FATTY ACID OXIDATION BY LIVER ENZYMES

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The enzymes which oxidize lower saturated fatty acids are difficult to study because of their great instability. It has been found (1) that butyric acid can be oxidized by liver tissue even after the cell structure has been destroyed. The oxidizing system is inactivated more rapidly in the absence of oxygen, and the addition of fumarate increases the rate of oxidation.

An enzyme preparation from rat liver, which oxidizes higher fatty acids, was described by Lang (2). Adenylic acid acted as a coenzyme and the reaction product was the corresponding unsaturated acid (3, 4). Previous work has been reviewed by von Euler (5) and Lang (2).

Further work on the oxidation of butyric acid and of other acids by washed preparations of liver has revealed the identity of some of the components of the oxidizing system.

EXPERIMENTAL

Methods—Butyric acid was estimated as previously described (1) after precipitation of the proteins with zinc sulfate and sodium hydroxide. Alkaline evaporation was omitted. Phosphate was estimated according to Fiske and Subbarow (6) after deproteinization with 1 volume of cold 5 per cent trichloroacetic acid. The phosphate liberated in 7 and 60 minutes by 1 N hydrochloric acid at 100° and by hypoiodite, according to Lohmann and Meyerhof (7), was also estimated. Oxygen uptake was measured with Warburg manometers.

Adenylic acid was prepared as described by Lohmann (8). Cytochrome from muscle extract (9) was dried with 4 volumes of cold acetone and kept in a desiccator (about 10 per cent pure). Solutions in 0.1 M sodium chloride were prepared each day.

Experiments were carried out in Erlenmeyer flasks with shaking at 25° and in air.

Inactivation of Enzyme System—Homogenized liver tissue is inactivated more rapidly in the absence of oxygen (1). Before an attempt was made to purify the system, it was considered convenient to find out whether oxygen acted on the coenzymes or on the enzyme proper. The experiment in Table I shows that the preparation which becomes inactive after 10 minutes anaerobically is reactivated by a heated extract of liver.

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If more time is allowed to elapse, the enzyme becomes inactive with or without oxygen and is not reactivated by the heated extract. However, this length of time is sufficient to obtain a partial purification if the temperature is kept low.

Preparation of Enzyme—Guinea pig liver removed immediately after death was washed with ice-cold water, cut with scissors, and again washed. The tissue was then transferred to a stainless steel tube closed at one end (2.5 × 25 cm.) containing 4 volumes of water. A steel ball (diameter 0.5 mm. smaller than the tube) fixed at the end of a rod was forced into the tube and moved vertically. After 60 double excursions of the ball an adequate rupture of the cells was obtained. The whole procedure was carried out at the lowest possible temperature without freezing. After 0.1 volume of 1 M magnesium chloride was added, the preparation was

TABLE I

Influence of Oxygen on Stability of System

Rat liver was homogenized in 1 volume of 0.1 M sodium chloride and filtered through muslin. One-half was kept at 25° in oxygen, the other half in a test-tube without oxygen during 10 minutes. 2 ml. portions were added to Erlenmeyer flasks containing 0.2 ml. of 1 M fumarate + 2 ml. of water or heated liver extract (5 minutes at 80° in 1 volume of water). Total volume, 4.2 ml.

Treatment of homogenized liver	Butyrate disappearance
	micromoles
Kept in O_2	8.7
" " " + heated extract	11.3
Without "	0.6
" " + heated extract	10.7

filtered through muslin and centrifuged 5 minutes at 6000 R.P.M. in a cooled centrifuge. The precipitate was suspended in 3 volumes of water, homogenized, and 0.1 volume of magnesium chloride was added. The suspension was again centrifuged and the washing repeated twice more. The precipitate after the fourth centrifugation was suspended in 3 to 4 volumes of 0.1 M fumarate or 0.03 M sodium chloride according to the experiments. The whole procedure lasted about 40 minutes. The activity and degree of purity of the preparation depend on the degree of cell rupture, on the volume of liquid used in washing, on the temperature, and on the time the preparation remains suspended in pure water.

If the cell rupture is insufficient, a clean preparation is obtained only after many washings. If it is excessive, the preparation becomes inactive.

The suspension of the precipitate in water produces not only hemolysis and the removal of many cell substances but also produces inactivation in

a few minutes. To prevent this inactivation only 20 to 40 seconds should elapse between the suspension in water and the addition of the magnesium chloride solution. The addition of salts preserves the activity and makes the centrifugation easier. Magnesium chloride is better than sodium chloride because it produces a greater insolubility of some active components.

At 0° the enzyme lasts 2 to 3 hours, less at a higher temperature. Freezing produces complete inactivation.

Owing to all these factors and to the variable composition of the liver, the resulting preparation varies somewhat in activity and purity. With one or two more washings a cleaner preparation is obtained, but the danger of inactivation is increased. Even with the procedure as described inactive preparations are sometimes obtained.

TABLE II

Components of Oxidation System

Disappearance of butyrate produced by 2.5 ml. of liver enzymes + 0.2 ml. of 1 M fumarate + 0.2 ml. of M/15 phosphate buffer of pH 7.7 + 0.1 ml. of 0.1 M magnesium chloride + 0.5 mg. of cytochrome *c* preparation + 1 mg. of adenylic acid + 17.3 micromoles of butyrate. Total volume, 6 ml.; 90 minutes at 25° in air.

	Butyrate disappearance	
	micromoles	*micromoles*
Complete system	8.2	8.9
No phosphate	1.9	2.6
" fumarate	3.0	2.6
" cytochrome *c*	−1.2	−1.2
" adenylic acid	−0.9	−1.0

Components of Oxidation System—The washed tissue is completely inactive by itself, but will oxidize butyrate when a heated extract of liver, kidney, or heart is added. The fractionation of these heated extracts led to the identification of several components, as is shown in Table II.

In the absence of phosphate or fumarate the system showed a slight activity, but not comparable with that of the complete system. In the absence of cytochrome or adenylic acid the preparation was completely inactive.

In these experiments the enzyme was prepared with magnesium chloride. In other experiments in which sodium chloride was used in the preparation it was found that the system was inactive in the absence of magnesium or manganese.

The optimum concentration of phosphate appears to be about 0.01 M, although the results varied somewhat with different preparations. With

0.002 M a slightly smaller disappearance of butyrate was obtained. This was the amount used in the experiments in which phosphate was estimated. The optimum concentration of fumarate under the same conditions was found to be 0.03 M. With greater or smaller concentrations there was a decrease in activity. Malate proved to be much less active than fumarate in increasing the rate of oxidation of butyrate, and succinate was inactive.

With varying amounts of adenylic acid the activity was as follows:

Adenylic acid, *mg*	0	0.2	0.5	1	2	4
Butyrate disappearance, *micromoles*	0	5	9.9	11	11.7	11.7

Adenylic acid can be replaced by adenyl pyrophosphate.

Table III shows the results of an experiment in which the oxygen uptake was also measured.

TABLE III

Components of Oxidation System

The composition of the system was as in Table II. The oxygen uptake was measured in 3 ml. aliquots and calculated for 6 ml. in micromoles of oxygen.

	Oxygen uptake		Butyrate disappearance
	With butyrate	No butyrate	
	micromoles	*micromoles*	*micromoles*
Complete system	45.0	33.2	9.5
No fumarate	2.6	10.0	1.0
" phosphate	30.2	21.2	3.6
" adenylic acid	10.8	11.8	−0.1
" cytochrome	5.0	4.3	0.1

The highest oxygen uptake was found in the complete system with butyrate. However, even without butyrate the oxygen uptake is rather high, owing presumably to the oxidation of fumarate. The relation between oxygen uptake (after subtraction of the blank) and butyrate disappearance is 1.2; *i.e.*, about 1 molecule of oxygen per molecule of butyrate. The butyrate oxidation in the absence of added phosphate was probably due to the impurity of the enzyme which contained 0.6 micromole of inorganic phosphate.

Oxidation of Other Acids—The oxygen uptake of some compounds related to butyrate was measured with and without adenylic acid (Table IV).

Butyrate and crotonate are the most rapidly oxidized. The latter also needs adenylic acid. Isocrotonate also gave an increase in oxygen uptake but smaller than did crotonate; the sample used was not pure and may have contained some crotonate; nevertheless the experiment shows that it is oxidized more slowly than crotonate. β-Hydroxybutyrate gave a small increase even in the absence of adenylic acid.

TABLE IV

Influence of Adenylic Acid on Oxidation of Butyrate and Related Compounds

Oxygen uptake in 30 minutes at 25° by 3 ml. of the complete system as in Table II with or without adenylic acid. Substrate concentration 0.003 M.

Substrate	Adenylic acid	Oxygen uptake
		microliters
None	−	42
"	+	101
dl-β-Hydroxybutyrate	−	53
"	+	126
Crotonate	−	24
"	+	185
Isocrotonate	−	23
"	+	139
Butyrate	+	172

TABLE V

Oxidation of Various Acids

Oxygen uptake in 30 minutes at 25° of 3 ml. of the complete system as in Table II with different acids (concentration, 0.003 M). The figures given represent the uptake in microliters after the uptake with no substrate which is given in the top row is subtracted. A negative sign indicates an oxygen uptake lower than with no substrate.

No substrate	83	94	102	155	107
Formate				−6	−11
Acetate			9	10	
Propionate	−5	−17			15
Lactate			7	6	
Pyruvate				22	−1
Butyrate	63	83	76	49	72
Isobutyrate		6		34	41
α-Bromobutyrate	−14				3
Valerate	50		80		
Isovalerate	2		12		30
Hexanoate		109			80
Heptanoate	58		99		
Octanoate	113	25			
Decanoate			−48	−141	
Dodecanoate			−80	−136	
Stearate		−25			5
Palmitate	−1	−66			
Oleate		−73	−84		

As Table V shows, the oxygen uptake was greatest with butyrate, valerate, hexanoate, heptanoate, and octanoate; less with isobutyrate and

isovalerate. Results with formate, acetate, lactate, pyruvate, propionate, and α-bromobutyrate were doubtful, while decanoate, dodecanoate, stearate, palmitate, and oleate showed a decrease in the oxygen uptake.

Changes in Phosphate—As shown in Table VI, in the complete system without butyrate there occurs an uptake of inorganic phosphate. This uptake is very small in the absence of adenylic acid. In the absence of cytochrome or fumarate there is an increase in inorganic phosphate which is liberated from the enzyme preparation which contains about 7 micromoles per ml. of bound phosphate.

TABLE VI

Changes in Inorganic Phosphate

The complete system was as in Table II. The results are given in micromoles.

	Initial	No butyrate	With butyrate	Butyrate disappearance
Complete system	15.6	6.2	11.8	14.6
No cytochrome	15.6	19.6	20.1	0.4
" fumarate	15.4	20.4	18.2	6.5
" adenylic acid	15.2	14.1	16.4	0.9

TABLE VII

Changes in Phosphate Fractions

The complete system was as in Table II. The results are given in micromoles. Each figure is the mean of duplicate estimations not differing by more than 3 per cent.

Complete oxidation system	Inorganic P		P hydrolyzed in 7 min. in N HCl at 100°	P hydrolyzed in 60 min. in N HCl at 100°	P liberated by hypoiodite	Titration with hypoiodite*	Butyrate disappearance
	Initial	Final					
With butyrate	15.3	16.5	0.9	1.2	0	7.3	7.5
No butyrate	15.0	12.1	1.9	5.7	4.7	12.2	0

* Corresponds to the iodine consumed by the samples in alkaline medium and calculated as pyruvic acid (6 equivalents of iodine per mole of pyruvic acid (7)).

When butyrate is present, the final amount of inorganic phosphate is larger than in the control with no butyrate. The difference is all accounted for by a greater formation of phosphopyruvic acid and of adenyl pyrophosphate, as is shown in Table VII. Tests for labile phosphate of the phosphocreatine type (10) or of the acetyl phosphate type of Lipmann (11) invariably gave negative results. All these experiments have been repeated many times with the same results.

With low concentrations of fumarate or when none is added as in Table VI there occurs a smaller disappearance of butyrate, while the increase in inorganic phosphate does not occur.

With acetate, β-hydroxybutyrate, and acetoacetate no changes in inorganic phosphate occurred, while with propionate, valerate, hexanoate, octanoate, decanoate, and stearate an increase in inorganic phosphate was found as with butyrate.

Inhibitors—Fluoride produced a complete inhibition of the disappearance of butyrate at 0.01 M, and about 80 per cent at 0.002 M. The same inhibition was obtained with iodoacetate. Arsenate also acted as an inhibitor, as did malonate. The latter effect is unexpected, because in liver slices malonate increases the spontaneous formation of ketone bodies and does not inhibit the oxidation of fatty acids. Methylene blue acted as an inhibitor at 0.01 per cent and had no effect at lower concentrations.

Oxidation Product—Many experiments have been carried out in order to measure the amount of acetoacetate and β-hydroxybutyrate formed. Variable results have been obtained, but as a rule hardly any acetoacetate was formed and the amount of β-hydroxybutyrate corresponded approximately to the amount of butyrate which disappeared. The oxygen uptake corrected for the blank corresponded to about 1 molecule per molecule of butyrate. However, owing to the lack of specificity of the method and to the variable results, it is considered that further work is necessary before the results are published.

DISCUSSION

The preparation of enzyme systems which are as labile as that which oxidizes butyrate is important, because it is likely that many physiological reactions are catalyzed by such systems. The procedure which is described may be useful in studies on tissue metabolism even if it yields impure preparations only, and often inactive products.

It is interesting that some of the components of the system which have been identified (fumarate, phosphate, magnesium, and adenylic acid) are also involved in the oxidation of carbohydrates and their derivatives. Perhaps this will give some clue on the relation between fat and carbohydrate metabolism.

The oxidation appears to be in some way coupled with a phosphorylation. Inorganic phosphate, adenylic acid, and magnesium which are known components of phosphorylating systems are needed for the oxidation. The specific inhibitors of phosphorylations (fluoride, iodoacetate) also inhibit the oxidation of butyrate. The changes which occur in the distribution of phosphate are difficult to understand. In the absence of fatty acids, fumarate is transformed into phosphopyruvate or into a substance that gives the same reactions, as has been found by Kalckar (12), and adenylic acid becomes phosphorylated. When butyric acid is oxidized, hardly any phosphopyruvate or adenyl pyrophosphate is formed. This does not appear to be due to an inhibition of fumarate oxidation, because the oxygen

uptake is high. With the other saturated acids (5 to 8 carbon atoms) the results are similar. Propionate, which does not increase the oxygen uptake and disappears at only about 20 per cent of the rate of butyrate, produces the same changes in phosphate as butyrate does. Decanoate and stearate, which decrease the oxygen uptake, also produced these changes.

The oxidation of fumarate appears to be necessary for obtaining the maximum speed of butyrate disappearance. The mechanism of this oxidation has been discussed by Lipmann (13). Succinate is oxidized by the system, but probably fumarate is not formed in sufficient amounts to produce an effect. Malate is less active than fumarate.

Our system does not oxidize higher fatty acids as does the enzyme preparation described by Lang *et al.*, although it contains adenylic acid and the method of preparation does not differ very much from theirs. However, the experimental conditions were slightly different because they measured the reduction time of methylene blue. Furthermore their enzyme is more stable and does not oxidize lower fatty acids; the addition of methylene blue to our enzyme did not enable it to oxidize higher fatty acids nor did it reactivate the enzyme after standing overnight in the ice box.

SUMMARY

The preparation of an enzyme system from liver which oxidizes lower saturated fatty acids is described. Inorganic phosphate, fumarate, cytochrome *c*, adenylic acid, and magnesium or manganese ions were found to be necessary components.

Fluoride, iodoacetate, arsenate, and malonate inhibit the oxidation.

The changes occurring in phosphate distribution have been studied.

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