CCLV. STUDIES IN FAT METABOLISM.

I. THE OXIDATION OF BUTYRIC, CROTONIC AND β-HYDROXYBUTYRIC ACIDS IN PRESENCE OF GUINEA-PIG LIVER SLICES.

By MAURICE JOWETT[1] and JUDA HIRSCH QUASTEL.

From the Biochemical Laboratory, Cardiff City Mental Hospital.

(*Received July 11th, 1935.*)

Until lately the study of the metabolism of fatty acids has depended on methods with which it is difficult to obtain quantitative results and comparisons.

Recently, however, Quastel and Wheatley [1933] have found that fatty acids are oxidised at considerable rates by slices of liver *in vitro* and give as one of their oxidation products acetoacetic acid, as in the body. Modern micromanometric technique can, therefore, now be applied to problems of fatty acid metabolism.

In the present work a study has been made of the kinetics of the oxidation of butyric, crotonic and β-hydroxybutyric acids by the liver of the guinea-pig, and this has led to a partial elucidation of the mechanisms by which these substances are oxidised to acetoacetic acid.

Experimental methods.

The manometric methods of Warburg [1926] have been used throughout. Measurements of respiration have been made by immersing tissue slices in a medium containing sodium β-glycerophosphate as buffer, 0·2 ml. of 6% KOH being present in a side-tube of the manometric vessel. Estimations of acetoacetic acid have been made in the solutions after removal of the tissue slices. The experimental period has usually been two hours at 37°. The mean rates for respiration refer to the period ¼ to 2 hours and those for acetoacetic acid formation to the period 0 to 2 hours.

In accordance with the notation introduced by Warburg, the respiration (Q_{O_2}) is defined as the number of μl. of oxygen (reduced to N.T.P.) absorbed per mg. dry weight of tissue per hour, and the rate of production of acetoacetic acid (Q_{Ac}) is similarly defined in terms of gas evolution. Our manometric method of estimating acetoacetic acid gives an evolution of one molecule of carbon dioxide per molecule of acetoacetic acid. Hence Q_{Ac} can be regarded as measured in terms of volumes of CO_2, or, according to the usual convention, as measured in terms of the volume which acetoacetic acid would occupy if it behaved as a perfect gas at N.T.P.

Media. All solutions have been so made up as to have an osmotic pressure approximately equal to that of 0·16 *M* NaCl. Uni-univalent salts employed are made up in stock solutions to 0·16 *M*, or to submultiples of this strength, dilutions being made with 0·16 *M* NaCl. Salts of other valency-types are made up to the appropriate strengths, *e.g.* sodium fumarate to 0·107 *M*. With solutions so

[1] Beit Memorial Research Fellow.

made up, the medium in which the tissue is immersed can readily be made isotonic by mixing the various stock solutions.

The volume of medium used in each vessel has been usually 3·2 ml., this choice of volume being convenient for performing dilutions. The volume is made up to 3·2 ml. with 0·16 *M* NaCl after all other desired substances are present.

The composition of a typical medium is as follows:

Sodium glycerophosphate	0·067 *M*
KCl	0·002 *M*
$CaCl_2$	0·001 *M*
Sodium butyrate	0·01 *M*
NaCl	0·051 *M*

In all media employed, unless otherwise stated, the concentration of K^+ is 0·002 *M*, and that of Ca^{++} 0·001 *M*.

Glycerophosphate buffer. Quastel and Wheatley [1933] found that phosphate inhibited partially the oxidation of butyric acid to acetoacetic acid by liver. Phosphate buffer solutions precipitate calcium ion and cannot be used in a medium of the Locke type. Following Quastel and Wheatley, we have therefore adopted sodium β-glycerophosphate as a buffer. The salt is brought to p_H 7·4 with HCl, and a 0·105 *M* solution is used, of which usually 1·9 ml. are present in a total volume of 3·2 ml.

According to Meyerhof and Kiessling [1933] $p_{K'_2}$ for β-glycerophosphoric acid is about 6·33; hence at p_H 7·4 the buffering action is weak, particularly in the direction of rising p_H. Quastel and Wheatley [1934] reported a drop in p_H of about 0·2 during experiments of 3 hours' duration with guinea-pig and rat liver slices. Glycerophosphate solutions undergo hydrolysis on keeping, with liberation of phosphate and fall of p_H. They may however be kept several days at 0° without much change.

Tissue slices. It has been found that the accuracy of the experiments depends mainly on the use of slices of similar thickness throughout any one experiment. The thickness of slices used varies from one experiment to another, but has usually lain between the limits 0·25–0·45 mm. Several slices are commonly employed in each vessel, since the total dry weight desired has usually been 15–25 mg. The use of several slices appears to help to smooth out differences of behaviour.

Livers. The livers of young guinea-pigs, mainly of weights 270–330 g., fed on bran, oats and greens, have been employed. The animals were bled from the throat before removing the livers, the blood of which is therefore partially removed.

Neutralisation of acids. All fatty and other acids are neutralised before use, solutions of the sodium salts being prepared.

Manometric estimation of acetoacetic acid. As was found by Quastel and Wheatley [1933], acetoacetic acid can be estimated manometrically by measurement of the volume of gas produced in its breakdown to acetone and carbon dioxide, aniline being used as a catalyst for the process.

Acetoacetic acid breaks down spontaneously in solution, giving the same end-products except in very alkaline solutions [Widmark, 1920]. The rate of breakdown is slow in neutral solutions but becomes faster in acid solutions. Below are given some data on this point calculated from the measurements of Widmark.

Values of the rate of decomposition have also been measured by us manometrically, and are summarised in the last column of the table. The agreement with the figures of Widmark is quite satisfactory.

Decomposition of acetoacetic acid at 37°.

k_{uni} (min.) (W.)	p_H	% decomposition In 15 min.	% decomposition In 2 hours	k_{uni} (J. and Q.)
$4{\cdot}5\ .10^{-3}$	<2·0	6·5	41·7	$4{\cdot}8.10^{-3}$
$1{\cdot}31.10^{-3}$	4·0	1·9	14·6	—
$5{\cdot}8\ .10^{-4}$	4·5	0·9	6·7	$5{\cdot}5.10^{-4}$
$2{\cdot}5\ .10^{-4}$	5·0	0·4	3·0	—
$8{\cdot}0\ .10^{-5}$	7–8	0·1	1·0	(2.10^{-4})

The table shows that acetoacetate formed in neutral solutions, as in our experiments with tissues, will not be appreciably decomposed during experiments of two hours' duration, by the homogeneous process here considered.

For the estimation of acetoacetic acid, the neutral buffered solutions used in tissue experiments must be acidified to a p_H of 5 or below before adding the aniline catalyst in order to avoid retention of carbon dioxide by the medium. The table shows that the solution can be kept at 37° for 15 min. at p_H 4·5 with less than 1 % loss of acetoacetic acid.

We have therefore usually employed the following technique. At the end of the experiment, the tissue slices are removed from the manometer vessels. The residual solution, usually 3·2 ml., is at once acidified by adding 0·3 ml. of a suitable solution of acetic acid or acetic-acetate buffer, which brings it to a p_H near 4·5 (B.D.H. "4·5" indicator may be used). 0·2 ml. of a solution of aniline hydrochloride containing 0·09 g. of the salt is run into a side-tube of the vessel[1]. The vessel, attached to its manometer with air as gas phase, is then shaken for 15 min. in the thermostat at 37° before the manometer is read and the aniline salt is added to the solution containing acetoacetic acid. The acetoacetic acid is now decomposed according to a unimolecular law, the precise rate depending on the buffering of the solution, which determines the p_H it assumes on addition of the more acid aniline hydrochloride solution. According to Widmark and Jeppsson [1922] the optimum p_H for the reaction is about 4·1, but we have not troubled to choose conditions which are exactly optimum. Under our conditions the acetoacetic acid is half decomposed in 5–6 min. and after 50 min. evolution of gas may be considered complete.

Addition of the concentrated aniline hydrochloride to solutions containing no acetoacetic acid results in evolution of gas (some 8 mm. of Brodie's fluid or 12μl. of gas under the conditions described), presumably due to expulsion of air and a "blank" correction, which need not be determined in every experiment, must be applied.

In tissue experiments where no acetoacetic acid was expected to be formed, the pressure changes obtained when corrected for "the blank" have sometimes given slightly negative values. This absorption is probably due to a very small oxygen uptake by tissue débris or extract, which continues after addition of aniline. Its magnitude has always been less than about 8 μl. and it has been neglected.

The CO_2 outputs from sodium acetoacetate solutions made by hydrolysing known amounts of ethyl acetoacetate have been measured and values up to 97–98 % of the theoretical have been obtained. Since the acetoacetate solutions are unstable even when kept at 0°, we take this to indicate a real and exact equivalence between CO_2 output and acetoacetic acid.

[1] If a side-tube of the vessel previously contained alkali (to absorb CO_2), the alkali is removed with filter-paper, and the side-tube rinsed with dilute acid and roughly dried, employing filter-paper again.

The method is suitable for measuring quantities of acetoacetic acid which give 100 μl. of CO_2 and upwards. For 10–20 μl. the accuracy is doubtful, but the errors will probably be of a similar order in estimations made in any one experiment.

A question to be considered is whether for the purposes of this work the manometric method is specific for acetoacetic acid, or whether other substances, and in particular other β-keto-acids, will react similarly with aniline.

Oxaloacetic acid reacts with aniline, but much more rapidly [Ostern, 1933], so that if quantities appreciable in relation to acetoacetic acid were present they could be detected by a rapid fall in the unimolecular constant of the reaction. No such indications of the formation of oxaloacetic acid have been found in this work.

This work, together with that of Quastel and Wheatley [1933; 1934], is apparently the first in which the formation of acetoacetic acid from fatty acids by the liver has been measured as such. Embden and Marx [1908] distilled their solutions after each experiment and obtained in the distillate an iodoform-yielding substance, presumed to be acetone and in some cases identified as acetone. Embden and Engel [1908] showed in some cases that the iodoform-yielding substance was preformed in the solutions only to a small extent and was mainly formed on heating. There was thus a strong presumption that acetoacetic acid is the product chiefly in question, but conceivably, as Hurtley pointed out [1916], it might be acetonedicarboxylic acid.

Respiration and acetoacetic acid production.

In Fig. 1 are given curves showing the manner in which the rate of acetoacetic acid production (Q_{Ac}) by liver slices varies with the concentration of the substrate in the medium. In the absence of added substrate, Q_{Ac} has a small but

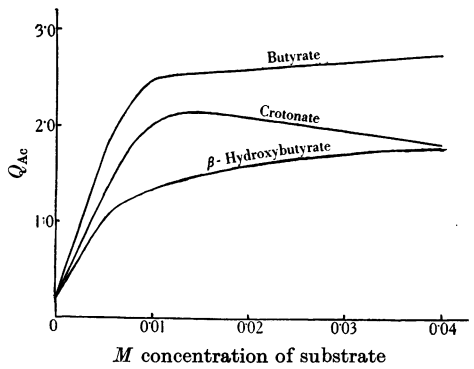


Fig. 1. Rates of acetoacetic acid production by guinea-pig liver. [Oxygen; glycerophosphate buffer.]

definite value. At low concentrations of fatty acid, Q_{Ac} increases approximately proportionally to the concentration. With butyric acid, Q_{Ac} reaches almost its highest value at a concentration of 0·01 M, with β-hydroxybutyric acid the saturation concentration is more nearly 0·03 or 0·04 M. With crotonic acid, however, Q_{Ac} passes through a maximum at a concentration of about 0·015–0·02 M. The curves are smoothed curves drawn by combining the results of several experiments.

The behaviour of butyric and β-hydroxybutyric acids is normal, whilst it appears that crotonic acid in some manner inhibits its own oxidation at high concentrations.

Influence of ionic environment on acetoacetic acid production.

The rate of production of acetoacetic acid is also influenced by the concentration of potassium and calcium ions in the medium, at least in the case of butyric and crotonic acids. Experiments are given in Table I showing the effect of varying (*a*) the potassium ion concentration, (*b*) the calcium ion concentration and (*c*) both potassium and calcium ion concentrations.

Table I. *Effect of varying potassium and calcium ion concentrations.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

		Concentrations (*M*)			
Exp.	Substrate (*M*)	$[K^+]$	$[Ca^{++}]$	Q_{O_2}	Q_{Ac}
1	Butyrate 0·01	0	0·001	7·43	2·52
		0·001	0·001	8·37	2·98
		0·002	0·001	9·13	3·10
		0·005	0·001	9·20	2·99
2	Butyrate 0·01	0·002	0	8·56	1·98
		0·002	0·001	8·70	2·93
		0·002	0·002	8·01	2·50
		0·002	0·004	7·86	2·54
3	Butyrate 0·01	0	0	7·24	1·64
		0·001	0·0005	9·50	3·32
		0·002	0·001	9·13	3·20
		0·005	0·0025	8·85	2·71
4	Crotonate 0·02	0·002	0	7·37	1·69
		0·002	0·0005	7·89	2·28
		0·002	0·001	7·92	2·35
		0·002	0·002	7·44	2·22
5	Crotonate 0·01	0	0	5·88	1·83
		0·0005	0·00025	7·57	2·34
		0·002	0·001	7·77	2·58
6	β-Hydroxybutyrate 0·01	0	0	4·25	1·46
		0·0006	0·0003	5·03	1·20
		0·002	0·001	4·89	1·39

The experiments show that with crotonic and butyric acids Q_{Ac} has optimum values when $[K^+]$ is about $0{\cdot}002M$ and $[Ca^{++}]$ is about $0{\cdot}001M$. These concentrations of potassium and calcium ions have therefore been maintained in the media throughout the rest of this work.

On the other hand, the production of acetoacetic acid from β-hydroxybutyric acid is not definitely influenced by the ionic environment.

It has been found that the rate of acetoacetic acid production from butyrate is approximately the same in a glycerophosphate medium as in Ringer's solution, when the initial p_H of both media is 7·4. It is therefore unlikely that β-glycerophosphate has any great specific action on the oxidation of fatty acids.

Effect of p_H variation on acetoacetic acid formation.

The effect of varying the p_H of the medium is shown in Table II, where in the several vessels employed in each experiment the same mixture of O_2 and CO_2 is present, and the bicarbonate concentration is varied. Bicarbonate-CO_2 here replaces glycerophosphate as buffer. The experiments show that, with all three

Table II. *Effect of* p_H *on rate of production of acetoacetic acid by guinea-pig liver.*

O_2+CO_2. p_H varied by variation of bicarbonate concentration and calculated roughly to midpoint of experiment by assuming $Q_{CO_2}=3{\cdot}5$.

Exp.	Substrate	Initial % CO_2	Relative values for Q_{Ac} at p_H 6·8	7·1	7·4	7·7	8·0
1	Butyrate 0·01 *M*	5	60	84	100	97	—
2	Butyrate 0·01 *M*	1·3	—	89	100	87	70
3	β-Hydroxybutyrate 0·01 *M*	5	54	72	100	78	—
4	β-Hydroxybutyrate 0·02 *M*	1·5	—	89	94	100	95
5	Crotonate 0·02 *M*	5	57	83	81	100	—
6	Crotonate 0·02 *M*	5	58	70	91	100	—
7	Crotonate 0·02 *M*	1·3	—	65	89	100	95
8	Crotonate 0·02 *M*	1·3	—	72	85	100	98

Hence approximate optimum p_H values are: butyrate 7·4–7·6; β-hydroxybutyrate 7·4–7·7; crotonate 7·7–7·9.

acids, the rate of formation of acetoacetic acid varies considerably with the p_H, the variation being of the same order as is shown in glycolysis [Warburg *et al.*, 1924].

In the case of butyrate and β-hydroxybutyrate, Q_{Ac} is maximum at about the physiological p_H, whilst in the case of crotonate the optimum is displaced definitely to the alkaline side.

Mean rates of acetoacetic acid formation.

In Table III are assembled data showing the mean rates of acetoacetic acid formation from the three acids. It is evident that the highest rate is given by butyric acid, followed closely by crotonic acid, whilst the rate from β-hydroxybutyric acid is appreciably lower. The rate of acetoacetic acid formation by the

Table III. *Mean rates of acetoacetic acid formation.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Substrate	Conc. (*M*)	No. of experiments	Range of values of Q_{Ac}	Mean Q_{Ac}	Average deviation from mean
—	—	42	0·00–0·68	0·20	±0·07
Butyrate	0·01	39	1·09–4·44	2·55	±0·46
Crotonate	0·01	14	0·79–3·02	2·21	±0·53
Crotonate	0·02	21	1·27–3·25	2·41	±0·36
dl-β-Hydroxybutyrate	0·01	10	1·15–1·99	1·43	±0·17
dl-β-Hydroxybutyrate	0·02	6	1·53–2·44	1·91	±0·27
	Animal starved 24 hours before killing.				
Butyrate	0·01	7	1·48–3·92	3·05	±0·48

Effect of size of liver on metabolism.

Wt. liver as % wt. animal	No. of experiments	Range of values	Mean values	Average deviation from mean
		Q_{Ac} from butyrate 0·01 *M*.		
2·6–3·6	12	1·97–3·67	2·61	±0·35
3·7–4·6	15	1·85–4·44	2·74	±0·45
4·7–6·9	11	1·09–2·82	2·14	±0·41
		Q_{O_2} without added substrates.		
2·8–3·6	13	3·67–5·93	4·84	±0·62
3·7–4·4	20	4·21–5·52	4·96	±0·35
4·5–7·2	20	3·85–6·84	5·39	±0·75

liver in absence of added fatty acid is low, too low for accurate measurement, and can for most purposes be neglected in considering the acetoacetic acid formation in presence of added fatty acid.

Q_{Ac} varies considerably from one liver to another. Experiments seem to indicate that the state of nutrition plays a part, for Q_{Ac} in the case of butyrate as substrate appears to be higher when the animal has been starved for 24 hours before being killed. This type of effect, which requires further investigation, may throw light on the question whether fatty acid substrates compete with carbohydrates for oxidation, or whether, as is sometimes suggested, "fats burn in the fire of the carbohydrates".

The size of the liver in young guinea-pigs is very variable. In considering this matter, we use the weight of the liver (wet weight) expressed as a percentage of the weight of the animal. The figures for 120 animals show a variation of this percentage between 2·6 and 7·2; 50% of the figures lie between 3·6 and 4·5, 75% between 3·3 and 5·1, and 90% between 3·1 and 5·6. The data given in Table III show that the weight of the liver is a factor in determining Q_{Ac}. In the case of butyrate as substrate heavy livers give lower values of Q_{Ac}. The same is true for crotonate, for which the data are not given.

Data on the respiration of liver in absence of added substrates have been calculated (Table III), which show that Q_{O_2} does not vary very significantly with the weight of the liver. The tendency is for larger livers to give higher values for Q_{O_2}, but the most definite finding is that the respiration of medium-sized livers is less variable than that of light or heavy livers. Further investigation is desirable to show whether or not the percentage of fat in the liver, which may influence considerably the dry weight (used in calculating metabolic quotients), varies with the size of the liver.

Correlation between acetoacetic acid production and increased respiration.

It is shown in Table IV that, when butyrate or crotonate is present in the medium, the respiration of the tissue is raised. There is found to be a very close correlation between the increase in the rate of production of acetoacetic acid, *i.e.* Q_{Ac}, and the increase in respiration, Q_{O_2}. The ratio between the two increases

Table IV. *Acetoacetic acid formation and oxygen consumption.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

	Without substrate			With substrate		
Exp.	Q_{O_2}	Q_{Ac}	Substrate	Q_{O_2}	Q_{Ac}	Extra Q_{Ac} / Extra Q_{O_2}
1	4·12	0·12	Butyrate 0·01 *M*	6·91	1·69	0·56
2	4·14	0·14	,,	7·10	2·07	0·65
3	4·66	0·18	,,	8·18	2·27	0·60
4	4·37	0·25	,,	8·32	2·71	0·62
5	4·53	0·20	,,	8·68	2·95	0·67
6	4·93	0·24	,,	9·25	3·05	0·65
7	4·66	0·19	,,	9·29	3·39	0·69
8	4·43	0·23	Crotonate 0·01 *M*	6·32	1·58	0·71
9	4·19	0·34	,,	7·22	2·41	0·68
10	5·12	0·18	,,	8·17	2·64	0·81
11	4·75	0·08	,,	8·41	2·65	0·70
12	4·33	0·13	,,	7·67	2·67	0·76
13	5·39	0·25	β-Hydroxybutyrate 0·01 *M*	6·11	1·36	1·5
14	4·66	0·19	,,	5·13	1·46	(2·7)
15	5·79	0·68	,,	6·84	1·99	1·25
16	4·93	0·24	β-Hydroxybutyrate 0·02 *M*	6·88	1·94	0·87
17	5·79	0·68	,,	7·45	2·44	1·06

is constant almost within the experimental error. Under the given conditions the ratio has the mean value 0·63 for butyrate and 0·73 for crotonate. Possibly also in the case of β-hydroxybutyrate the variation does not exceed experimental error.

The value of the ratio, however, depends on the concentration of the substrate (Table V). It is noteworthy that on continuing to increase the substrate concentration beyond the point at which Q_{Ac} has reached its highest value, the respiration continues to rise. There is an indication here that the oxidation of the substrates follows two different paths, one to acetoacetic acid and another to some other product or products.

Table V. *Acetoacetic acid formation and oxygen consumption.*

Guinea-pig liver. Glycerophosphate buffer. Oxygen.

Exp.	Substrate (M)	Q_{O_2}	Q_{Ac}	$\frac{\text{Extra } Q_{Ac}}{\text{Extra } Q_{O_2}}$
1	—	4·33	0·13	—
	Crotonate 0·005	7·26	1·66	0·52
	,, 0·01	7·67	2·67	0·76
	,, 0·02	8·53	2·83	0·64
	,, 0·04	8·64	2·38	0·52
2	Butyrate 0·0025	8·34	0·81	—
	,, 0·005	9·22	1·68	—
	,, 0·01	10·1	2·49	—
	,, 0·02	10·7	2·60	—

Relative rates of acetoacetic acid production from butyric and crotonic acids.

A series of experiments has been made in which the actions of each liver on butyric and crotonic acids have been compared (Table VI). It will be seen that a close parallelism exists between the values of Q_{Ac} observed for the two acids. A liver that gives a low value with one acid gives a low value for the other. Crotonic

Table VI. *Comparative rates of acetoacetic acid formation from butyric and crotonic acids.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.
Butyrate when present is at a concentration of 0·01 M.

Exp.	Conc. of crotonate when present (M)	Q_{Ac} in presence of Crotonate	Q_{Ac} in presence of Butyrate	Ratio	Mean ratio
1	0·01	0·79	1·09	0·72	
2	,,	1·37	1·67	0·82	
3	,,	1·57	2·01	0·78	
4	,,	2·10	2·77	0·76	
5	,,	2·20	2·32	0·95	
6	,,	2·62	3·12	0·84	0·81
7	0·015	1·72	1·85	0·93	
8	,,	1·91	2·24	0·85	
9	,,	2·12	2·32	0·91	0·90
10	0·02	1·96	2·02	0·97	
11	,,	2·23	2·43	0·92	
12	,,	2·33	2·50	0·93	
13	,,	2·34	2·77	0·84	
14	,,	2·36	2·45	0·96	
15	,,	2·43	2·73	0·89	
16	,,	2·46	2·81	0·88	
17	,,	2·87	3·27	0·88	
18	,,	3·25	3·67	0·89	0·91

acid gives lower values for Q_{Ac} than does butyric acid, and at a given concentration the ratio of the values of Q_{Ac} is almost constant, having a maximum value (0·9) at the concentration of crotonate which gives maximum values of Q_{Ac} (0·015–0·02 M, Fig. 1). This close parallelism between the values of Q_{Ac} is a strong indication of some similarity in the mechanism of oxidation of the two acids.

Competition between substrates.

Results are given in Table VII of some experiments on the respiration and acetoacetic production when two of the acids are present together.

When β-hydroxybutyrate and butyrate are present together, the rates of production of acetoacetic acid from the two substances are not additive. Yet

Table VII. *Competition between the acids as substrates.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

	Concentration (M) in medium of				
Exp.	Butyrate	Crotonate	β-Hydroxy-butyrate	Q_{O_2}	Q_{Ac}
1	—	—	—	4·66	0·19
	—	—	0·01	5·13	1·46
	0·01	—	—	9·29	3·39
	0·01	—	0·01	9·52	3·93
2	—	—	—	4·93	0·24
	—	—	0·02	6·88	1·94
	0·01	—	—	9·25	3·05
	0·01	—	0·02	9·57	3·59
3	0·01	—	—	11·4	4·44
	0·01	0·01	—	11·0	3·82
	0·01	0·02	—	11·5	3·59
	0·01	0·04	—	10·8	2·71
4	—	—	—	4·19	0·34
	0·01	—	—	8·45	2·80
	—	0·01	—	7·22	2·41
	0·01	0·01	—	7·58	2·35

although Q_{Ac} from butyrate is approximately maximum, the value of Q_{Ac} can be raised by the presence of β-hydroxybutyrate. It is evident, therefore, that the production of acetoacetic acid from these two substances may proceed, at least partially, by quite independent paths.

On the other hand, when crotonate is added to butyrate, Q_{Ac} is lowered. The experiments agree with the view that, when both acids are present, crotonate exerts on the oxidation of butyrate the same inhibitory action that it exerts on its own oxidation at sufficiently high concentrations. There is no evidence that the acids are oxidised to acetoacetic acid by independent paths.

It may be mentioned here that under anaerobic conditions guinea-pig liver does not oxidise butyric, crotonic or β-hydroxybutyric acid appreciably to acetoacetic acid. This was shown by experiments similar to those already recorded, except that nitrogen replaced oxygen in the gas phase. The highest value of Q_{Ac} found was 0·14, which does not exceed the experimental error.

The action of inhibitors.

The action of a number of substances likely to inhibit the oxidation of one or more of the fatty acids was investigated largely with a view to finding differential effects on one or more of the acids, which might give information regarding the mechanisms of the oxidations.

It will be seen (Table VIII) that among the substances examined are several unsaturated compounds, which, it was thought, might inhibit particularly the oxidation of crotonic acid. This however was not found to be the case, although there is one possible exception.

With all substances examined which cause much inhibition of acetoacetic acid formation, the effects on crotonic acid and butyric acid are very similar, with the qualification that as a rule the inhibition is slightly greater for crotonic acid (Table VIII).

The inhibitory action of propionate on oxidation of butyrate had already been found by Quastel and Wheatley [1933]. It is now found that oxidation of crotonate is inhibited to a similar extent, whilst that of β-hydroxybutyrate is only very slightly lowered. Propionate is itself oxidised by the liver without acetoacetic acid formation.

Acrylate[1] exerts an inhibitory action on acetoacetic acid formation similar to, but weaker than, that of propionate and like propionate increases the respiration of guinea-pig liver, but to a smaller extent.

Allyl alcohol, already known as a powerful respiratory poison, inhibits the respiration of liver strongly, its action being progressive with time. It lowers Q_{Ac} in presence of crotonate and butyrate still more strongly, inhibition being still in evidence at a concentration of $0{\cdot}0003\,M$. Observations suggest that at low concentrations the alcohol is strongly taken up by the tissue, since the inhibition seems to depend on the weight of tissue present. Allyl alcohol is not therefore convenient for quantitative experiments.

Several powerful inhibitors have been found in the aromatic series. Cinnamic, β-phenylpropionic and benzoic acids all cause considerable inhibitions of acetoacetic acid formation from butyric and crotonic acids at concentrations of $0{\cdot}0005\,M$, the inhibition of oxidation of crotonic acid being greater than of butyric acid. The oxidation of β-hydroxybutyric acid is much less inhibited. The aromatic acids mentioned above do not diminish the respiration of the liver in presence of the fatty acids to a greater extent than would be expected from the diminution in production of acetoacetic acid, *i.e.* on the basis of the relation between respiration and acetoacetic acid production which has already been found (Table IV). The diminution in respiration is in fact usually less than would be expected from the ratios previously given. As far as our experiments go, they indicate that weak solutions of the aromatic acids in question affect no other respiratory processes in the liver than the oxidation of fatty acids to acetoacetic acid.

Our experiments give no indication that these aromatic acids are themselves undergoing appreciable destruction in the liver, as according to the work of Knoop and Dakin they do in the intact organism. If the acids disappeared during our experiments, it might be expected that their inhibitory effect on respiration (due to their inhibitory effect on fatty acid oxidation) would be lessened, and an increase would take place in the respiration relative to the "control". No such phenomenon was observed. It was also observed in one experiment (Table VIII) that the inhibitory effect of benzoate on acetoacetic acid production is independent of the weight of tissue present, which indicates that benzoate is neither very greatly adsorbed nor destroyed by quantities of tissue such as we employ in our work.

Tropic (α-phenyl-β-hydroxypropionic) acid is much less toxic to acetoacetic acid production than are cinnamic and β-phenylpropionic acids.

Fluoride is found to be a strong inhibitor of acetoacetic acid formation from

[1] Not a pure preparation.

Table VIII. *Action of inhibitors.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

	% decrease in Q_{Ac} due to inhibitor, Substrate (0·01 *M*)		
	Butyrate	Crotonate	β-Hydroxybutyrate
Effect of propionate (0·01 *M*)			
Exp. 1	47	—	9
„ 2	—	63	6
„ 3	71	74	—
Effect of acrylate (0·02 *M*)	43	57	6

	0·02 *M* crotonate			
Allyl alcohol (*M*) ...	0·04	0·006	0·001	0·0003
Effect of allyl alcohol				
% decrease in Q_{O_2}	80	75	59	3
% decrease in Q_{Ac}	100	100	94	23

	% decrease in Q_{Ac}, Substrate (0·01 *M*)		
Conc. (*M*)	Butyrate	Crotonate	β-Hydroxybutyrate
Effect of cinnamate			
0·01	—	84	—
0·005	72	83	23
0·002	71	87	—
0·0005	63	88	11
0·0001	18	32	(− 9)
Effect of β-phenylpropionate			
0·0005	54	70	—
Effect of benzoate			
0·01	—	90	—
0·0002	—	78	—
0·001	40	63	
0·0005	—	39	—
Effect of tropate			
0·02	22	—	10
0·005	9	—	25

	% decrease in Q_{Ac}	
	Crotonate	Butyrate
Effect of fluoride		
0·02*	90	—
0·01	82	—
0·005	75	—
0·0031	—	70
0·003	68	—
0·0025	75	79, 66
0·0015	31	—
0·00075	18	—

	% effect on Q_{Ac}	
	Butyrate	Crotonate
Effect of fumarate		
0·02–0·05	+12	− 9
	+ 4	−18
	+ 2	− 8

* In this solution calcium fluoride precipitated at room temperature, which was not observed in weaker solutions.

Table VIII (*cont.*).

Inhibition in relation to weight of tissue slices.

Dry wt. tissue mg.	Conc. of benzoate *M*	Q_{Ac} in presence of butyrate	% inhibition
28·9	0	1·69	—
15·6	0·002	1·13	33
19·8	0·002	1·12	34
44·2	0·002	1·10	35

crotonic and butyric acids. The effect persists down to concentrations of 0·001 *M*. The stronger solutions of fluoride have also a considerable and progressive inhibitory action on respiration, but weaker solutions (say 0·0025 *M*) inhibit respiration no more than would be expected from their effect in diminishing oxidation of the fatty acids. The action of fluoride in weak solutions is probably therefore fairly specific. The inhibitory action on acetoacetic acid formation is exerted at concentrations similar to those at which fluoride inhibits glycolysis [Dickens and Šimer, 1929].

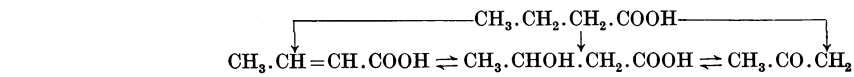
A slight differential effect on the production of acetoacetic acid from butyric acid and crotonic acid is shown by strong solutions of fumarate. Fumarate slightly increases Q_{Ac} in presence of butyrate and decreases it slightly in presence of crotonate. This is the only case we have observed where a substance affects the rates in opposite directions, but the effect does not lie much outside experimental error. Fumarate is itself oxidised by guinea-pig liver.

The mechanisms of the oxidations.

It has frequently been suggested that the mechanisms of oxidation of butyric, crotonic and β-hydroxybutyric acids to acetoacetic acid in the liver are related. Friedmann [1908] suggested that β-hydroxybutyric acid occupied an intermediary place between crotonic and acetoacetic acids, and Hurtley [1916] proposed the scheme:

Butyric → crotonic → β-hydroxybutyric → acetoacetic acid.

The most elaborate scheme, which Dakin appears to have favoured [1922], allows three different paths for butyric acid:



The arguments used in favour of these various mechanisms are mainly chemical analogies of more or less value, and none of them is decisive.

Our experiments with inhibitors (propionate and cinnamate, Table VIII) show that the oxidation of β-hydroxybutyric acid is hardly affected by conditions which inhibit considerably the oxidation of butyric and crotonic acids to acetoacetic acid. We can conclude with certainty that neither butyric nor crotonic acid is an intermediary in the oxidation of β-hydroxybutyric acid to acetoacetic acid by guinea-pig liver.

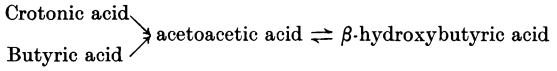
The discovery by Quastel and Wheatley [1934] that ascorbic acid has little effect on acetoacetic acid production by rat liver from β-hydroxybutyric acid, whilst it accelerates acetoacetic acid production from butyric and crotonic acids, provides some evidence that the same statement is true for rat liver.

Let us next consider the view, advocated by several authors, that β-hydroxybutyric acid is an intermediary in the oxidation of butyric and crotonic acids to acetoacetic acid. The only clear evidence we have found in the literature bearing

on this view tells against it, this being the evidence due to Marriott [1914], who stated that when butyrate was injected into a fasting dog a rise in the aceto-acetic acid content of the blood preceded a rise in the β-hydroxybutyric acid content.

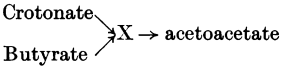
Our own experiments are also very definitely against the view that β-hydroxy-butyric acid is an intermediary. In the first place, we have found some additivity between the acetoacetic acid production from butyric acid and β-hydroxy-butyric acid (Table VII). In the second place, we find that the rate of acetoacetic acid production from β-hydroxybutyric acid is definitely lower than from crotonic or butyric acid (Fig. 1 and Table III). An examination of the curves and figures provides quite decisive evidence that β-hydroxybutyric acid cannot be an obligate intermediary between crotonic or butyric acid on the one hand and acetoacetic acid on the other. It remains possible that a part of the crotonic or butyric acid passes through β-hydroxybutyric acid as an intermediary, but there is little need to complicate our views with this possibility, for which there is no positive evidence.

Another view which has been advanced is that crotonic and β-hydroxy-butyric acids are interconvertible directly by addition or loss of water. We are aware of no evidence which is inconsistent with the alternative view that in the liver crotonic acid is first oxidised to acetoacetic acid and then reduced to β-hydroxybutyric acid. We prefer the second view, which accounts for the fact that the conversion of crotonic acid into β-hydroxybutyric acid by minced dog liver [Friedmann and Maase, 1913] requires the presence of oxygen. Our conclusions up to the present may be summarised by the scheme:



We have not yet considered the evidence that acetoacetic acid is convertible into β-hydroxybutyric acid by the liver but may state that adequate evidence is available.

We turn next to a more difficult problem, the relation between the mechanisms of oxidation of butyric and crotonic acids to acetoacetic acid. Our experiments have shown numerous similarities between the rates of the processes and make it fairly certain that a relation exists. For instance, a close numerical relation is found between the rates in different livers (Table VI), which would hardly be likely to exist if the processes were entirely distinct. The reactions are also competitive—when both acids are present the rate is never higher than when one is present alone (Table VII), which is an indication that the modes of oxidation have some feature in common. We will consider the problem first on the usual lines of classical kinetics, according to which if a relation exists, it must, we consider, take one of the three forms:

1. Butyrate → crotonate → acetoacetate.
2. Crotonate → butyrate → acetoacetate.
3. 

The weight of the evidence is against the first possibility. Under all circumstances the rate of formation of acetoacetic acid from butyric acid is greater than that from crotonic acid when both are present at their optimum concentrations. This is shown by numerous figures already given, *e.g.* in Table VI. The effects of inhibitors (Table VIII) on the oxidation of crotonic acid tend to be greater than

on that of butyric acid, which cannot be explained on the first scheme. Nor would it be anticipated that a higher concentration of crotonic acid than of butyric acid would be required to attain the optimum rate.

The second scheme, namely

crotonate → butyrate → acetoacetate

is much more plausible, for the rate of oxidation of butyrate to acetoacetate is always faster than that of crotonate.

One set of facts causes apparent difficulty. Reference to Table II shows that at p_H 7·7–8·0 the rate of oxidation of crotonic acid rises above the level at p_H 7·4, whilst the rate for butyric acid falls below the level at p_H 7·4. Setting the values for p_H 7·4 at 100 for butyric acid and 90 for crotonic acid, values true for a glycerophosphate medium (Table III), it appears that at p_H 7·7–8·0 the rate of oxidation of crotonic acid should be greater than that of butyric acid. But direct comparison of the rates of oxidation of the two acids to acetoacetic acid, at p_H 7·9 in Ringer's solution, shows that even under these conditions butyric acid is oxidised the more rapidly.

Crotonate, however, exerts a secondary inhibitory action on its own oxidation and also on that of butyrate (Table VII), the rate of oxidation of which is lowered to that of crotonate. Taking into account this inhibitory effect, we consider it probable that crotonate is oxidised as fast as butyrate, when both are present at optimum concentrations. We must suppose, therefore, that crotonate is reduced quickly enough to saturate the butyrate-oxidising enzyme, a supposition for which there is no evidence.

It might have been expected that the reduction of crotonate to butyrate would be inhibited by substances that do not affect the oxidation of butyrate. We have, however, found no very striking evidence in favour of greater inhibition of crotonate oxidation than of butyrate oxidation, although certain evidence in this direction exists.

We conclude that this mechanism may be correct, but the evidence is insufficient to establish it.

We turn to the third scheme, which represents the possibility that crotonate and butyrate are oxidised to acetoacetate through a common intermediary. It has already been shown that this cannot be β-hydroxybutyrate, and no other substance suggests itself as a likely intermediary.

In these three mechanisms which have just been considered, the intermediary substances have been considered to be formed as ordinary chemical individuals. A fourth mechanism can be proposed, according to which both crotonate and butyrate are adsorbed by the same enzyme, and both are transformed to acetoacetate while remaining adsorbed on the enzyme surface. In such a process the intermediary stages will not exist as separate molecules, but only in combination with the enzyme. It may be suggested that the reactions which take place are as follows:

$$CH_3.CH_2.CH_2.COO' \rightarrow CH_3.\overset{\times}{C}.CH_2.COO' + 2H$$

$$CH_3.CH=CH.COO' \rightarrow CH_3.\overset{\times}{C}.CH_2.COO'$$

$$CH_3.\overset{\times}{C}.CH_2.COO' \xrightarrow[\text{or H acceptor}]{\text{active O}} CH_3.CO.CH_2.COO'$$

A similar method of representing the activation of butyric acid has already been put forward by Quastel [1926]. According to this view, butyrate is desaturated to a product identical with an activated crotonate molecule. The theory is

able to explain the same facts as the second scheme, and also accounts for our failure to find substances which inhibit the oxidation of crotonate to any definite extent without also inhibiting oxidation of butyrate. On the basis of the theory, the facts indicate that crotonate has a lower affinity for the enzyme than has butyrate. We should account most readily for the approximate equality of rates of oxidation of the two acids by assuming that the second stage in the process, which is identical for the two acids, is a slower reaction than the first stage. The evidence is insufficient to decide between the second and fourth schemes proposed. We, however, favour the latter, which in its suggestion of a process taking place entirely on the surface of one enzyme is in line with certain facts related to the oxidation of higher fatty acids, which are discussed in the following paper.

Summary.

1. The rates of oxidation of butyric, crotonic and *dl*-β-hydroxybutyric acids to acetoacetic acid by slices of guinea-pig liver in the presence of oxygen have been investigated.

2. The rate of acetoacetic acid production (Q_{Ac}) varies in the normal manner as a function of substrate concentration with butyric and β-hydroxybutyric acids, but passes through a maximum value when crotonic acid is the substrate.

3. The rate of acetoacetic acid production from butyric and crotonic acids varies with the potassium and calcium ion concentrations in the medium and passes through a maximum value.

4. The optimum p_H for the oxidation is close to the physiological value for butyric and β-hydroxybutyric acids but lies a little to the alkaline side (7·7–7·9) for crotonic acid.

5. Q_{Ac} in presence of butyrate is slightly greater than in presence of crotonate under optimum conditions. The ratio of the rates is fairly constant when measured under definite conditions. Q_{Ac} in presence of β-hydroxybutyrate is considerably lower.

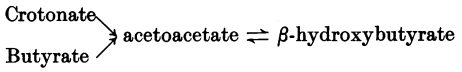
6. There is a definite correlation between the increase in respiration and the increase in Q_{Ac} brought about by the acids.

7. Experiments with mixtures of the acids show that there is competition between butyric and crotonic acids for oxidation, but partial additivity with β-hydroxybutyric acid.

8. Benzoate, cinnamate and phenylpropionate inhibit strongly the oxidation of butyric and crotonic acids to acetoacetic acid. At low concentrations (0·001 M) they appear to inhibit specifically the oxidation of fatty acids.

9. Cinnamate and propionate inhibit the oxidation of β-hydroxybutyric acid to a much smaller extent than the oxidations of butyric and crotonic acids.

10. The evidence supports the scheme of reaction:



11. It is possible that crotonate passes through butyrate as an intermediary. It is rather more probable that the process of oxidation to acetoacetate takes place at one and the same enzyme, which effects the complete process with both butyrate and crotonate.

One of the authors (M. J.) wishes to express his thanks to the Medical Research Council for a personal grant. The authors also wish to thank this body for a grant in aid of equipment.

REFERENCES.

Dakin (1922). Oxidations and reductions in the animal body, 2nd ed. (Longmans, Green and Co., London.)
Dickens and Šimer (1929). *Biochem. J.* **23**, 936.
Embden and Marx (1908). *Beitr. chem. Physiol. Path.* **11**, 318.
—— and Engel (1908). *Beitr. chem. Physiol. Path.* **11**, 323.
Friedmann (1908). *Beitr. chem. Physiol. Path.* **11**, 371.
—— and Maase (1913). *Biochem. Z.* **55**, 450.
Hurtley (1916). *Quart. J. Med.* **9**, 301.
Marriott (1914). *J. Biol. Chem.* **18**, 241.
Meyerhof and Kiessling (1933). *Biochem. Z.* **267**, 315.
Ostern (1933). *Z. physiol. Chem.* **218**, 160.
Quastel (1926). *Biochem. J.* **20**, 166.
—— and Wheatley (1933). *Biochem. J.* **27**, 1753.
—— —— (1934). *Biochem. J.* **28**, 1014.
Warburg (1926). Über den Stoffwechsel der Tumoren. (Berlin.)
—— Posener and Negelein (1924). *Biochem. Z.* **152**, 309.
Widmark (1920). *Acta Med. Scand.* **53**, 393.
—— and Jeppsson (1922). *Skand. Arch. Physiol.* **42**, 43.