METABOLISM OF GLUCOSE BY BUTYRIBACTERIUM RETTGERI[1]

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Butyribacterium rettgeri has been reported to carry out a butyric acid fermentation of lactate in which the yield of carbon dioxide is unusually low and the yield of volatile fatty acids is high (Barker and Haas, 1944). Tracer experiments demonstrated that carbon dioxide is used extensively by the organism for the synthesis of acetate and that acetate is converted to butyrate (Barker *et al.*, 1945). A study of the nutrition of the organism showed that it required a new growth factor, called the BR factor, which has since been found to be replaceable by lipoic acid (Kline and Barker, 1950; Kline *et al.*, 1952). The present paper is concerned with several aspects of the metabolism of glucose by *B. rettgeri*.

MATERIALS AND METHODS

Three strains of the bacterium were used: (1) *B. rettgeri*, strain L, a strain adapted to grow on a lactate medium. This strain requires 48 hours at 37 C to reach maximum growth on a medium containing BR factor (Kline *et al.*, 1952) with lactate, glucose, or pyruvate as substrate. Its growth on glucose is stimulated by the presence of the BR factor. Little if any lactate is formed when the organism is grown on a semisynthetic medium (Kline and Barker, 1950) containing glucose, BR factor, and one per cent phosphate. (2) *B. rettgeri*, strain G2, a strain obtained from strain L by 10 to 12 passages in a glucose medium containing no BR factor (Kline, 1950). Maximum growth is obtained within 18 hours. Growth is not stimulated by the addition of BR factor. Lactate is formed in small but significant amounts from glucose. (3) *B. rettgeri*, strain G3, which is similar to strain G2 except that it produces much larger yields of lactate, 40 to 60 per cent of the glucose fermented.

The general conditions for growth of the organism and the chemical methods used are described elsewhere (Pine and Barker, 1954).

RESULTS AND DISCUSSION

Products of glucose fermentation. The main products of glucose fermentation are carbon dioxide, acetate, butyrate, and lactate. Some hydrogen, never exceeding 0.75 mole per mole of glucose and generally much less, is formed also. Acetoin, pyruvate, and possibly glycerol have been detected in small amounts in some experiments. Formate, propionate, and caproate could not be detected either by fractional distillation of 25 g of mixed fermentation fatty acids or by silica gel partition chromatography (Elsden, 1946). No other neutral volatile products or organic acids were formed in appreciable amounts.

The lactic acid formed by *B. rettgeri* is mainly the DL-isomer. This was determined by isolation and examination of the crystalline zinc salt by the procedure of Pederson *et al.* (1926). The first and second crops of crystals contained 18.6 and 18.1 per cent water, and showed specific rotations, $(\alpha)_D$ = 1.2 and 0.5. Authentic DL-zinc lactate contains 18.2 per cent water whereas the D- or L-isomer contains 12.8 per cent water and has an optical rotation $(\alpha)_D = \pm 8.25°$. The small positive optical rotation observed with the isolated zinc salt may indicate the presence of D-lactate or some other optically active compound. Recrystallization was not attempted because it would result in removal of the more soluble optically active isomers.

Quantitative data on products of glucose and pyruvate decomposition by cell suspensions are given in table 1. As in the fermentation of lactate (Barker and Haas, 1944), the most noteworthy feature of both the glucose and pyruvate fermentations is the low yield of carbon dioxide and the higher yields of acetic and butyric acids. In most carbohydrate fermentations the molar

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TABLE 1

Fermentation of glucose and pyruvate by washed cells of Butyribacterium rettgeri, strain G3

A washed cell suspension containing 1.43 mg of cell nitrogen, 50 μM potassium phosphate pH 7.1, 2 μM Na_2S, and 10 μM glucose or 20 μM lithium pyruvate, in 2.2 ml of water was incubated in a Warburg vessel. Gas phase, H_2. Temp 37 C. 0.2 ml of 6 N H_2SO_4 was tipped from a side arm after 60 minutes. Hydrogen was determined with one vessel containing KOH in the center well, and carbon dioxide production in other flasks was calculated by difference. Other products were estimated by chemical methods.

COMPOUND	GLUCOSE FERMENTATION	PYRUVATE FERMENTATION
	μM	μM
Substrate fermented	10.0	14.5
Carbon dioxide	4.8	8.3
Hydrogen	7.4	1.0
Lactate	10.7	0.3
Acetate	8.8	12.4
Butyrate	2.9	0.6
Carbon recovery, per cent	100.0	84.0
Redox index	0.98	1.01
C_1/C_2 ratio	0.33	0.61

ratio of C_1 to C_2 plus derived C_4 products is unity because the C_1 and C_2 compounds are formed by decomposition of pyruvate. In *B. rettgeri* fermentations, this ratio usually lies between 0.2 and 0.6, indicating a large reutilization of carbon dioxide or possibly other C_1 compounds for acetate synthesis (Barker *et al.*, 1945). In the glucose fermentation of table 1, the C_1/C_2 ratio is 0.33 and the calculated carbon dioxide utilization is 1.39 moles per mole of glucose, after correcting for the conversion of glucose to lactate. This calculation is based on the assumption that two moles of carbon dioxide are in fact formed per mole of glucose converted to acetate and its derivatives. The carbon dioxide fixed is then equal to the expected carbon dioxide production, estimated from glucose decomposition, less the observed carbon dioxide accumulation. In the pyruvate fermentation the C_1/C_2 ratio was 0.61 and the calculated carbon dioxide fixation was 0.25 mole per mole of pyruvate. The smaller carbon dioxide fixation with pyruvate as compared with glucose cannot be attributed to the higher oxidation state of the former. Calculations for both fermentations indicate that the actual amounts of hydrogen available for the reduction of carbon dioxide and acetate were approximately the same. The larger carbon dioxide fixation with glucose could be the result of the greater production of energy rich compounds or of an essential intermediate.

Lactate formation. In several glucose fermentations, carried out with somewhat different media, the yield of lactate was observed to vary widely from zero to over one mole per mole of glucose decomposed. One factor affecting the yield of lactate appears to be the pH of the medium. Table 1 shows that the yield of lactate increases toward the end of the fermentation when the pH is low. Other experiments also indicate that considerably more lactate is formed below pH 6 than above. However, the observed variations in the yield of lactate are much greater than can be accounted for by the pH effect, and therefore we studied the effect of the concentration of iron, which has been shown by Waring and Werkman (1944) and Pappenheimer and Shaskan (1944) to influence markedly the yield of lactate in other bacteria.

In order to remove iron present as an impurity in the medium, we have added α,α'-dipyridyl. This compound forms a stable complex with ferrous iron, and Hickey (1945) has shown that the iron in the complex is unavailable to bacteria. With the yeast extract-yeast autolysate media used in this investigation, the addition of 20 μg per ml of dipyridyl suppresses growth, whereas 15 μg permit slow growth. The inhibitory effect of as much as 50 μg per ml of dipyridyl can be reversed completely by the addition of 0.24 mg per ml of $FeSO_4 \cdot 7H_2O$.

The influence of low and high levels of iron on the products of glucose fermentation is shown in table 2. It can be seen that a low level of iron results in the conversion of 73 per cent of the glucose to lactic acid, whereas with an adequate iron supply the yield is 13 per cent. The rest of the glucose is converted mainly to carbon dioxide, acetate, and butyrate. These results indicate that in *B. rettgeri*, as in *Aerobacter indologenes* (Waring and Werkman, 1944) and *Clostridium perfringens* (Pappenheimer and Shaskan, 1944), iron has an effect in the conversion of pyruvate to acetate and carbon dioxide. Absence of an adequate supply of iron presumably leads to an accumulation of pyruvate and favors its reduction to lactate. A considerable accumulation of

pyruvate, observed in several experiments with low iron media, supports this interpretation. Since the yield ratios of acetate to butyrate and of carbon dioxide to C_2 derivatives were nearly independent of the iron level, it may be concluded that under the conditions of this experiment, the conversions of acetate to butyrate and of carbon dioxide to acetate were not limited by lack of iron. In other experiments done with a glucose adapted strain of *B. rettgeri* (strain G3), the ratio of C_1 to C_2 derivatives was significantly higher when the iron supply was suboptimal (table 3). This suggests that iron is in some way involved in carbon dioxide fixation.

Waring and Werkman (1944) have shown that iron deficiency reduces the ability of *A. indologenes* to decompose formate. The same is true of *B. rettgeri*. Cell suspensions derived from a high iron medium readily converted formate to hydrogen and carbon dioxide, whereas low iron cells were completely inactive.

Exchange reactions between lactate and acetate and carbon dioxide. In view of the fact that iron deficiency increases the conversion of glucose to lactate, apparently by interfering with the breakdown of pyruvate, it was of interest to see how iron deficiency or excess affects the exchange of carbon between lactate and carbon dioxide or acetate (Pine and Barker, 1954). Experiments were done with growing cultures of strain G3 which were allowed to ferment glucose in the presence of C^{14}-labeled carbon dioxide or acetate. The results obtained with labeled carbon dioxide, which will not be presented in detail, showed that approximately the same amount of carbon dioxide carbon is incorporated into the carboxyl group of lactate irrespective of whether the medium contains a low or high level of iron. The 6.36 mM of lactate produced in the high iron medium contained 44.5 per cent of the added C^{14} in its carboxyl group, whereas the 7.25 mM of lactate produced in the low iron medium contained 73 per cent of the added C^{14} in the same position. The same quantity of glucose was decomposed in both cultures, but the incubation period was about twice as long in the low iron culture.

To study the exchange of acetate carbon with lactate, glucose was fermented in the presence of acetate-1-C^{14} and acetate-2-C^{14} in separate experiments in high iron media and in the presence of acetate-1,2-C^{14} in a low iron medium. It was found with the high iron media that 13 and 12 per cent of the C^{14} added as acetate-1-C^{14} and acetate-2-C^{14}, respectively, were incorporated into lactate, whereas in the iron deficient medium 60 per cent of the C^{14} added as acetate-1,2-C^{14} appeared in lactate. The yield of lactate (8.16 mM) in the low iron medium was about twice those (4.25 and 3.79 mM) in the high iron media.

TABLE 2

Effect of iron on the fermentation of glucose by Butyribacterium rettgeri, strain L

The medium contained the following components in g per 100 ml of distilled water: yeast autolysate 0.3, yeast extract (Difco) 0.3, glucose 1, K_2HPO_4 1, $(NH_4)_2SO_4$ 0.05, $MgSO_4 \cdot 7H_2O$ 0.02, cysteine·HCl 0.05, and H_2SO_4 to pH 7.4. The low Fe culture contained 10 μg of α,α'-dipyridyl per ml. To the high Fe culture was added 0.12 mg of $FeSO_4 \cdot 7H_2O$ per ml. The cultures were incubated anaerobically for 72 hours at 37 C and then were analyzed.

PRODUCT	YIELD IN mM PER 100 mM GLUCOSE FERMENTED	
	Low Fe culture	High Fe culture
Carbon dioxide	8.8	26
Acetate	38.0	150
Butyrate	7.4	22
Lactate	146.0	26

TABLE 3

Fermentation of glucose-1-C^{14}

50 ml of synthetic medium (Kline and Barker, 1950) containing 1 per cent glucose, 2 per cent potassium phosphate, and 0.004 per cent $FeSO_4 \cdot 7H_2O$ or no added iron, and no BR factor was inoculated with *Butyribacterium rettgeri*, strain G3. The cultures were incubated anaerobically for 36 hours at 37 C.

COMPOUND	ADDED IRON		NO ADDED IRON	
	Quantity	Specific activity	Quantity	Specific activity
	mM	*cpm/μM*	*mM*	*cpm/μM*
Glucose fermented	2.67	15.0	2.60	15.0
Carbon dioxide	0.92	0.30	0.52	0.72
Acetate	0.96		0.39	
COOH		1.05		1.50
CH_3		5.28		3.39
Butyrate	0.86	9.80	0.23	11.0
Lactate	2.13		3.05	
COOH		0.90		1.00
CHOH		0.23		0.16
CH_3		4.83		5.29
C_1/C_2 ratio	0.34		0.61	

In the above experiments a rather large yield of lactate was obtained even with an adequate supply of iron. This contrasts with earlier results obtained with the same media and suggests that some modification may have occurred in the organism. Nevertheless in both experiments more lactate was formed without than with added iron. In spite of the increased yield of lactate and the decreased formation of carbon dioxide and fatty acids as a result of the low level of iron, the incorporation of carbon from carbon dioxide and acetate into lactate was as great or greater under these conditions. The explanation for this unexpected relation is not obvious. The possibility is that a limited accumulation of pyruvate, as a result of iron deficiency, actually facilitates the exchange of carbon between lactate and acetate. It is also possible, though less likely, that a metabolic pathway from lactate to acetate exists that does not involve pyruvate. Further work is required to clarify this problem.

Fermentation of glucose-1-C^{14}. Data on the fermentation of glucose-1-C^{14} by a glucose adapted strain in low and high iron media are given in table 3. It can be seen that in both experiments the methyl groups of lactate and acetate have the highest specific activities as would be expected if the glucose is broken down by glycolysis. The low incorporation of C^{14} in carbon dioxide is also consistent with this mechanism. However, it is apparent that other types of reactions are occurring to an appreciable extent since approximately 20 per cent of the C^{14} are found in nonmethyl positions. The nature of the reactions responsible for the randomization of C^{14} cannot be determined from the available data.

SUMMARY

Butyribacterium rettgeri ferments glucose with the formation of DL-lactate, acetate, butyrate, carbon dioxide, and hydrogen. The yield of lactate is dependent upon several factors including the iron supply in the medium. Iron deficient cells form much more lactate than iron sufficient cells. However, the exchanger of carbon between lactate and its oxidation products, acetate and carbon dioxide, is accelerated rather than reduced by iron deficiency.

The lactate and acetate formed from glucose-1-C^{14} are predominantly methyl labeled. About 20 per cent of the C^{14} appear in other carbon atoms. The results suggest that glycolytic reactions provide the main pathway for glucose decomposition.

REFERENCES

BARKER, H. A., AND HAAS, V. 1944 *Butyribacterium*, a new genus of gram-positive, nonsporulating anaerobic bacteria of intestinal origin. J. Bact., **47**, 301–305.

BARKER, H. A., KAMEN, M. D., AND HAAS, V. 1945 Carbon dioxide utilization in the synthesis of acetic and butyric acids by *Butyribacterium rettgeri*. Proc. Natl. Acad. Sci. U. S., **31**, 355–360.

ELSDEN, S. R. 1946 An application of silica gel partition chromatogram technique to the estimation of volatile fatty acids. Biochem. J. (London), **40**, 252–256.

HICKEY, R. J. 1945 The inactivation of iron by 2,2′-dipyridine and its effect on riboflavin synthesis by *Clostridium acetobutylicum*. Arch. Biochem., **8**, 439–447.

KLINE, L. 1950 A new growth factor required by *Butyribacterium rettgeri*. Ph.D. dissertation, University of California.

KLINE, L., AND BARKER, H. A. 1950 A new growth factor required by *Butyribacterium rettgeri*. J. Bact., **60**, 349–363.

KLINE, L., PINE, L., GUNSALUS, I. C., AND BARKER, H. A. 1952 Probable identity of the growth promoting factor for *Butyribacterium rettgeri* with other biologically-active substances. J. Bact., **64**, 467–472.

PAPPENHEIMER, A. M., AND SHASKAN, E. 1944 Effect of iron on carbohydrate metabolism of *Clostridium welchii*. J. Biol. Chem., **155**, 265–275.

PEDERSON, C. S., PETERSON, W. H., AND FRED, E. B. 1926 The forms of lactic acid produced by pure and mixed cultures of bacteria. J. Biol. Chem., **68**, 151–164.

PINE, L., AND BARKER, H. A. 1954 Tracer experiments on the mechanism of acetate formation from carbon dioxide by *Butyribacterium rettgeri*. J. Bact., **68**, 216–226.

WARING, W. S., AND WERKMAN, C. H. 1944 Iron deficiency in bacterial metabolism. Arch. Biochem., **4**, 75–87.