METABOLIC REACTIONS OF PASTEURELLA PESTIS

II. The Fermentation of Glucose

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Received for publication September 7, 1954

In paper I of this series the mechanism of acetate oxidation by *Pasteurella pestis* has been described (Santer and Ajl, 1954). This communication deals with certain aspects of the metabolism of glucose and, to a lesser extent, of ribose and pyruvate, all of which can give rise to acetic acid. These experiments are a part of an investigation of the over-all metabolic pathways and toxin production of the plague bacillus.

The genus *Pasteurella* is characterized in *Bergey's Manual* (Breed *et al.*, 1948) as "a group of microorganisms which ferment carbohydrates with little or no gas production". Doudoroff (1943), however, reported that the strain of *P. pestis* which he employed produced, in addition to the usual fermentation products, appreciable amounts of carbon dioxide when grown in media containing glucose. Despite the fact that the latter were growth experiments in complex media, he obtained excellent carbon recoveries although the oxidation-reduction (O/R) indices were considerably removed from the expected value of one. Quantitative data dealing with the anaerobic metabolism of resting cells of this organism are completely lacking.

METHODS

Washed cells of *P. pestis*, strain Tjiwidej, harvested from trypticase soy broth after 24 hours of aerobic or anaerobic growth at 30 C were used for manometric studies. Resting cells were incubated with appropriate substrates in Warburg cups and gassed with oxygen-free nitrogen to maintain anaerobic conditions. During the course of the experiment carbon dioxide evolution was determined quantitatively by following pressure changes of the manometer fluid. At the end of the incubation period, residual carbon dioxide was liberated on tipping the sulfuric acid contained in one of the side arms of the Warburg vessel. In experiments with 1-C^{14}-glucose, NaOH was present in the center well to trap the evolved CO_2. At the end of the incubation period the deproteinated cells were centrifuged off, and the supernate analyzed for fermentation products.

The following analytical procedures were employed: Residual glucose was determined by the method of Nelson (1944). The colorimetric methods of Friedemann and Haugen (1943) and Barker and Summerson (1941) were used for the estimation of pyruvate and lactate, respectively. Ethanol was determined quantitatively by the procedure of Friedemann and Kaas (1936). Acetate and formate were estimated by titration of the total steam volatile fraction. The concentration of acetate was obtained by subtracting the formate content after determining the latter manometrically with mercuric acetate (Pickett, 1943). The conventional succinoxidase method was used for succinate determination. The orcinol method of Mejbaum (1939) was used for pentose and pentose phosphate detection. Methods for the determination of carbon 14 were essentially similar to those described by Ajl and Kamen (1951).

EXPERIMENTAL RESULTS

Untreated, resting cells of *P. pestis* oxidized glucose and, to a lesser extent, ribose. Some of the phosphorylated hexoses, however, were not metabolized by such unaltered cell suspensions. Lyophilized preparations of the same organism oxidized all of the C_6 phosphorylated sugars including gluconic acid. Results are exhibited in figure 1. From these data it appeared that the phosphorylated hexoses as well as gluconic acid participated in glucose breakdown since all of the compounds mentioned in figure 1 have long

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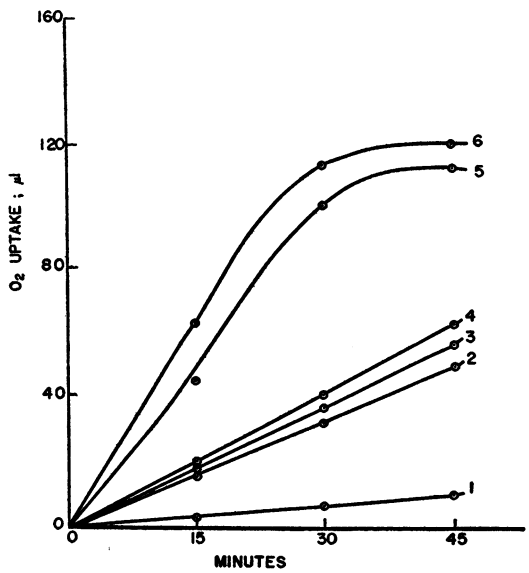

Figure 1. Oxidation of hexoses and hexose phosphates by dried preparation of *Pasteurella pestis*. Flasks contained 0.5 ml of 0.5 M phosphate buffer, pH 6.9, 50 µM of substrate, 45 mg (dry weight) of *P. pestis*, and 0.3 ml of NaOH in center well. Total volume 2.8 ml. Temperature 33 C. 1—endogenous; 2—fructose-6-PO_4; 3—fructose-1-6-$DiPO_4$; 4—glucose-6-PO_4; 5—gluconic acid; 6—glucose.

been recognized as intermediates is hexose metabolism.

Nonproliferating, resting cells of *P. pestis* also exhibited considerable fermentative abilities. Complete fermentation analyses were performed on glucose, ribose, and pyruvic acid. The findings are shown in table 1. The chief products of glucose fermentation were lactic, acetic, and formic acids, ethanol, and carbon dioxide. Small amounts of succinate and pyruvate were also detected. Ribose gave rise to acetate, formate, and ethyl alcohol. No CO_2 production was noted. Pyruvate yielded formic and acetic acids, carbon dioxide, and lactate. The carbon recovery in each case was nearly complete, and the O/R index likewise approached the expected value of one.

In an effort to determine the origin of CO_2 in glucose fermentation, 1-C^{14}-glucose was incubated in the presence of *P. pestis* under completely anaerobic conditions and the evolved gas trapped in alkali. These experiments were performed with cells grown both aerobically and anaerobically at various pH's and in a variety of buffers. Under

TABLE 1

Products of glucose, ribose, and pyruvic acid fermentation by Pasteurella pestis

Products	µM/100 µM of Substrate Fermented		
	Glucose	Ribose	Pyruvate
Carbon dioxide	18	—	8
Lactate	88	traces	8
Acetate	50	134	92
Formate	30	109	84
Succinate	5	—	—
Pyruvate	3	traces	—
Ethanol	41	59	—
Carbon recovery per cent	87.2	99	100
O/R Index	0.9	0.92	—

Each reaction vessel contained 100 µM of substrate, 0.5 ml of 0.5 M phosphate buffer, pH 6.9, 1 ml of a 12 per cent suspension of freshly harvested *P. pestis*, and 0.2 ml of 12 N H_2SO_4 in side arm. Total volume of reactants 3 ml. Time of incubation 3 hours. Temperature 33 C. Conditions anaerobic.

no conditions was the evolved CO_2 appreciably radioactive. Typical results are shown in table 2. It is of interest to note that when as many as 80 µM of glucose containing 10^5 cts/min had been dissimilated, only 800 cts/min were recovered in the CO_2. In other words, less than one per cent of the glucose was decarboxylated under these experimental conditions. These data further indicate that the CO_2 produced from glucose under anaerobic conditions came from a source other than the conversion of glucose to a C_3 sugar. It is to be noted that *P. pestis* will not produce CO_2 anaerobically from any of its glucose fermentation products indicated in table 1 with the exception of pyruvate. It was found that the enzyme formic hydrogenlyase which is responsible for the breakdown of formate to CO_2 and H_2 is completely absent from either aerobically or anaerobically grown *P. pestis*.

The production of CO_2 from glucose under anaerobic conditions deserves further comment. If carbon dioxide evolution and glucose disappearance are plotted, the data shown in figure 2 are obtained. It appears that during the early stages of the experiment, the disappearance of the glucose follows very closely the production of CO_2. After 40 minutes, however, glucose continues

TABLE 2

Radioactivity of evolved CO_2 during the fermentation of 1-C^{14}-glucose by Pasteurella pestis

Expt. No.	Glucose Concentration		Δ Glucose	Total Activity of Glucose		Δ Radioactivity of Glucose	Initial Specific Activity of Glucose	Total Activity of Evolved CO_2	Specific Activity of Evolved CO_2
	Initial	Final		Initial	Final				
	μM	μM	μM	cts/min	cts/min	cts/min	cts/min/μM	cts/min	cts/min/μM
1	200	75	125	25 × 10^4	156,250	93,750	1,250	690	51
2	200	80	120	25 × 10^4	150,000	100,000	1,250	800	55

Total volume of reactants 2.8 ml. Each vessel contained 1.0 ml of a 12 per cent suspension of *P. pestis* grown with slight aeration, 0.5 ml of 0.5 M phosphate buffer, pH 6.9, 0.3 ml of NaOH in center well. Time of incubation 3 hours under N_2. Temperature 33 C.

to disappear without a concomitant evolution of gas.

The complete distribution of radioactivity in the products of 1-C^{14}-glucose fermentation is shown in table 3. Lactate, acetate, ethanol, and succinate contained the bulk of the activity. Formate remained completely inactive, and very little activity was recovered in the evolved CO_2. These findings are in accord with the operation of the Embden-Meyerhof scheme for the anaerobic dissimilation of glucose. If a hexose monophosphate shunt were operative, the evolved CO_2 should have been highly active while the lactate, acetate, and ethanol should have contained little or no activity as Gunsalus and Gibbs (1952) found with the heterofermentative lactic acid bacterium *Leuconostoc mesenteroides*.

TABLE 3

Distribution of radioactivity in products of 1-C^{14}-glucose fermentation by resting cell suspensions of Pasteurella pestis

Product	Total Counts
	cts/min
Lactate	38,000
Acetate	21,000
Formate	Bkgd
Ethanol	17,000
CO_2	690
Succinate	1,200
Pyruvate	Not determined

Initial activity of glucose 93,750 cts/min. Final activity of products recovered 77,890. Recovery 83 per cent.

Experimental conditions as described in table 2.

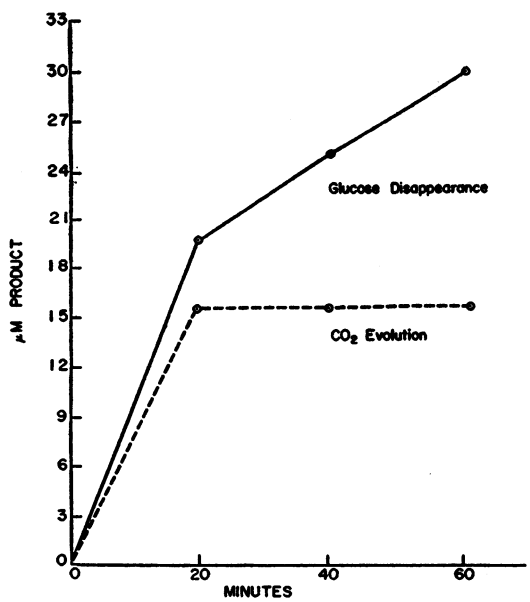

Figure 2. Comparative rates of CO_2 evolution and glucose disappearance during the fermentation of glucose by *Pasteurella pestis*. Flasks contained 50 μM of glucose, 0.5 ml of 0.5 M phosphate buffer, pH 6.9, 1 ml of a 12 per cent suspension of *P. pestis*, and 0.3 ml of H_2SO_4 in side arm. Conditions anaerobic. Temperature 33 C. Time of incubation as indicated.

To provide additional evidence for the operation of the Embden-Meyerhof scheme in glucose fermentation by *P. pestis*, the specific activity of the formed succinate was compared with the initial specific activity of the 1-C^{14}-glucose. It is to be noted that this scheme requires that all the glucose be initially converted to pyruvic acid. If this occurs, each molecule of glucose yields two molecules of C_3 keto acid. With the hexose labeled in the one position, one C_3 unit will be radioactive while the other one will not. The formed C_3 units will mix, and their specific activity will be one-half of the specific activity of the initial

TABLE 4

Comparison between the initial specific activity of 1-C^{14}-glucose and experimentally formed succinate during a fermentation by Pasteurella pestis

Expt. No.	Specific Activity of Initial Glucose	Specific Activity of Succinic Acid Produced*
	cts/min/μM	*cts/min/μM*
1	810	377
2	810	407

* The succinate in these experiments was isolated from typical fermentation runs performed in essentially the same manner as described in table 2.

glucose. If the C_3 unit now condenses with relatively inactive CO_2 to form succinate, the specific activity of the latter C_4 acid should also be one-half of the specific activity of the 1-C^{14}-glucose. This was found to be the case (table 4). It is quite clear from the data presented that the specific activity of the succinate, in both experiments, is precisely one-half of the specific activity of the initial hexose.

DISCUSSION

Resting cells of *P. pestis* appear to ferment glucose almost exclusively via the Embden-Meyerhof series of reactions. All attempts to demonstrate the hexose monophosphate shunt system failed. The data with the 1-C^{14}-glucose bear out this contention rather clearly. If glucose were fermented, even in part, by this alternate pathway, the evolved CO_2 should have become appreciably radioactive. However, no more than 0.7 per cent of the total activity of the glucose metabolized was converted to CO_2. Further, the distribution of radioactivity in the products of 1-C^{14}-glucose fermentation and in particular the specific activity of the formed succinate are completely consistent with the notion that the conventional glycolytic scheme operates. If 1-C^{14}-glucose was metabolized by the hexose monophosphate shunt, none of the fermentation products would have become radioactive. Instead, all products (with the exception of formate) became appreciably active, and most of the activity could be recovered in the fermentation compounds. On the other hand, crude and partially fractionated cell-free extracts of this bacterium do contain enzymes of the shunt system, and their significance will be discussed in a later communication of this series (Santer and Ajl, *in preparation*).

Quantitative analysis of the products of pyruvic acid dissimilation suggests that it is metabolized via two pathways, i.e., a dismutation reaction, whereby two moles of the C_3 keto acid are converted to one mole each of acetate, lactate, and CO_2, and a "phosphoroclastic split" of the *Escherichia coli* type (Utter and Werkman, 1944), whereby a mole of pyruvate yields one mole each of acetate and formate. One hundred μM of pyruvate yielded 84 and 92 μM of formate and acetate, respectively, and 8 μM each of CO_2 and lactate. From the dismutation alone 8 μM of CO_2 and 8 μM of lactate were obtained. Subtracting the 8 μM of acetate formed as a result of the dismutation, 84 μM are left which were produced from the acetate-formate split. This value, in turn, is equivalent to the 84 μM of formate that were actually found.

The CO_2 produced during the fermentation of glucose arises from the dismutation of pyruvic acid. Since 100 μM of pyruvate from the dismutation yielded 8 μM of CO_2, 100 μM of glucose should have yielded twice that amount of CO_2 or 16 μM of the keto acid. The amount of CO_2 produced falls within this expected range. In addition, the absence of formic hydrogenlyase in these cells coupled with the 1-C^{14}-glucose data further supports the contention that the major source of CO_2 is carbons 3 and 4 of the glucose molecule as a result of the dismutation of pyruvate.

It was noted in a previous section that although CO_2 evolution stops, glucose continues to disappear during the course of a normal fermentation. The precise cause for this finding is not yet clear. However, it is conceivable that during the early stages of the fermentation the over-all equilibria of the reactions involved are such that the dismutation of pyruvate with its concurrent CO_2 production is favored. As the fermentation proceeds, and lactate arising from sources other than from the dismutation begins to accumulate, the equilibrium is shifted in such a manner that the acetate-formate split predominates and CO_2 evolution ceases.

SUMMARY

The over-all fermentation pattern of glucose by *Pasteurella pestis* has been described. Experiments with 1-C^{14}-glucose reveal that the

Embden-Meyerhof scheme operates in the conversion of the hexose to its end products. All attempts to demonstrate an operative hexose monophosphate shunt failed. The CO_2 produced during the fermentation arises from the dismutation of pyruvate.

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