BUTYRATE OXIDATION BY LIVER ENZYMES

By LUIS F. LELOIR and JUAN M. MUÑOZ

(From the Institute of Physiology of the Faculty of Medical Sciences, Buenos Aires, Argentina)

(Received for publication, November 1, 1943)

It has been found (1) that lower saturated fatty acids are oxidized by a system formed by a preparation of liver enzymes with the addition of adenylic acid, cytochrome *c*, inorganic phosphate, magnesium ions, and fumarate. In the absence of added fatty acids phosphopyruvate or a similar substance accumulated, whereas this did not occur in the presence of fatty acids.

This paper describes experiments which were undertaken in order to study the possibility of replacing fumarate by other components of the citric acid cycle, the formation of phosphopyruvate and its influence on the oxidation of butyrate, and the relation between oxidation products and oxygen uptake.

EXPERIMENTAL

Methods

The general technique was the same as that described previously (1). The concentration of magnesium chloride used for preparing the enzyme was 0.5 M instead of 1 M. Experiments were carried out in 50 cc. Erlenmeyer flasks filled with oxygen and shaken at 80 oscillations per minute. It was found that in air the reaction rate may be limited by the rate of oxygen diffusion.

The following methods of preparation were used: adenylic acid, Lohmann (2); phosphopyruvic acid, Kiessling (3); 3-phosphoglyceric acid, Neuberg and Kobel (4); sodium pyruvate, Robertson (5). The sample of sodium α-glycerophosphate was from The British Drug Houses, Ltd. All substances were added as sodium salts.

Methods of estimation were as follows: phosphate, after Fiske and Subbarow (6); butyrate after Muñoz and Leloir (1); ketone bodies after Edson (7); phosphopyruvate after Lohmann and Meyerhof (8) and by estimating pyruvate before and after 1 hour's hydrolysis in 1 N HCl at 100°. A colorimetric method with 2,4-dinitrophenylhydrazine was used (9).

Some Properties of Enzyme System—The activity of the system is maintained for more than 90 minutes at 25° in oxygen when all the components and substrate are present. If butyrate, adenylic acid, or cytochrome is added after 10 to 15 minutes incubation, there occurs a complete inactiva-

tion. If pyruvate instead of butyrate is added, the activity is maintained. It appears that the system is more stable when certain substrates are being oxidized. Many unsuccessful attempts have been made to stabilize the system by other procedures.

Microscopic examination shows that the preparation contains nuclei and many cell particles.

The preparation appears yellowish white and a spectroscopic examination shows a band at about 560 mμ, corresponding probably to cytochrome *b*, and a fainter band at about 600 mμ, corresponding to cytochrome *a*. No cytochrome *c* is detectable. As these bands appear on reduction, they can be used in ascertaining the activity of the system.

Action of Different Substances on Butyrate Oxidation—It was previously stated (1) that fumarate could not be replaced by succinate. As is shown

TABLE I

Action of Succinate, Malate, Citrate, and Glutamate on Oxidation of Butyrate

Disappearance of butyrate (micromoles) produced by 2.5 ml. of liver enzymes + 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 + 0.1 ml. of 0.1 M sodium α-glycerophosphate + 17 micromoles of butyrate. Total volume, 6 ml. 90 minutes at 25° in oxygen.

Additions	200 micromoles	20 micromoles	2 micromoles
Succinate	1.8	8.9	8.5
Fumarate	8.6	9.4	8.4
Malate	0	9.6	8.0
Citrate	1.2	8.3	8.4
Glutamate	6.1	7.4	3.4
None, 2.4, 1.7			

in Table I, this is true only for higher concentrations (0.03 M). The same is true with malate and citrate. When medium concentrations (0.003 M) are used, succinate, fumarate, malate, citrate, and glutamate are all active. At still lower concentrations (0.0003 M) they are all active, but glutamate is only slightly active. Aspartate showed no action. The amount of butyrate which disappears may be 3 or 4 times greater than the amount of added succinate, fumarate, malate, or citrate. This fact is a clear indication that these substances act catalytically. In the experiment shown in Table I α-glycerophosphate was added because it was found that in some preparations it accelerated the disappearance of butyrate.

Formation of Phosphopyruvate—It was previously found (1) that phosphopyruvate or a similar substance accumulated when fumarate was added, but not when fatty acids were also present. The formation of phospho-

pyruvate from substances which are active in promoting butyrate oxidation was therefore studied. As is shown in Table II, phosphopyruvate was formed from succinate, fumarate, malate, citrate, and also in smaller amounts from glutamate. In the presence of fluoride (0.02 M) the formation is practically the same in all cases, the only difference being that there occurs a greater disappearance of inorganic phosphate. There is also a greater uptake of inorganic phosphate with all the additions as compared with the control.

TABLE II

Formation of Phosphopyruvate

Composition of the system: 2.5 ml. of liver enzymes + 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 + 0.2 ml. of 0.1 M additions. Total volume, 6 ml. 90 minutes at 25° in oxygen. The results are given in micromoles.

Additions	Inorganic P	P liberated by		Pyruvic acid	
		$HgCl_2$	Hypoiodite	Before	After 60 min. at 100° in N HCl
None	18.3	0	0.4	0	1.5
" + 0.02 M NaF	8.6	0.7	0.1	0	1.9
Succinate	12.6	3.7	3.2	0	5.3
" + 0.02 M NaF	2.9	4.9	4.0	1.6	8.5
Fumarate	11.1	4.9	4.2	0	6.8
" + 0.02 M NaF	2.3	5.5	4.8	1.0	8.4
Malate	12.1	3.5	3.7	0	5.3
" + 0.02 M NaF	2.9	4.9	4.2	0.6	7.2
Citrate	8.2	5.8	5.3	0	8.2
" + 0.02 M NaF	3.3	4.2	3.3	2.6	8.0
Glutamate	14.4	1.5	1.4	0	2.5
" + 0.02 M NaF	5.1	1.9	1.1	0	3.6

The estimation of phosphopyruvate by the hypoiodite and mercuric chloride methods gave practically the same results. The amount of pyruvate liberated by acid hydrolysis gave higher values, presumably because of some interfering substance, but the results were parallel with those obtained by other methods. No pyruvate was present before hydrolysis except in the presence of fluoride plus succinate, fumarate, malate, and citrate, when a small amount accumulated. Smaller amounts of succinate and fumarate, while still promoting the oxidation of butyrate, do not lead to the accumulation of detectable amounts of phosphopyruvate.

The addition of 3-phosphoglycerate produced a formation of phosphopyruvate which was completely suppressed by 0.02 M fluoride. This shows

that enolase is inhibited by that concentration of fluoride and therefore that phosphopyruvate may be formed by a mechanism in which enolase is not involved. No phosphopyruvate was formed from lactate or pyruvate.

Components Necessary for Formation of Phosphopyruvate—As is shown in Table III, no phosphopyruvate is formed from fumarate in the absence of cytochrome *c*, adenylic acid, or inorganic phosphate. Adenylic acid is also necessary for phosphopyruvate formation from succinate or citrate.

Action of Malonate on Phosphopyruvate Formation—As malonate is known to inhibit succinic dehydrogenase, it was considered that it might be useful as a means of finding out which substance is the immediate precursor of phosphopyruvate. If it were fumarate, an inhibition of phosphopyruvate formation from succinate but not from fumarate would be expected. However, it was found (Table IV) that malonate inhibits phosphopyruvate formation from succinate, fumarate, and citrate approximately to the same

TABLE III

Components Necessary for Formation of Phosphopyruvate

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

	Inorganic P	P liberated by hypoiodite	Pyruvic acid liberated by acid
Complete system	9.3	5.8	6.5
No cytochrome	18.9	0.3	0.4
" adenylic acid	16.1	0.9	0.6
" phosphate	3.4	1.0	1.6
" fumarate	19.0	0.6	0.5

degree. At 0.001 M there is almost no inhibition and with a higher concentration (0.005 M) formation of phosphopyruvate is almost completely inhibited.

Influence of Phosphopyruvate on Butyrate Disappearance—As phosphopyruvate is formed from the compounds which promote butyrate oxidation, the action of synthetic phosphopyruvate was studied. Curiously enough it was found that phosphopyruvate is active only in a medium containing bicarbonate (Tables V and VI). If phosphate buffer was used and the carbon dioxide was absorbed with alkali, hardly any disappearance of butyrate occurred. This effect is not due to differences in the pH of the medium, as was proved by measurements with a glass electrode. Moreover, the bicarbonate buffer did not affect the butyrate oxidation when fumarate instead of phosphopyruvate was used.

The rate of butyrate and phosphopyruvate disappearance with and without bicarbonate was studied (Table V). Phosphopyruvate disappeared

within 20 minutes in the presence of bicarbonate and butyrate. However, butyrate continued to disappear even after phosphopyruvate was not detectable any more. In the absence of bicarbonate the disappearance of phosphopyruvate was slower and hardly any butyrate was oxidized.

It was found that the disappearance of butyrate in the presence of phosphopyruvate occurred only with cytochrome *c*, adenylic acid, and bicarbonate present (Table VI). Without added inorganic phosphate there was

TABLE IV

Action of Malonate on Phosphopyruvate Formation

The complete system was as in Table II. The results are given in micromoles of phosphate liberated by hypoiodite.

	Malonate concentration			
	0	0.001 M	0.003 M	0.005 M
Succinate	2.8	2.6	1.3	0.1
Fumarate	2.0	2.0	1.2	0.8
Citrate	6.3	4.7	2.2	1.1

TABLE V

Influence of Carbon Dioxide on Butyrate and Phosphopyruvate Disappearance

Composition of the system: 2.5 ml. of liver enzymes + 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 + sodium phosphopyruvate + 16.9 micromoles of butyrate. Series A, + 0.3 ml. of 0.15 M sodium bicarbonate; gas, 2 per cent carbon dioxide in oxygen. Series B, no bicarbonate; oxygen; CO_2 absorbed with alkali. Total volume, 6 ml. The results are given in micromoles.

		Time					
		0 min.	10 min.	20 min.	30 min.	60 min.	90 min.
Series A (with CO_2)	Butyrate	16.9	16.8	15.4	14.5	11.7	9.5
	Phosphopyruvate	1.8	0.3	0	0	0	0
Series B (no CO_2)	Butyrate	16.9	16.7	16.6	15.6	14.7	15.0
	Phosphopyruvate	2	1.2	1.4	1.0	0.6	0.1

a small disappearance, but the enzyme preparation already contained a certain amount of phosphate. In the experiment shown in Table VI the estimations of phosphopyruvate were carried out after 40 minutes of incubation, whereas butyrate was estimated after 90 minutes, as the former compound disappears faster than the latter.

Phosphopyruvate disappears faster when butyrate is oxidized. The absence of any of the components necessary for the oxidation slows down the

rate of disappearance. In the absence of bicarbonate, phosphopyruvate generally produces an inhibition of the small butyrate oxidation which occurs when no phosphopyruvate is added.

Influence of Pyruvate and Lactate—Both these substances increase the rate of butyrate oxidation. However, in the majority of experiments they were less active than fumarate or phosphopyruvate plus carbon dioxide. Their action, in contrast to that of phosphopyruvate, is not influenced by the presence of carbon dioxide. In some experiments the disappearance

TABLE VI

Disappearance of Butyrate and Phosphopyruvate

Composition of the complete system as in Table V, Series A. Time of incubation at 25°, 40 minutes for phosphate estimation, 90 minutes for butyrate. The results are given in micromoles.

	Butyrate disappearance	Inorganic P	Phosphopyruvate
Complete system (initial)		15.1	5.5
" "	9.1	20.6	1.8
No cytochrome	0	20.2	3.3
" phosphate	6.7	9.8	0.5
" adenylic acid	0	19.1	2.4
" phosphopyruvate	1.2	17.5	0.4
" bicarbonate	0	17.8	4.6
" butyrate		19.5	3.5

TABLE VII

Oxygen Uptake and Formation of Ketone Bodies

Complete system as in Table II; 2 micromoles of fumarate; 90 minutes at 25°. The results are given in micromoles.

	Oxygen uptake	CO_2 formed	Δ butyrate	Aceto-acetate	β-Hydroxy-butyrate
Complete system, no butyrate	2.4	2.2		0	0.8
Same + 18 micromoles butyrate	43.4	23.2	9.6	6.8 7.2*	5.8

* Aniline method.

produced by addition of lactate or pyruvate was increased by bicarbonate, but the blank was affected to the same extent.

Oxygen Uptake and Reaction Product—In a previous paper (1) the oxygen uptake was measured in the system with a high concentration of fumarate. Under these conditions the oxygen uptake of the blank with no butyrate was rather high. After subtraction of this blank value, it was found that about 1 molecule of oxygen per molecule of butyrate was used up. The amount of acetoacetate formed was small.

In the later experiments, in which a low concentration of fumarate was used, the oxygen uptake of the blank was much lower. This gives more significance to the results, as it is doubtful whether the oxygen uptake of the blank should be subtracted from the uptake with butyrate.

The results of an experiment in which both oxygen uptake and the ketone bodies formed were determined are reproduced in Table VII. The amount of acetoacetate formed per molecule of butyrate was 0.7 molecule, and there was good agreement between the aniline and the modified Van Slyke methods. About 0.5 molecule of β-hydroxybutyrate was formed. Therefore it can be considered that all the butyrate which disappears is transformed into ketone bodies. The oxidation of butyrate to acetoacetate should require 1 molecule of oxygen. The uptake of oxygen actually found was about 4 molecules per molecule of butyrate. These high values, ranging from 3 to 4, have been observed in many experiments. The carbon dioxide formed was about 2.2 molecules per molecule of butyrate.

The identity of the substance which is oxidized together with butyrate has not been established.

DISCUSSION

The rate of butyrate oxidation is increased by substances which intervene in the Krebs citric acid cycle. The same substances give rise to phosphopyruvate. The latter substance is also active, but only in the presence of carbon dioxide. It is difficult to ascertain which is the active substance, because enzymes are present which catalyze their interconversion. For phosphopyruvate it appears probable that there might occur a carboxylation to a phosphorylated C_4 compound. A carbon dioxide fixation on a phosphorylated compound has been suggested by Werkman and Wood (10). As to the type of reaction between the active compound and butyrate nothing is known. Butyrate appears to be oxidized to acetoacetate without an intermediary formation of β-hydroxybutyrate. Most preparations formed acetoacetate at a higher rate from butyrate than from β-hydroxybutyrate and in some enzyme preparations β-hydroxybutyrate was not oxidized, while butyrate was. Similar results were obtained by Jowett and Quastel (11) in liver slices.

The high oxygen uptake and carbon dioxide formation show that some other substance is oxidized together with butyrate. Either there is a coupled reaction or butyrate acts by maintaining the activity of some part of the system necessary for the oxidation of other substrates.

SUMMARY

Succinate, fumarate, malate, citrate, and glutamate increase the rate of butyrate oxidation by preparations of liver enzyme. These same sub-

stances give rise to a formation of phosphopyruvate. The latter was also active, but only in the presence of carbon dioxide, whereas pyruvate and lactate were less active. No phosphopyruvate was formed in the absence of adenylic acid, cytochrome *c*, or inorganic phosphate. Malonate at different concentrations equally inhibited phosphopyruvate formation from succinate, fumarate, or citrate.

Butyrate was nearly all recovered as acetoacetate, but the amount of oxygen used was greatly in excess for this reaction.

BIBLIOGRAPHY

1. Muñoz, J. M., and Leloir, L. F., *J. Biol. Chem.*, **147**, 355 (1943).
2. Lohmann, K., *Biochem. Z.*, **254**, 381 (1932).
3. Kiessling, W., *Ber. chem. Ges.*, **68**, 597 (1935).
4. Neuberg, C., and Kobel, M., *Biochem. Z.*, **263**, 219 (1933).
5. Robertson, W. B., *Science*, **96**, 93 (1942).
6. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
7. Edson, N. L., *Biochem. J.*, **29**, 2082 (1935).
8. Lohmann, K., and Meyerhof, O., *Biochem. Z.*, **273**, 60 (1934).
9. Horvath, S. M., Consolazio, W. V., and Dill, D. B., Syllabus of methods of the Fatigue Laboratory, Harvard University, Boston, 108 (1942).
10. Werkman, C. H., and Wood, H. G., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **2**, 135 (1942).
11. Jowett, M., and Quastel, J. H., *Biochem. J.*, **29**, 2143 (1935).