XCIV. FATTY ACID OXIDATION IN LIVER

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FATTY acid oxidation in tissues has been studied by measuring the oxygen uptake or the reaction products (acetoacetic and β-hydroxybutyric acids). The quantitative importance of β-oxidation cannot be ascertained in this way, as a fraction of the acids might undergo some other type of oxidation, such as the ω-oxidation of Verkade & van der Lee [1934].

In Knoop's classical theory the successive elimination of a two-carbon substance is required. This substance has been supposed to be acetic acid, but has never been identified.

The tissue slice technique of Warburg has been applied to the study of this problem by Edson [1935, 1, 2; 1936], Jowett & Quastel [1935, 1, 2, 3], Edson & Leloir [1936], Mazza [1936], Cohen & Stark [1938], etc. Combining this method with microestimations of fatty acids we have endeavoured to obtain more quantitative results for the oxidation of normal fatty acids with 1–8 C atoms.

Whereas liver slices rapidly oxidize fatty acids, ground tissue or enzyme preparations have never shown any such activity. For this reason it has been supposed that fatty acid oxidation is in some way dependent on cell structure. We have found it possible to prepare a cell-free liver "brei" which will oxidize butyric acid, but attempts to isolate the enzyme system have so far failed, presumably owing to a rapid inactivation by reduction of some of the components.

METHODS

Flasks as described by Krebs [1933] were filled with 30 ml. $NaHCO_3$-Ringer solution [Krebs, 1933] and with rat liver slices (about 200 mg. dry wt.). After mixing the contents thoroughly, a 12 ml. initial sample was withdrawn; the flasks were then gassed with O_2+5 % CO_2 and shaken 2 hr. at 37·5°.

Bicarbonate was estimated as previously described [Leloir & Muñoz, 1937].

Ketone bodies. Acetoacetic acid was estimated by both the manometric and modified Van Slyke methods as described by Edson [1935, 1]. The amount of NaOH given by Edson for the Rupp titration is slightly too small and may give rise to errors. It is better to double the amount, and then use a double quantity of acetic acid.

Every estimation was carried out in duplicate, the agreement being good (5 %). The agreement with the manometric method was also good (difference less than 10 %), but only with amounts larger than 50 μl.

Fatty acids. All the existing methods for fatty acid estimation require relatively large amounts of acid. In order to obtain greater accuracy and a shorter time of distillation we have used small volumes. Distillation was carried out after treating the samples with copper-lime reagent, because glucose can give rise to the formation of distillable acid. For estimating acids of 3 to 8 C atoms in the presence of acetic acid, we have taken advantage of the fact that the latter is not oxidized by dichromate. Interfering volatile substances were

eliminated by alkaline evaporation in the presence of HgO. Decanoic acid can also be estimated in this way, but it was not used in our experiments because of the insolubility of its Ca salt.

The details of the fatty acid estimations are as follows.

Precipitation of proteins. In experiments with liver slices the liquid can be directly treated with copper-lime in the amounts given by Edson [1935, 1]. For liver brei, proteins were precipitated with 1 ml. 10 % $ZnSO_4$ per ml. brei and NaOH, the amount of which was ascertained by titrating the zinc sulphate in the presence of phenol red to an orange yellow. The liquid was then diluted 7 times and filtered. Zn cannot be used with octanoic acid as it forms an insoluble salt. Hg and Cu octanoates are also insoluble, but the latter redissolves on adding $Ca(OH)_2$ and recovery is quantitative.

Samples for acidimetric titration were directly distilled, whereas those for dichromate oxidation were treated as follows:

Elimination of interfering substances. The sample (6–8 ml.) after deproteinization was measured into a test tube (15 × 140 mm.), followed by 0·2 ml. 2·5 *N* NaOH, 0·2–0·3 g. powdered HgO (yellow) and a small piece of porous porcelain. It was then placed in a boiling salt water bath (105°), the test tube rack being suspended in such a way that the tubes were only partly immersed, so that their boiling could be easily controlled.

Boiling was continued until the samples were evaporated to half volume (about 1–2 hr.), the liquid being then ready for distillation. HgO has also been used by Friedemann [1938] in order to remove aldehydes, formic, pyruvic and crotonic acids, etc.

Distillation. An all glass apparatus similar to that described by Nicloux *et al.* [1934] was used. As it is very important that the rate of distillation should be reproducible and constant, electric heating was used, the column was covered with cotton wool and the flask surrounded by a wide glass tube.

6 ml. of the sample were measured into the distillation flask, followed by 2 g. anhydrous Na_2SO_4, 1 ml. H_2SO_4 (2 vol. conc. H_2SO_4 to 1 vol. H_2O) and a capillary tube to avoid bumping. Crystallization occurs before the end of the distillation if smaller amounts of H_2SO_4 are used, but not under the given conditions. Distillation takes about 15 min. and was interrupted when 5 ml. distillate had collected in a 15 × 140 mm. pyrex test tube.

Acidimetric titration. The contents of the test tube are boiled in an open flame for 10 sec. in the presence of a small crystal of $BaCl_2$. This removes the CO_2 and detects the presence of H_2SO_4, any trace of which would cause the estimation to be discarded. The solution is then titrated with 0·01 *N* NaOH and phenolphthalein.

Dichromate oxidation. Oxidizing solution: 2·45 g. $K_2Cr_2O_7$ are dissolved in 1 l. of conc. H_2SO_4 (heat until white fumes appear). 10 ml. of this solution are sufficient for oxidizing up to 2 ml. 0·01 *N* hexanoic acid. For the same amount of octanoic acid the solution should contain double the amount of $K_2Cr_2O_7$.

To 5 ml. distillate contained in a test tube, 10 ml. of $K_2Cr_2O_7$-H_2SO_4 are added. The liquid is allowed to fall directly on the surface of the distillate so that immediate mixing occurs. A blank with distilled water is run at the same time. The tubes are covered with a small beaker and then immersed in a boiling water bath for 1 hr. The contents of the tubes are then quantitatively transferred into a 250 ml. Erlenmeyer flask, using about 100 ml. water. After adding 1 ml. 10 % KI the liberated I_2 is titrated with 0·025 *N* $Na_2S_2O_3$.

Calculation. The ml. thiosulphate used in titrating the blank minus those used for the unknown are multiplied by 2·5 × 22·4 and divided by the oxidation equivalent (Table I). This gives the amount of fatty acid in μl.

Table I. *Acidimetric and oxidimetric estimations of fatty acid solutions*

Acid solution ml.	Titration with 0·01 *N* NaOH (ml.) Direct	Distilled	Titration with 0·01 *N* $Na_2S_2O_3$ (ml.) Direct	Distilled	ml. $Na_2S_2O_3$ / ml. NaOH
			Acetic		
1·0	0·975	0·924	—	—	—
1·0	0·969	0·935	—	—	—
2·0	1·908	1·862	—	—	—
2·0	1·910	1·797	—	—	—
			Propionic		
0·5	0·465	0·431	4·86	5·11	—
0·5	0·461	0·424	5·04	5·08	11·9
1·0	0·922	0·875	9·80	10·10	—
1·0	0·925	0·865	9·90	9·75	11·4
2·0	1·775	1·695	19·80	19·95	—
2·0	1·790	1·735	20·00	19·60	11·5
			Butyric		
0·5	0·438	0·455	8·10	8·56	—
0·5	0·443	0·473	8·09	8·32	17·3
1·0	0·918	0·932	15·93	16·10	—
1·0	0·927	0·948	15·98	15·98	17·1
2·0	1·850	1·859	31·50	31·80	—
2·0	1·854	1·870	31·55	31·20	16·9
			Valeric		
0·5	—	0·461	—	11·04	—
0·5	—	0·458	—	10·91	23·9
1·0	0·985	0·956	21·83	21·67	—
1·0	0·991	0·935	21·40	21·51	22·8
2·0	—	1·875	—	42·50	—
2·0	—	1·862	—	42·40	23·8
			Hexanoic		
0·5	—	0·430	—	11·13	—
0·5	—	0·454	—	11·40	25·6
1·0	—	0·920	—	22·65	—
1·0	—	0·938	—	22·65	24·65
2·0	—	1·830	—	43·80	—
2·0	—	1·860	—	43·20	23·6
			Heptanoic		
0·5	—	0·432	—	14·30	—
0·5	—	0·455	—	13·80	32·0
1·0	—	0·900	—	26·30	—
1·0	—	0·894	—	26·70	29·5
2·0	—	1·800	—	52·60	—
2·0	—	1·760	—	51·70	29·2
			Octanoic		
0·5	—	0·413	—	18·0	—
0·5	—	0·420	—	17·63	42·7
1·0	—	0·795	—	32·9	—
1·0	—	0·787	—	34·0	42·4
2·0	—	1·483	—	65·9	—
2·0	—	1·438	—	—	45·0

The results obtained by applying these methods to pure solutions are shown in Table I. 95 % of the acetic acid is recovered after distillation, and recovery is quantitative for the other acids within the titration error. Results of the $K_2Cr_2O_7$ oxidation show errors not exceeding 10 %, which is satisfactory for work with liver slices. There are some differences in the oxidation equivalents, these errors being specially due to the acidimetric titration. With small amounts of acids this error becomes greater; and with higher fatty acids which are insoluble in water, the formation of the Na salt takes some time and requires strong shaking. This explains the too high value obtained in the oxidation equivalent when 2 ml. of octanoic acid were used (Table I).

Formic acid. Distillation under the described conditions is not quantitative (about 70 %), therefore estimations with $HgCl_2$ were carried out on the samples after copper-lime treatment. The method was used as described by Riesser [1915] but with smaller amounts. In a test tube with a ground glass stopper, 5 ml. of the filtrate were carefully neutralized (phenol red), and 1 ml. of the $HgCl_2$ reagent added ($HgCl_2$ 300 g., Na acetate 300 g., NaCl 80 g., per l.). The tubes were then heated in a salt water bath (105°) for 40 min. After cooling, 0·5 ml. glacial acetic acid, 1 ml. saturated KI and 2 ml. 0·03 *N* I_2 were added. The tubes were shaken, and after complete solution of the calomel the excess I_2 was titrated with 0·01 *N* $Na_2S_2O_3$.

With pure solutions the results are reproducible within 10% with amounts ranging from 30 to 300 μl. (0·06–0·6 mg.).

Units. Results are given in μl., the acids being considered as perfect gases at N.T.P.[1] (22·4 μl. = 1 μmol.). Q represents μl. of substance formed per mg. tissue (dry wt.) per hr.

Experimental results

One of the difficulties in the interpretation of the results is that in the control there is always a spontaneous formation of ketonic acids and that it is impossible to know if this continues at the same rate when a substrate is added. This also applies to the measurements of $NaHCO_3$. Liver slices with no substrate produce a decrease in $NaHCO_3$, less than half of which is due to ketonic acids. The rest is not due to lactic acid or to a distillable acid. Perhaps it is due to a fixation of base (K) by the liver slices.

For this reason we shall often refer to the corrected Q. This is the value obtained by subtracting the value of Q given by a control with no substrate. Measuring as we have done in every case the distillable acid, acetoacetic and β-hydroxybutyric acids and $NaHCO_3$, we can get a rough idea of the formation of a non-distillable non-ketonic acid.

Slices in the presence of, e.g., Na butyrate, consume the butyrate ion and an increase in $NaHCO_3$ occurs; ketonic acids are formed decreasing the $NaHCO_3$, and if any other acid is formed it will also decrease $NaHCO_3$. We should then have:

$$-Q_{\text{bic. (corr.)}} = Q_{\text{dist. ac.}} + Q_{\text{ketonic ac.}} + Q_{NN}.$$

Q_{NN} would therefore represent the non-distillable non-ketonic acid. Naturally, as this is calculated indirectly, Q_{NN} will only be significant when its value is large.

Formic acid. Liver slices without substrate give rise to the formation of a substance which is estimated as formic acid (see Table II, Nos. 1 and 2). This amounts to about 24 μl. per ml., giving a Q_{formic} of 1·27 and 0·75. The method of estimation used is far from specific and we cannot assert that this substance is really formic acid.

On adding formic acid to liver slices a small disappearance occurs: $Q_{\text{formic}} = -1{\cdot}1$ and $-0{\cdot}06$. Subtracting the spontaneous formation, the values for the disappearance ($-Q$) would be 2·37 and 0·81 respectively. Ketonic acid formation is not modified and the acid disappearance is in good agreement with the changes in $NaHCO_3$: $Q_{\text{bic.}}$ (corrected) 2·44 and 0·65. The velocity of disappearance of formic acid is therefore small, and if it were formed from added fatty acids we should expect it to accumulate to a certain extent in the medium. As we shall see later, this is not the case.

[1] We have continued using μl. because it is the unit used by all those who have worked with tissue slices, but it would be more correct to use μmol.

Table II. *Liver slices from rats starved 24 hr.*

Bicarbonate Ringer. Gas O_2+5 % CO_2. Δ indicates the difference in composition (in μl.) of 1 ml. medium before and after 2 hr. at 37·5°

No.	Volume of medium ml.	Dry wt. of slices (mg.)	Substrate	Bicarbonate Δ	Bicarbonate Q	Acid by oxidation Δ	Acid by oxidation Q	Distillable acid Δ	Distillable acid Q	Acetoacetic acid Δ Titr.	Acetoacetic acid Δ Manom.	β-Hydroxybutyric acid Δ	Total ketonic acids Δ	Total ketonic acids Q
1	18·0	188	Formate 0·0118 *M*	−20	−0·96	−23	−1·1	—	—	25	33	21	46	+2·2
	18·0	170	None	−64	−3·40	+24	+1·27	—	—	27	33	23	50	+2·65
2	17·4	276	Formate 0·008 *M*	−26	−0·82	−2	−0·06	—	—	6	8	7	13	+0·44
	17·4	278	None	−47	−1·47	+24	+0·75	—	—	4	9	8	12	+0·38
3	18·8	254	Acetate 0·0136 *M*	+75	+2·8	—	—	−144	−5·3	12	18	20	32	+1·2
	18·8	234	None	−45	−1·81	—	—	0	0	3	—	7	10	+0·4
4	20·8	246	Acetate 0·0107 *M*	+55	+2·3	—	—	−91	−3·8	26	29	12	38	+1·7
	20·8	219	None	−26	−1·2	—	—	0	0	7	6	16	23	+1·1
5	20·8	228	Acetate 0·0284 *M*	+49	+2·2	—	—	−112	−5·1	46	51	19	65	+3·0
	20·8	222	None	−44	−2·2	—	—	0	0	13	20	13	26	+1·2
6	18·0	235	Propionate 0·0125 *M*	−15	−0·57	−58	−2·22	−46	−1·80	5	9	9	14	+0·54
	18·0	251	None	−25	−0·90	0	0	0	0	3	4	7	9	+0·25
7	18·0	273	Propionate 0·01 *M*	−32	−1·0	−27	−0·89	−9	−0·33	12	21	13	25	+0·84
	18·0	270	None	−37	−1·23	0	0	0	0	5	11	12	16	+0·57
8	18·0	184	Butyrate 0·0135 *M*	−14	−0·7	199	−9·8	−175	−8·6	100	100	56	155	+7·6
	18·0	191	None	−26	−1·2	0	0	0	0	4	12	10	14	+0·7
9	18·0	226	Butyrate 0·0133 *M*	−24	−0·9	−242	−9·7	−237	−9·5	158	157	71	229	+9·1
	18·0	198	None	−19	−0·9	0	0	0	0	+8	+10	+10	+17	+0·5
10	19·0	206	Butyrate 0·0177 *M*	−12	−0·6	−167	−7·7	−187	−8·6	119	120	65	+184	+8·5
	19·0	188	None	−29	−1·5	0	0	0	0	4	10	1	5	+0·3
11	17·4	292	Valerate 0·0090 *M*	−80	−2·38	−68	−2·03	−47	−1·40	6	17	17	24	+0·71
	17·4	245	None	−72	−2·57	0	0	0	0	4	8	13	17	+0·60

12	17·4	244	Valerate 0·0086 *M*	−52	−1·86	−63	−2·25	−52	−1·86	19	24	19	38	+1·36
	17·4	250	None	−42	−1·46	0	0	0	0	6	10	10	16	+0·56
13	17·4	190	Hexanoate 0·0087 *M*	−97	−4·45	−164	−7·55	−137	−6·28	144	147	99	243	11·1
	17·4	195	None	−35	−1·56	0	0	0	0	24	29	18	42	1·82
14	18·0	151	Hexanoate 0·0121 *M*	−61	−3·63	−97	−5·8	−81	−4·8	102	107	39	+141	+8·4
	18·0	178	None	−43	−2·17	0	0	0	0	25	32	15	+40	+2·0
15	18·0	226	Hexanoate 0·0117 *M*	−64	−2·55	−120	−4·77	−125	−4·97	87	89	84	+170	+6·37
	18·0	233	None	−48	−1·85	0	0	0	0	4	9	10	+14	+0·54
16	17·4	201	Heptanoate 0·0095 *M*	−49	−2·12	−65	−2·81	−42	−1·86	22	28	15	38	+1·64
	17·4	228	None	−72	−2·72	0	0	0	0	22	29	11	33	+1·26
17	17·4	256	Heptanoate 0·0103 *M*	−95	−3·23	−91	−3·1	−23	−0·78	13	21	23	36	+1·22
	17·4	244	None	−56	−2·00	0	0	0	0	6	13	10	16	+0·57
18	17·4	213	Octanoate 0·01 *M*	−138	−5·6	−156	−6·4	−110	−4·5	+106	+111	+92	+198	+8·1
	17·4	175	None	−51	−2·7	0	0	0	0	4	8	4	8	0·4
19	17·4	160	Octanoate 0·0089 *M*	−174	−9·4	−131	−7·1	−88	−4·8	+112	+116	+122	+234	+12·8
	17·4	114	None	−86	−4·7	0	0	0	0	9	17	+6	+15	+0·8
20	17·4	196	Octanoate 0·0096 *M*	−116	−5·2	−155	−6·9	−96	−4·3	+144	+137	+87	+231	+10·2
	17·4	246	None	−44	−1·6	0	0	0	0	+25	+20	+14	+38	+1·3
21	17·4	234	Octanoate 0·0090 *M*	−154	−5·7	−144	−5·4	−98	−3·7	+73	+74	+165	+238	+8·9
	17·4	230	None	−55	−2·1	0	0	0	0	+5	+10	+12	+18	+0·7
22	17·5	244	Octanoate 0·008 *M*	−123	−4·42	−121	−4·34	−70	−2·51	45	49	133	178	+6·39
	17·5	269	Acetate 0·022 *M*	+121	+3·96	0	0	−205	−6·69	14	23	16	30	+0·98
23	17·5	221	Octanoate 0·095 *M*	−94	−3·72	−117	−4·64	−88	−3·49	63	68	115	178	+7·05
	17·5	236	Acetate 0·0125 *M*	+90	+3·34	0	0	−125	−4·64	7	—	8	15	+0·56
24	17·5	237	Octanoate 0·0079 *M*	−97	−3·58	−128	−4·72	−71	−2·62	98	100	116	215	+7·87
	17·5	244	Acetate 0·0125 *M*	—	—	0	0	−134	−4·81	15	22	15	30	+1·08

Acetic acid. Acetic acid disappears at a rate more than twice that of formic acid.

The values of $-Q_{\text{acetic}}$ obtained by distillation and titration with NaOH were 5·3, 3·8 and 5·1; corresponding $Q_{\text{bic.}}$, 4·6, 3·5 and 4·4. $Q_{\text{keto.}}$ (corrected) amounted to 0·8, 0·6 and 1·8.

Calculating with these results the non-distillable, non-ketonic acid ($Q_{\text{NN}} = -Q_{\text{bic.}} - Q_{\text{dist.}} - Q_{\text{keto.}}$) we obtain −0·1, −0·3 and −1·1. Therefore, when acetic acid disappears, there is no formation of any other acid except acetoacetic and β-hydroxybutyric.

It is clear from these experiments that the increase in ketonic acids only accounts for a small fraction of the acetic acid which disappears. The amount of acetic acid which disappears is 6·6, 6·3 and 2·8 times greater than the ketonic acids formed (mol. per mol.).

The mechanism of this reaction has been discussed by Krebs & Johnson [1937]. They give good evidence that the first step is a condensation of acetic with pyruvic acid, acetopyruvic acid being formed. The latter is then transformed into ketonic acids.

Acetic acid increases Q_{O_2} by 2–4 units and is therefore probably oxidized. If the oxidation were direct, the only possible intermediary would be glycollic acid, which would then be oxidized to glyoxylic and this acid might give 2 mol. of formic acid or be oxidized to oxalic acid. But this does not occur in liver, as is proved by the experiment in Table III in which the changes in bicarbonate and ketonic acids were measured.

Table III. *Liver slices in bicarbonate Ringer*

	$Q_{\text{bic.}}$	$Q_{\text{keto.}}$
No substrate	−1·30	0·34
Acetate 0·02 *M*	+2·69	1·38
Glycollate 0·02 *M*	−2·92	0·19
Oxalate 0·02 *M*	−1·23	0·78

This experiment shows that the acetate ion disappears, producing an increase in base ($Q_{\text{bic.}}$). In the presence of glycollate this increase in base does not occur; on the contrary there is a slight acidification which might be due to oxidation to oxalate. Oxalic acid is not oxidized, for if this were the case it would give two basic equiv. per mol.

In another identical experiment formic acid was also estimated, no difference being found between the flask with no substrate and that with glycollic acid.

If acetic acid disappears by condensation with another substance one would expect that the addition of that substance would increase the rate of disappearance. Experiments in this direction were not quite satisfactory, because our method was not capable of detecting very small changes. Nevertheless we have tried many substances (C_4 dicarboxylic acids, glycine, aspartic acid, insulin, dry thyroid, glucose, fructose, lactate, citrate, liver and yeast extracts etc.) without finding any appreciable increase in the rate of disappearance.

Malonic acid inhibits acetic acid disappearance (*M*/50 malonate decreases the $-Q_{\text{acetic}}$ from 5·1 to 2·1).

Propionic acid. The rate of disappearance is small ($Q_{\text{propionic}} = -2·22$ and −0·89); decrease in distillable acid, −1·80 and −0·33. $Q_{\text{bic.}}$ (corrected) = 0·33 and 0·23: increase in ketonic acids = 0·29 and 0·27.

As propionic acid is metabolized slowly we have not tried to determine what is the first reaction product.

Butyric acid. Of all the acids studied butyric is oxidized most rapidly ($-Q_{\text{butyric}} = 9{\cdot}8$, 9·7 and 7·7, Table II). The corresponding values of $Q_{\text{keto.}}$ were 6·9, 8·6 and 8·2. Therefore 70, 89 and 106 % of the butyric acid was transformed into ketonic acids.

The values of $Q_{\text{bic.}}$ (corrected) were 0·5, 0 and 0·9. This shows that only a small amount is totally oxidized (5, 0 and 12 % respectively).

Valeric acid. Experiments with valeric acid (Nos. 11 and 12, Table II) gave the following results: $Q_{\text{valeric}} = -2{\cdot}03$ and $-2{\cdot}25$. $Q_{\text{dist. ac.}} = -1{\cdot}40$ and $-1{\cdot}86$. $Q_{\text{bic.}}$ (corrected) $= +0{\cdot}2$ and $-0{\cdot}40$. Q_{keto} (corrected) $= 0{\cdot}11$ and 0·8. The difference between the values obtained by titration with NaOH and by oxidation are too small to be significant. The amount of non-distillable non-ketonic acid would be 1·09 and 1·46; values to which no importance can be given owing to the indirect way in which they are calculated.

Hexanoic. The rate of disappearance of hexanoic acid, as measured by the oxidation method, was $-Q_{\text{hexanoic}} = 7{\cdot}55$, 5·8 and 4·77; and as measured by distillation and titration with NaOH: $-Q_{\text{dist. ac.}} = 6{\cdot}28$, 4·8 and 4·97. The difference between these values (1·27, 1·0 and 0) is attributed to a small accumulation of acetic acid.

If we suppose that each molecule of hexanoic gives rise to one of ketonic acid and one of acetic, the $Q_{\text{ketonic ac.}}$ should be equal to the Q_{hexanoic} plus the amount of ketonic acids which are formed from acetic acid.

The values found for the $Q_{\text{ketonic ac.}}$ (corrected) were 9·28, 6·4 and 5·83. They are larger than the Q_{hexanoic}, the excess being: 1·7, 0·6 and 1·06. These values are of the order of those found for acetic acid which can increase the $Q_{\text{ketonic ac.}}$ by 1 or 2 units. Moreover, the amount of acetic acid formed should be equal to the $-Q_{\text{hexanoic}}$. Of this, part accumulates in the medium (1·27, 1·0 and 0) and the rest (6·28, 4·8 and 4·97) would disappear. The latter values are of the order of those found for the disappearance of added acetic acid.

The values of $-Q_{\text{bic.}}$ (corrected) were 2·89, 1·46 and 0·80. From these we can calculate the non-distillable non-ketonic acid ($-0{\cdot}11$, $-0{\cdot}14$ and $-0{\cdot}06$). These small values not only show that no fixed acid is formed but also that there is a good agreement between the different methods of estimation.

Heptanoic (Exps. 15 & 16, Table II). Values obtained for the disappearance of heptanoic were $-Q_{\text{heptanoic}} = 2{\cdot}81$ and 3·1; $-Q_{\text{dist. ac.}} = 1{\cdot}86$ and 0·78. The difference between these values (0·95 and 2·32) would indicate the accumulation of a distillable acid which is not oxidized with dichromate (acetic).

The increases in the $Q_{\text{ketonic ac.}}$ were 0·38 and 0·65. The $Q_{\text{bic.}}$ (corrected) $= +0{\cdot}60$ and $-1{\cdot}23$. The non-distillable non-ketonic acid would be 0·88 and 1·36. All these results may be interpreted by the classical β-oxidation: 2 mol. acetic acid and 1 mol. propionic acid being formed from each mol. of heptanoic acid.

The lanthanum reaction, using the technique described for octanoic acid, was carried out in three experiments. The final sample of the flask containing slices and heptanoic acid gave a positive reaction. The reaction loses in this case some of its value because the positive result could be due to propionic acid.

Octanoic acid. This acid disappears at a greater rate than any of the odd numbered acids. $-Q_{\text{octanoic}} = 6{\cdot}4$, 7·1, 6·9 and 5·4. The corresponding $Q_{\text{ketonic ac.}}$ (corrected) $= 7{\cdot}7$, 12, 8·9 and 8·2. Therefore each mol. octanoic acid gives rise to 1·2, 1·7, 1·3 and 1·5 mol. ketonic acid (Exps. 18, 19, 20 and 21, Table II).

The amount of acetic acid formed would be ($Q_{\text{acetic}} = Q_{\text{octanoic}} - Q_{\text{dist. ac.}}$) 1·9, 2·3, 2·6 and 1·7. The $Q_{\text{bic.}}$ values (corrected) were: $-2{\cdot}9$, $-4{\cdot}7$, $-3{\cdot}6$ and $-3{\cdot}6$.

The calculation of the amount of non-distillable non-ketonic acid gives negative values (−0·3, −2·5, −1·0 and −0·9).

According to the classical β-oxidation each mol. octanoic acid should give one of ketonic acid and two of acetic. Therefore Q_{ketonic} should be equal to the $-Q_{\text{octanoic}}$ and double this amount of acetic should be formed. But in our experiments the $Q_{\text{ketonic ac.}}$ exceeds the Q_{octanoic} by 1·3, 4·9, 2·0 and 2·8. Some of these values are considerably greater than the amount of ketonic acids that arise from acetic acid and cannot be attributed to experimental errors as every estimation was carried out in duplicate. Moreover, the amount of acetic acid formed should be double the $-Q_{\text{octanoic}}$, that is 12·8, 14·2, 13·8 and 10·8; of this a part (Q = about 2) accumulates in the medium and the rest should disappear. But we have seen that acetic disappears at most at a rate of Q = 4–6. If we suppose that each mol. of octanoic acid is split into two of ketonic acid the Q_{ketonic} should be double the Q_{octanoic} and this does not explain the experimental results.

We may then suppose that octanoic acid can be oxidized by both mechanisms, a fraction (a) would give 2 mol. ketonic acid, and the rest (b) would give 1 mol. ketonic acid and 2 mol. acetic acid.

To test this hypothesis we carried out another set of experiments (Nos. 22, 23 and 24) in which acetate was added to the control, enabling us to ascertain for the liver specimen how much acetate disappears and the amount of ketonic acids which are formed from it.

The amount of octanoic acid which is oxidized by the mechanisms (a) and (b) is calculated as follows: $Q_{\text{octanoic}} = a + b$ (I). The amount of ketonic acids formed will be (II) $2a + b + Q_{\text{ketonic}}$ (control) (this represents the amount formed spontaneously and from acetic acid).

Replacing in (II) the value of (b) in (I) we obtain:

$$a = Q_{\text{ketonic}} - Q_{\text{octanoic}} - Q_{\text{ketonic}} \text{ (control)}.$$

On applying this equation to the experimental results we obtain the values given in Table IV.

Table IV

$-Q_{\text{octanoic}}$	a	b	$\frac{b}{-Q_{\text{octanoic}}}$
4·34	1·07	3·27	0·75
4·64	1·85	2·79	0·60
4·72	2·07	2·65	0·56

We can now calculate the amount ($2b$) of acetic acid formed from octanoic acid. We know by experiment how much acetic acid accumulates and we also know the rate at which acetic acid disappears in that liver (from the control). We can then compare the values for acetic acid formation calculated from the Q_{octanoic} and the Q_{ketonic} with the values obtained from the accumulated acetic acid: ($Q_{\text{dist. ac.}} - Q_{\text{octanoic}}$) plus the acetic acid which disappears in the control.

The $-Q_{\text{acetic}}$ of the control = 6·69, 4·64 and 4·81. Accumulated acetic = 1·83, 1·15 and 2·10. The sum of these values would be the acetic acid formed from octanoic = 8·5, 5·7 and 6·9 (calc. values ($2b$) = 6·5, 5·6 and 5·3). The agreement is good if we consider that the acetic acid formed is calculated in an indirect way.

Acetic acid formation from octanoic acid. In the experiments with octanoic acid we have found a difference of 1 or 2 units in the Q as measured by oxidation and as measured by titration with NaOH. This we have attributed to an accumulation of acetic acid. These results may also be attributed to the formation of acids with less C atoms (formic, butyric or hexanoic).

Formic acid formation is excluded because estimations showed no difference between slices with and without octanoic acid. Butyric acid disappears faster than octanoic so that its accumulation is not probable.

Lanthanum reaction. In order to confirm the fact that acetic acid is really formed we have used the lanthanum reaction. Acetic acid does not give the lanthanum reaction in the presence of octanoic acid, which must be removed as its less soluble Ag salt before carrying out the test.

The liquid to be examined was treated with copper-lime, the alkaline filtrate was evaporated to dryness in a water bath; the residue was extracted with hot water (6 ml.) and distilled. The distillate (5 ml.) was carefully neutralized, solid Ag_2SO_4 added and the solution was boiled and then cooled to $-2°$ and filtered. The filtrate was distilled and the lanthanum reaction [Krüger & Tschirch, 1930] applied to the distillate.

As in all the experiments, liver slices (200 mg. dry wt.) were suspended in 30 ml. of $NaHCO_3$-Ringer in two flasks. One of them contained octanoate (0·01 *M*) and the other no substrate. An initial sample of 12 ml. was withdrawn from each and the rest left 2 hr. at 37°. Of these four samples treated in the same manner only one gave a positive lanthanum reaction. This corresponded to the final sample of the flask with octanoate.

That acetic acid responsible for the positive lanthanum reaction does not arise from the action of alkali on acetoacetic acid was proved by adding β-hydroxybutyric acid to some of the controls. Although a considerable formation of acetoacetic acid occurred, the La reaction was always negative.

Oxidation of butyric acid in a cell-free "brei"

Preparation of the "brei". We have used a similar procedure to that described by Potter & Elvehjem [1936]. A bulb is blown at the end of a capillary tube in such a way that it fits with less than 0·3 mm. clearance in a strong test tube (16 × 150 mm. or 22 × 200 mm. according to the amount of tissue). The liver of one or two recently killed rats is weighed, cut in small portions with scissors and put in the test tube which already contains the cooled alkaline buffer (31 g. $Na_2HPO_4, 2H_2O$ + 8 g. KCl per l.; 1·53 ml. per g. liver). With the test tube in a freezing mixture, the piston is introduced and worked up and down energetically. When the contents are frozen the operation is continued out of the freezing mixture. After thawing, the tube is again put in the freezing mixture. The procedure is continued for 10 min., 0·5 *M* KH_2PO_4 (0·87 ml. per 10 ml. of alkaline buffer) is added and the liquid is filtered through muslin. During all these manipulations the contents of the tube are aerated with O_2.

Microscopical examination[1] of the "brei" so obtained shows the absence of liver cells, only some nuclei and white and red blood cells being visible. (Blood has no oxidative activity.) The resulting *p*H is optimal for the preparation, addition of small amounts of acid or alkaline buffer decreasing the activity.

Activity measurements can only be made by estimating butyric acid. The O_2 uptake without added substrate is so great that it cannot be used to determine the rate of butyric acid oxidation.

Butyric acid added to this "brei" at 25° in O_2 disappears. That this disappearance is due to oxidation is proved by estimation of acetoacetic and β-hydroxybutyric acids. The "brei" is rapidly inactivated in absence of O_2: it is sufficient to leave it in a test tube at room temperature for 15–30 min. without bubbling O_2 through it, in order to obtain complete inactivation.

[1] We are indebted to Dr Porto for the microscopical study of our preparations.

Succinic, fumaric, malic and citric acids added to the "brei" increase the disappearance of butyric acid but exert no action once the "brei" has been inactivated by anaerobiosis.

Table V. *Cell-free liver "brei" 7 ml. (about 700 mg. dry wt.). 2 hr. at 25°. Gas O_2*

	Butyric μl.	Δ μl.	Aceto-acetic acid μl.	β-Hydroxy-butyric acid	Total ketonic acid μl.	Ketonic / Butyric
Butyrate added	3029	—	—	—	—	—
"Brei" + butyrate	2370	−742	788	420	+796	1·07
„ + no substrate	83	—	137	412	—	—
„ + butyrate + fumarate	2159	−924	125	835	+640	0·69
„ + fumarate (0·01 *M*)	74	—	5	195	—	—

In Table V we give the results of one of three exp. with fumaric acid. It shows that the "brei" with no substrate forms a certain amount of ketonic acids. When butyric is added it disappears and there is a corresponding increase in total ketonic acids. When both butyric and fumaric acids are added more butyric disappears (sometimes 50 % or more), but the ketonic acid formation does not increase so much. With butyric acid alone the relation $\frac{\text{total ketonic}}{\text{butyric acid}}$ was 1·07 and when fumaric was also present it was only 0·69.

Moreover, when fumaric is present more of the ketonic acid appears in the reduced state. The relation $\frac{\beta\text{-hydroxybutyric}}{\text{acetoacetic}}$ is 0·5 with butyric acid alone and 5·3 when fumaric acid is also present.

Discussion

The methods described are suitable for the type of experiments for which we have used them, and with slight modifications might be useful for other purposes. They are good for acids with 3–8 C atoms, the oxidation with dichromate being more accurate than acidimetric titration. One important point is that acetic acid does not interfere in the oxidation method.

Measurements of the rate of disappearance of saturated fatty acids show a net difference between the odd and even series. This difference is also observed in the ketogenesis, but is not clear from measurements of O_2 uptake: the increases in Q_{O_2} [data of Edson, 1935, 1] being C_1, 1·1; C_2, 3·8; C_3, 0·6; C_4, 4·0; C_5, 2·1; C_6, 1·7; C_7, 2·4; C_8, 1·3. By measurement of the disappearance of the acids we have obtained C_1, 1·5; C_2, 5; C_3, 2; C_4, 9; C_5, 2; C_6, 6; C_7, 3; C_8, 6.

Formic acid is presumably oxidized completely to CO_2 and H_2O. Liver slices without any added substrate give rise to a small amount of a substance which is estimated as formic acid (as was observed in liver perfusion by Toenniessen & Brinkmann [1938]). Owing to the lack of specificity of the method we have used we cannot be sure if it is in fact formic acid. The formation of this substance does not increase in the presence of octanoate.

Acetic acid disappears at a much higher rate and, as bicarbonate increases proportionally, we may deduce that no other acid accumulates. Oxalic and glycollic acids do not give an increase in base; therefore they cannot be intermediaries in the disappearance; formic acid can also be excluded as its oxidation is too slow.

The amount of ketonic acids formed from acetic acid only accounts for about 20 % of that disappearing (mol. per mol.). We have not been able to find out how the rest of the acetic acid is metabolized. If it were by condensation with

another substance we might expect an increased disappearance on adding that substance but this was not observed in our experiments. The fact that there is an increase in O_2 uptake with acetic acid indicates that a part of it is oxidized, although probably not directly.

The rate of disappearance of propionic acid is low and therefore we have not tried to find out how it is oxidized.

Butyric is the acid which is most rapidly oxidized by the liver. Most of it (80 or 90 %) is β-oxidized, but we cannot completely dismiss the possibility of a very small fraction undergoing some other type of change.

Valeric disappears at about the same rate as propionic acid. The small increase in ketonic acid formation already observed by Edson [1935,1] and Jowett & Quastel [1935, 2] might arise from the acetic acid formed. Results obtained with hexanoic and heptanoic acids can be quite well interpreted by β-oxidation.

With octanoic acid, results are rather more complicated. This acid gives more acetoacetic + β-hydroxybutyric than is required by a successive β-oxidation, but not enough to account for the molecule breaking up into two 4 carbon units. If we suppose that a fraction of octanoic acid follows each of these possibilities, the experimental results can be well interpreted.

This type of oxidation of fatty acids has previously been suggested by Jowett & Quastel [1935, 2], although the theory was supported by somewhat indirect evidence. According to Jowett & Quastel the fatty acid molecule (e.g. octanoic) would undergo a simultaneous oxidation at the 2, 4 and 6 C atoms. The triketo-acid formed, which might only exist in combination with the enzyme, can then break down, giving 2 mol. acetoacetic or 1 mol. of acetoacetic and 2 mol. of acetic acid. This interesting hypothesis explains satisfactorily the results we have obtained. In our experiments about 30 % of the octanoic acid would be split into 2 mol. acetoacetic. Moreover, Butts *et al.* [1935] and Deuel *et al.* [1936] have found that feeding rats with measured amounts of the salts or the ethyl esters of hexanoic to tetradecanoic acids leads to the elimination of twice the expected amount of ketonic acids.

When octanoic acid is oxidized by liver slices, a certain amount of acetic acid accumulates. This was ascertained by estimation and by the lanthanum reaction.

Attempts to isolate the enzyme system which oxidizes butyric acid have failed. Precipitates obtained with acetone, ammonium sulphate and acetic acid are inactive and are not activated by adding "kochsaft" or "brei" from liver or muscle. As the inactivation is presumably due to a reduction by the substrates present in the liver, we have also investigated if the presence of oxidants would activate the preparation. However, H_2O_2, ferricyanide, iodate, quinone etc., all acted as inhibitors, and in fact nearly everything tried acted as an inhibitor.

The activating effect of dicarboxylic acids on the "brei" is difficult to understand. Szent-Györgyi [1937] has observed a similar action on the O_2 uptake of pigeon muscle "brei", fumarate acting as an activator but having no action if added after a certain time.

The anaerobic inactivation of the "brei" appears in our case to be the inverse of what is known to occur with other enzymes. Thus papain, cathepsin and succinic dehydrogenase [Hopkins *et al.* 1938] are inactivated by mild oxidizing agents.

Summary

A micromethod for the estimation of fatty acids by distillation and oxidation with dichromate is described.

The action of liver slices on normal fatty acids with 1–8 C atoms was studied.

The rates of disappearance ($-Q$) of the different acids are: formic 1·5; acetic, 5; propionic, 2; butyric, 9; valeric, 2; hexanoic, 6; heptanoic, 3; octanoic, 6.

Glycollic and oxalic acids are not intermediaries in acetic acid disappearance. The ketonic acids formed only account for about 20 % of the acetic acid consumed. Butyric acid is almost completely (80–90 %) oxidized in the β-position.

Hexanoic and heptanoic acids also seem to follow classical β-oxidation.

Octanoic acid appears to be oxidized, a part giving 2 mol. of ketonic acid and another part giving 2 mol. of acetic and 1 mol. of ketonic acid. Acetic acid was identified by the lanthanum reaction.

Butyric acid oxidation can be obtained in a cell-free liver "brei". This preparation is rapidly inactivated, especially under anaerobic conditions.

C_4 dicarboxylic acids appear to exert an activating action on the "brei". They decrease the amount of total ketonic acids formed and increase the reduction of acetoacetic acid.

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