42	0.36	0.45	0.39	0.33
Butyric	0.004	285	0.55	0.49	0.36	0.38	0.34	0.28
Butyric	0.005	357	0.40	0.40	0.27	0.31	0.33	0.23
Hexanoic	0.0025	179	1.00	0.92	0.61	0.86	0.81	0.55
Octanoic	0.0015	107	0.79	0.84	0.75	0.55	0.64	0.60
Decanoic	0.0015	107	0.84	0.68	0.65	0.48	0.48	0.48
Decanoic	0.002	143	0.92	0.83	0.80	0.64	0.64	0.64

Yields of acetoacetic acid.

In presence of malonate.								
Hexanoic	0.0025	179	1.32	1.18	1.07	0.89	0.85	0.82
Octanoic	0.002	143	1.51	1.28	—	0.88	0.92	—
Decanoic	0.002	143	1.27	1.14	—	0.70	0.74	—
Decanoic	0.002	143	1.35	1.19	—	0.63	0.69	—

Yields of total ketones.

Mols. per mol. of fatty acid.								
Butyric	0.0029	223	0.36	0.35	0.28	0.22	0.26	0.21
Hexanoic	0.0023	179	1.29	1.26	1.06	1.16	1.15	0.98
Octanoic	0.002	142	1.14	1.27	1.21	0.96	1.11	1.11
Decanoic	0.002	156	0.86	0.85	—	0.66	0.69	—

livers. These figures show a yield of 0.12–0.18 mols. of acetone + acetoacetic acid per mol. of fatty acid, uncorrected for the “spontaneous” production by the liver. It was not proved or even suggested in these experiments that the fatty acids added were completely removed by the liver. The low values of the figures have however made Leathes and Raper [1925] sceptical of the importance of ketone bodies in fatty acid metabolism, and it is only evident now that acetoacetic acid is a quantitatively important product of fatty acid oxidation.

An even more interesting figure than the yield of acetoacetic acid is the yield of total ketones from the fatty acids. A few experiments have been made to determine total ketone yields on similar lines to those carried out with acetoacetic acid, and they are summarised in Table XI. The medium in these experiments was Ringer's solution (5% CO₂, 0.025 *M* NaHCO₃). In the “blank” vessels, *i.e.* those to which no fatty acid was added, the quantity of total ketones found as mercury precipitate did not exceed 0.44 mg., which corresponds to a value of Q_{ket} of 0.09.

The Van Slyke method is probably inaccurate for the small precipitates in question, and we have assumed a “blank” value $Q_{ket}=0.2$ for the purpose of calculating “corrected” values for the yield of total ketones. The experiments give us values of 1.15–1.3 mols. of total ketones from hexanoic acid, 1.1–1.3 from octanoic acid, and 0.7–0.85 from decanoic acid. The value found with butyric acid, 0.2–0.35, is probably rather too low, in view of the higher yield of acetoacetic acid previously found.

We cannot state with certainty that any of the values definitely exceeds one molecule of total ketones per molecule of fatty acid, and the results may be

summarised as follows: hexanoic and octanoic acids each yield about one molecule of ketone bodies, decanoic acid slightly less and butyric acid $\frac{1}{2}$ – $\frac{1}{3}$ molecule.

The yield of ketone bodies from butyric acid is much less than that from the higher acids, and it is therefore very unlikely that the higher acids yield ketone bodies exclusively by a mechanism by which they first break down to butyric acid.

It is possible that a proportion of the fatty acid is broken down by a path that does not lead to ketone body formation, and the greater yields of ketone bodies which have been found with fatty acids higher than butyric acid may be explained on the following lines: (a) acetic acid may be one of the products of breakdown of higher fatty acids, and this may be an additional source of acetoacetic acid, or (b) octanoic and decanoic acids may break down into two units containing four carbon atoms and thereby yield two molecules of acetoacetic acid¹.

It is unlikely, however, that the production of acetic acid from hexanoic acid will explain the very considerable difference between the yields of acetoacetic acid from this acid and from butyric acid, for this would necessitate the complete conversion of acetic acid into acetoacetic acid.

This section may be concluded by giving some values (Table XII) for the acetoacetic acid formed per molecule of fatty acid added, which were obtained in experiments not specially designed for obtaining complete removal of the fatty acid. The figures given are therefore lower than the actual yields that could be obtained, but may serve for the present to indicate minimum values of the yield of acetoacetic acid, for some cases where a better approximation is not yet available. The figures are sufficient to show that guinea-pig liver produces acetoacetic acid from heptanoic and nonanoic acids to a small but definite extent. It is also clear that rat liver produces acetoacetic acid from the even-numbered fatty acids to a considerable extent.

Table XII. *Yields of acetoacetic acid.*

(Not maximum values.)				Yield, mols. acetoacetic acid per mol. fatty acid	
Liver	Acid	Initial conc. <i>M</i>	μ l. fatty acid	Uncorrected	Corrected for blank
Guinea-pig	Acetic	0.005	357	0.08	0.04
"	Valeric	0.005	357	0.06	0.04
"	Heptanoic	0.007	500	0.18	0.14
"	Nonanoic	0.002	143	0.31	0.16
Rat	Acetic	0.01	714	0.16	0.08
"	Butyric	0.01	714	0.45	0.34
"	Octanoic	0.006	428	0.29	0.23
"	Decanoic	0.0025	178	0.88	0.58

Acid-base changes during fatty acid oxidation.

A series of experiments is summarised in Table XIII in which the change in the bicarbonate concentration of the Ringer's solution employed as medium is used to measure the production or disappearance of fixed acid during the oxidation of fatty acids by guinea-pig liver. The symbol Q_s , defined on similar lines to the others employed, denotes acid production. As is usual, no attempt has

¹ The probability of this latter mode of breakdown is enhanced by the conclusion drawn, in work recently published by Butts *et al.* [1935], that the intact rat can break down octanoic acid to two molecules of ketone bodies. These authors consider that in the rat butyric and hexanoic acids yield about one molecule of ketone bodies.

Table XIII. *Acid production during fatty acid oxidation.*

Guinea-pig liver.
Ringer's solution: O₂ + 1.5% CO₂, 0.009 M NaHCO₃.

Exp.	Added substrate	Conc. M	Q _s	Q _{Ac}	Q _{ket}	$\frac{Q_s}{Q_{Ac}}$	$\frac{Q_s}{Q_{ket}}$
1	—	—	+0.29	0.19	—	—	—
2	—	—	+0.40	0.25	—	—	—
3	—	—	+0.25	0.09	—	—	—
4	—	—	+0.35	—	—	—	—
5	—	—	+0.51	0.14	—	—	—
6	—	—	+0.38	—	—	—	—
5	Acetate	0.005	-0.39	0.38	—	-1.0	—
4	Acetate	0.009	-1.23	—	—	—	—
6	Propionate	0.009	-3.25	—	—	—	—
7	Propionate	0.01	-2.35	—	—	—	—
3	Butyrate	0.006	-0.04	3.06	—	-0.01	—
8	Butyrate	0.01	-0.17	—	—	—	—
9	Valerate	0.01	-1.86	0.95	2.11*	-2.0	-0.9*
2	Hexanoate	0.006	+2.20	2.93	—	+0.8	—
10	Hexanoate	0.0075	+1.40	1.50	2.94	+0.9	+0.5
11	Heptanoate	0.007	+0.52	0.80	1.30	+0.6	+0.4
12	Octanoate	0.004	+2.50	2.22	—	+1.1	—
13	Octanoate	0.004	+1.95	1.75	—	+1.1	—
1	Octanoate	0.004	+1.97	2.03	—	+1.0	—
14	Octanoate	0.006	+0.63	0.73	0.79	+0.9	+0.8
15	Octanoate	0.006	+0.91	1.22	1.37	+0.7	+0.7
16	Decanoate	0.0025	+1.10	0.81	1.55	+1.4	+0.7

* See footnote to Table IX.

been made to correct values of Q_s for the buffering power of the tissue slices, or for the very slight buffering due to the bicarbonate-carbonate system, and the results will all be numerically a little too low.

The results are illuminating, although not very numerous. It may first be noted that there is evidence of a definite parallelism between Q_s and Q_{Ac} in the cases where several experiments have been done with any one acid. The parallelism provides some evidence that the acid-base changes are directly connected with fatty acid oxidation and are not due to a secondary stimulation or inhibition of other processes in the liver.

Hexanoic, octanoic and decanoic acids all show acid production definitely exceeding the small acid production by liver in the absence of added fatty acid. Butyric acid on the other hand shows a slight disappearance of acid as compared with the control.

The reactions

which occur with butyrate lead to no acid-base change, while the reaction

which probably occurs to a small extent, leads to disappearance of fixed acid. It may be concluded that any other type of oxidation which butyrate may undergo does not lead to much nett appearance or disappearance of acid groups.

The reactions occurring with the higher even-numbered acids may on two views at least be expected to lead to production of acid.

(1) The reaction with hexanoic acid may be

and on the same lines the higher acids would give two or more molecules of acetic acid. It must be made clear that there is no evidence whatever, of which

we are aware, that acetic acid is a product of fatty acid oxidation. That any acid at all is produced during the oxidation of fatty acids by liver, beyond acetoacetic and β -hydroxybutyric acids, is a fact which has not been shown prior to the present work.

The hypothesis that acetic acid is formed is not easy to test from our data. Reactions such as the one formulated lead to quite definite ratios between the acid production that should be observed and the formation of acetoacetic acid + β -hydroxybutyric acid. With hexanoic acid the ratio Q_s/Q_{ket} should be 1, with octanoic 2, with decanoic 3. The position is complicated by the fact that part of the acetic acid hypothetically formed would react further, and in such a way as to lower the expected ratios below the values given.

Acetic acid itself forms acetoacetic acid, and furthermore, if the process is a condensation,

fixed acid will disappear. Disappearance of fixed acid is observed, in fact, with acetic acid in liver, but its amount is probably greater than can be accounted for by the rate of acetoacetic acid formation, and it may be that some of the acetic acid is oxidised completely, with disappearance of fixed acid.

In spite of the complications, it would be expected on the hypothesis that the ratio Q_s/Q_{ket} would rise as we pass from hexanoic to octanoic and from octanoic to decanoic acid. The observed ratios (0.5, 0.7, 0.7), however, do not lead to a definite conclusion. Attempts to isolate acetic acid as a product of fatty acid oxidation should settle the question. A few experiments we have made in this direction have as yet given no definite results.

(2) It has been found by Verkade and van der Lee [1934, 1] that when human subjects are fed on glycerides made from pure saturated fatty acids, the reaction

takes place in the body to some extent when $n+2$ has values of 8 to 12, for small quantities of the dibasic acid are excreted in the urine. Such a process as that formulated would lead to the observation of fixed acid production in our experiments. It is, however, unknown whether the production of dibasic acids takes place in the liver or elsewhere in the body. The yields of dibasic acid in the urine are also small in relation to the quantity of fatty acid ingested—octanoic acid giving up to 0.18% and decanoic acid up to 2.2% of the yield theoretically possible—and it is therefore unknown whether a considerable proportion of the fatty acid is oxidised in this way in the human body. From our results on the total ketone production from hexanoic acid, we conclude that hexanoic acid at least does not undergo oxidation in guinea-pig liver to dibasic acid as a final product to any appreciable extent, hence it is unlikely that the acid production observed in this case can be due to the formation of dibasic acids.

The reaction formulated, however, together perhaps with degradation to lower dibasic acids [Verkade and van der Lee, 1934, 2], cannot at present be excluded as a possible partial path of oxidation for a number of the fatty acids.

Let us now consider the problem of the oxidation of the odd-numbered acids. It will be seen that when propionic acid is oxidised, there is a considerable disappearance of fixed acid. This may be due to complete oxidation of some of the propionic acid, and perhaps, as already suggested [Dakin, 1922], lactic and pyruvic acids are intermediaries in the oxidation.

With valeric acid fixed acid also disappears, whilst with heptanoic acid there is apparently slight acid production. With both these acids formation of acetoacetic acid is higher than would be expected if acetic acid is the inter-

mediary that gives rise to it. It is probable that valeric acid gives rise directly to some acetoacetic acid but the disappearance of fixed acid suggests that a more important mode of oxidation may be through propionic and acetic acids or their partially oxidised derivatives. The reaction may be primarily:

the propionic acid undergoing complete oxidation, the nett result being a disappearance of fixed acid but rather less than in the case of propionic acid alone owing to the presence of the hypothetical acetic acid.

When heptanoic acid is oxidised we imagine the same process extended, so that a molecule of propionic acid and two molecules of acetic acid are formed. The nett result of this process would be greater fixed acid production than in the case of valeric acid. Acetoacetic acid production from heptanoic acid cannot be explained as dependent upon the formation of acetic acid.

It will have been observed in Table XIII, that where a weakly buffered Ringer's solution was used, the production of acetoacetic acid from valeric and heptanoic acids compared with those from the even-numbered acids seems high relative to the figures previously obtained in a glycerophosphate medium. It occurred to us that this phenomenon might be due to a relatively low p_{H} in these experiments, caused by the greater acidifying effect of respiratory carbon dioxide when the initial carbon dioxide content of the gas mixture is low (1.5 %). We have therefore compared (Table XIV) the relative activities of the odd- and even-numbered acids in more strongly buffered media and have obtained some indication that at a lower p_{H} the acetoacetic acid production of the odd-numbered acid is raised relatively to that of a neighbouring even-numbered acid.

Table XIV. *Effect of p_{H} on relative acetoacetic acid production from odd- and even-numbered acids.*

Guinea-pig liver. Ringer's solution, 95 % O_2 + 5 % CO_2 .

Exp.	Initial [HCO_3]	Approx. p_{H}	Q_{Ac} in presence of				
			No addition	Valerate 0.01 M	Hexanoate 0.0075 M	Heptanoate 0.007 M	Octanoate 0.006 M
1	0.025	7.4	—	0.49	2.25	0.61	1.52
2	0.0125	7.1	0.10	0.84	2.03	0.92	—

The effect of benzoate on the oxidation of fatty acids.

We have already shown in Part I that benzoate at low concentrations inhibits the oxidation of butyric and crotonic acids to acetoacetic acid by guinea-pig liver. The same substance has been examined here with regard to its inhibiting action on the formation of acetoacetic acid from fatty acids by rat liver.

As will be seen from Table XV, 0.002 M benzoate lowers Q_{Ac} by some 50 % (a) when there is no substrate added, (b) when acetic acid is present and (c) when the substrate is butyrate. It should be noted that the respiration in the absence of added substrates is unaffected by benzoate at concentrations up to 0.04 M . Benzoate at low concentrations is evidently quite a specific poison for tissue processes.

Benzoate, however, in contrast to its effect on Q_{Ac} in presence of acetate and butyrate, has a much smaller effect on the production of acetoacetic acid from hexanoic, octanoic and decanoic acids (Table XV).

As a result, when the acetoacetic acid productions in the presence of benzoate from the various fatty acids are compared in any one experiment it is found that Q_{Ac} from butyrate is lower than that from hexanoic, octanoic and decanoic acids.

Table XV. *Effect of benzoate on formation of acetoacetic acid.*

Rat liver. Oxygen. Glycerophosphate buffer.

Substrate (<i>M</i>)	% decrease in Q_{Ac} in presence of 0.002 <i>M</i> benzoate		
	53	32	46
—	51	63	56
Acetate 0.01	63	48	45
Butyrate 0.01	13		
Hexanoate 0.007	18	20	
Octanoate 0.006	5		
Decanoate 0.0025			

Values of Q_{Ac} in presence of 0.002 M benzoate.

Exp.	Butyrate	Hexanoate	Octanoate	Decanoate
1	2.11	4.01	—	—
2	1.70	—	3.45	—
3	1.81	—	3.44	—
4	2.66	—	—	3.79

Values of Q_{Ac} in presence of benzoate.

Exp.	Concentration benzoate (<i>M</i>)	Butyrate 0.02 <i>M</i>	Hexanoate 0.007 <i>M</i>	Octanoate 0.006 <i>M</i>	Decanoate 0.0025 <i>M</i>
5	0	4.70	4.90	4.68	3.07
6	0.005	2.62	4.22	4.57	3.12
7	0.0075	1.77	3.09	3.97	3.10
8	0.01	2.59	3.92	4.09	4.01

Same as above in relative units. Values of Q_{Ac} in presence of benzoate.

Exp.	Concentration benzoate	Butyrate	Hexanoate	Octanoate	Decanoate
5	0	153	160	152	(100)
6	0.005	84	135	146	(100)
7	0.0075	57	100	128	(100)
8	0.01	65	98	102	(100)

When the concentration of benzoate is increased, it is possible to find conditions where, similarly, Q_{Ac} from hexanoate is lower than that from octanoic acid. Probably conditions of benzoate concentration could be found under which decanoic acid would form acetoacetic acid faster than do octanoic and hexanoic acids.

In the absence of benzoate the values of Q_{Ac} are approximately equal with butyrate, hexanoate and octanoate, but rather lower with decanoate. The behaviour in absence of benzoate may thus be compatible with the β -oxidation mechanism according to which decanoic acid is transformed successively into octanoic, hexanoic and butyric acids before acetoacetic acid is formed. The behaviour in presence of benzoate, however, is very difficult to explain on this theory of fatty acid breakdown.

Before considering the subject further, we should note that it has also been found that the same alteration in relative rates with the different fatty acids by benzoate is shown when we measure the total ketone production instead of acetoacetic acid production. Reference to Table XVI shows in the case of rat liver that the findings are the same, and that the total ketone production from butyric acid can be lowered below the level shown by the other fatty acids.

These results clearly compel us to abandon the mechanism proposed by Embden. The formation of ketone bodies from the higher fatty acids appears not to be necessarily preceded by their degradation to lower fatty acids and

Table XVI. *Total ketone production in presence of benzoate.*Ringer's solution (95 % O₂ + 5 % CO₂; 0.025 *M* NaHCO₃).

	Q_{ket} in presence of			
	Butyrate 0.02 <i>M</i>	Hexanoate 0.007 <i>M</i>	Octanoate 0.006 <i>M</i>	Decanoate 0.0025 <i>M</i>
Rat liver, 0.01 <i>M</i> benzoate	1.54	2.48	3.13	1.95
Rat liver, 0.015 <i>M</i> benzoate	2.28	3.08	2.63	—
Guinea-pig liver, 0.01 <i>M</i> benzoate	1.39	0.87	—	—
Guinea-pig liver, 0.01 <i>M</i> benzoate	0.85	0.54	0.61	0.71

finally to butyric acid before ketone bodies are formed. No escape from this conclusion seems possible. In our experiments butyric acid is the acid with which optimum conditions are most easily obtained, since excessive concentrations do not affect the oxidation to ketone bodies. It is much more certain that conditions are optimum for butyrate than for the higher fatty acids, and yet in presence of benzoate it forms ketone bodies at a lower rate than do the higher fatty acids.

It remains to consider our experiments further in the light of one or two possibilities.

(1) It is possible that a part of the ketone bodies from the higher fatty acids arises through acetic acid which may be split off from them as an intermediary. Our experiments with acetic acid show however that acetoacetic acid production from it is as much inhibited by benzoate as is that from butyrate, and is in any case small. The view that acetic acid is an intermediary is therefore of little importance in this connection.

(2) In the case of octanoic and higher acids, it is conceivable that a molecule of fatty acid might give rise to two or more molecules of acetoacetic acid, and that one of these molecules might be formed through butyric acid as an intermediary. On this view the inhibition of ketone body formation in the case of octanoic acid should be at least half as great as with butyric acid.

In the case of guinea-pig liver the inhibitory effect of benzoate does not vary so much from one fatty acid to another (Tables XVI and XVII), and we cannot conclude from our experiments on this tissue with benzoate that the Embden mechanism is invalid. Apparently fluoride, like benzoate, inhibits acetoacetic acid production from the higher fatty acids rather less than from butyric acid.

Table XVII. *Effect of inhibitors on guinea-pig liver.*Exp. 1. (Ringer's solution: 5 % CO₂; 0.025 *M* NaHCO₃.)

Conc. butyrate (<i>M</i>)	0.01	0.01	—	—
Conc. octanoate (<i>M</i>)	—	—	0.01	0.01
Conc. benzoate (<i>M</i>)	—	0.002	—	0.002
Q_{Ac}	1.78	0.74	1.79	0.90
% decrease in Q_{Ac}	—	58	—	50

Exp. 2. (Glycerophosphate buffer.)

Conc. butyrate (<i>M</i>)	—	0.01	0.01	—	—
Conc. octanoate (<i>M</i>)	—	—	—	0.006	0.006
Conc. fluoride (<i>M</i>)	—	—	0.0031	—	0.0031
Q_{O_2}	4.66	8.18	5.67	9.25	6.12
Q_{Ac}	0.18	2.27	0.67	1.93	0.84
% decrease in Q_{Ac}	—	—	70	—	57

Exp. 3. (Glycerophosphate buffer.)

Conc. butyrate (<i>M</i>)	—	—	0.01	0.01
Conc. arsenite (<i>M</i>)	—	0.001	—	0.001
Q_{O_2}	4.53	1.96	8.68	2.23
Q_{Ac}	0.20	0.02	2.95	0.40

An experiment in Table XVII shows in an interesting way how much more specific fluoride and benzoate are in their actions on acetoacetic acid production than is arsenite. Arsenite inhibits acetoacetic acid production strongly, but the respiration also is greatly inhibited, showing that other tissue-processes are greatly affected.

THE MECHANISMS OF THE OXIDATIONS.

The β -oxidation theory of Knoop proposes that fatty acids are oxidised in a series of stages, in each of which the β -carbon atom of a fatty acid is attacked in such a manner that loss of two carbon atoms follows. The experiments of Embden led him to suggest that for the even-numbered fatty acids the stages led through the following substances:

decanoic acid $\rightarrow$ octanoic acid $\rightarrow$ hexanoic acid $\rightarrow$ butyric acid $\rightarrow$ acetoacetic acid.

Embden found that the odd-numbered acids formed no acetoacetic acid, which is the result anticipated if these acids also undergo β -oxidation.

We have found, in accordance with Embden, that the rate of production of acetoacetic acid from fatty acids varies in an alternate manner, the even-numbered acids producing much more acetoacetic acid than their odd-numbered neighbours. A new result is that, with guinea-pig liver at least, the odd-numbered fatty acids—apart from formic and propionic acids—also give rise to small but significant amounts of acetoacetic acid.

It is therefore likely that β -oxidation, if it occurs at all, is not the only type of oxidation which takes place with the odd-numbered acids.

The fatty acids produce β -hydroxybutyric acid as well as acetoacetic acid. The hydroxy-acid is probably produced by reduction of acetoacetic acid, for this has been shown to be true in the case of crotonic and butyric acids [Jowett and Quastel, 1935, 1]. The production of total ketones is therefore in all probability the best measure of the gross acetoacetic acid production.

The increase in respiration of guinea-pig liver brought about by odd-numbered acids is greater than that due to their even-numbered neighbours, which suggests that the odd-numbered acids are more completely oxidised. This more complete oxidation would be expected if odd-numbered acids yield a three-carbon acid which is readily oxidised by the liver, in contrast to acetoacetic acid, which is little oxidised.

The yields of total ketone bodies obtained from the even-numbered acids of 4 to 10 carbon atoms with guinea-pig liver are of the order of one molecule of ketone bodies per molecule of fatty acid.

So far, then, we have a number of facts which are in general accord with the β -oxidation theory, although there is a suggestion that the odd-numbered acids possess also another mode of oxidation which may not be quantitatively of great importance. The facts next to be considered may also be interpreted on the β -oxidation theory, if we add to this an assumption regarding the nature of the product formed from the two carbon atoms lost in each stage of oxidation. We have, in fact, found that fixed acid is produced during the oxidation of the higher fatty acids by guinea-pig liver. The acid (or acids) has not yet been identified. If it should be acetic acid, or another 2-carbon acid, our results regarding acid production are in qualitative agreement with the predictions of the β -oxidation theory. These results have already been discussed.

The suggestion that acetic acid is a product of fatty acid oxidation leads to the query whether acetic acid might not be the source of the acetoacetic acid formed by the higher odd-numbered fatty acids. Since these acids form acetoacetic acid at a higher rate than does acetic acid with guinea-pig liver, it is

probable that the explanation does not suffice, and that β -oxidation will not explain the acetoacetic acid production of odd-numbered acids.

Considering more quantitatively the yields of ketone bodies obtained from the even-numbered fatty acids with guinea-pig liver, we see that butyric acid gives a lower yield than do the higher fatty acids. Here, again, we may ask whether the higher yield obtained from the higher acids may not be due to their yielding acetic acid, which may be a source of additional ketone bodies. From our data, we consider that this possible explanation of the higher yields is inadequate. Now, according to the β -oxidation theory, butyric acid may give a higher yield of ketone bodies than do the higher fatty acids but cannot give a lower yield. Taking into account acetic acid as a possible intermediary, we find the position little altered. We, therefore, consider that the facts regarding yields are incompatible with the β -oxidation theory.

A more striking discrepancy still is found in the effect of benzoate on the rates of acetoacetic acid and total ketone body production from the even-numbered fatty acids. The rates are much more strongly inhibited in the case of butyrate than with the higher fatty acids. In the absence of an inhibitor the rates of acetoacetic acid production from the 4-, 6- and 8-carbon acids are approximately equal. In the presence of benzoate the 6-, 8- and 10-carbon acids yield acetoacetic acid and total ketones at definitely higher rates than does butyric acid. Since the production from acetic acid is low, and is as strongly inhibited by benzoate as that from butyric acid, it does not avail to suggest that acetic acid is an intermediary and is causing an apparent discrepancy with the β -oxidation theory. We must therefore conclude that the even-numbered fatty acids are not broken down through butyric acid as an intermediary. Nor, according to our data, can hexanoic acid be an intermediary for the higher acids.

We are therefore forced to abandon the β -oxidation theory. In attempting to formulate a new hypothesis to replace it, we will first point out that at the present stage such a hypothesis must differ from the β -oxidation theory more in the mechanism of oxidation which it proposes than in the nature of the products which it supposes to be formed.

We propose the view that fatty acids undergo, at a common enzyme, an oxidation throughout the fatty chain, alternate carbon atoms being affected. The oxidised product then breaks down. We may term this a theory of "multiple alternate oxidation"¹.

At the present time, the detailed application of this theory is necessarily somewhat speculative. The oxidised intermediary substances, which may exist only in combination with the enzyme, may be supposed to be capable of breaking down in a number of ways.

The theory is illustrated below by the supposition that an even-numbered acid, octanoic acid, undergoes oxidation at its β -, δ - and ζ -carbon atoms before the chain breaks, and that an odd-numbered acid, valeric acid, may undergo either β -oxidation or oxidation at the α - and γ -carbon atoms.

(1) Even-numbered acid:

¹ A somewhat similar view was put forward by Hurtley [1916].

(2) Odd-numbered acid:

(a) β -oxidation (b) $\alpha\gamma$ -oxidation

The detailed working out of our hypothesis is still, as just pointed out, a matter of speculation. We cannot dismiss the possibility that even-numbered fatty acids may undergo oxidation at their α -, γ -, ϵ -... carbon atoms, or the possibility that ω -oxidation may occur with the higher fatty acids to some considerable extent.

We can do little more now than present the hypothesis that oxidation proceeds along the whole chain of a fatty acid molecule at alternate carbon atoms, with the ultimate formation of acetoacetic acid and other acid products. Oxidation at the β -, δ -... carbon atoms is considered to be the major process in the oxidation of fatty acids containing four or more carbon atoms.

SUMMARY.

1. An investigation has been made of the formation of acetoacetic and β -hydroxybutyric acids, resulting from the oxidation of the normal saturated fatty acids containing from two to ten carbon atoms, in the presence of rat and guinea-pig liver. Van Slyke's method for estimating ketone bodies is used on a micro-scale.

2. Acetoacetic acid is probably the only β -keto-acid produced in significant quantities by the oxidation of fatty acids.

3. Acids of four, six and eight carbon atoms produce acetoacetic acid most rapidly. The ten-carbon acid is slightly less active and acetic acid considerably less active.

4. Acids of five, seven and nine carbon atoms produce acetoacetic acid at small but significant rates with guinea-pig liver. Propionic acid does not form acetoacetic acid.

5. The greater increase in the respiration of liver brought about by the odd-numbered fatty acids suggests that these are more completely burned to carbon-dioxide and water than their even-numbered neighbours.

6. In the case of guinea-pig liver, about one molecule of ketone bodies is produced per molecule of hexanoic or octanoic acid, and rather less is produced with decanoic acid. The yield of ketone bodies from butyric acid is considerably lower.

7. With all fatty acids higher than valeric acid, oxidation results in the formation of fixed acid, which is greater with the even-numbered fatty acids than with the odd-numbered fatty acids. The nature of the fixed acid produced,

which is neither acetoacetic nor β -hydroxybutyric acid, has not been determined. The oxidation of propionic and valeric acids results in disappearance of fixed acid.

8. Sodium benzoate inhibits the acetoacetic acid and total ketone body production from butyric acid to a greater extent than from the higher fatty acids in the presence of rat liver. The inhibition decreases on ascending the series of even-numbered acids.

9. The results are discussed in relation to the theory of successive β -oxidation, which is shown to be inadequate to explain the facts. A hypothesis of "multiple alternate oxidation" is put forward, according to which the fatty acids are supposed to be oxidised at alternate carbon atoms along the whole length of the chain before breakdown to acetoacetic and other acids takes place.

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