Carbohydrate Metabolism in *Pasteurella multocida*. II. Mechanism of Pyruvate Oxidation.* (28959)

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Evidence has been presented(1), for the operation of both the pentose and glycolytic pathways in *Past. multocida*, the former being the main route of glucose metabolism in this organism. Pyruvate oxidation is essential to complete glucose utilization through both the Embden-Meyerhof and pentose pathways, hence the corresponding mechanism has been investigated. Oxidation of the Krebs cycle intermediates by *Past. multocida*(2) lends support to the hypothesis that the cycle is the final oxidation pathway.

Materials and methods, unless stated otherwise were as described previously(1). *Preparation of disrupted-cell suspensions.* 3 g (wet weight) of packed cells were suspended in 50 ml 0.3 M phosphate buffer, pH 7.6 and ground at 3°C with 45 g glass powder in a Virtis homogenizer for 8 minutes at 40000 rpm. After decantation, the supernatant was centrifuged twice at 4000 *g* and the sediment discarded. The supernatant was centrifuged at 25000 *g*, the precipitate washed twice in the centrifuge at 25000 *g* with 0.3 M phosphate buffer and finally suspended in the same buffer (15 ml per 700 mg of bacteria fragments). The residue contained about 20% of unbroken cells, as shown by phase contrast microscopy. *Reagents.* Sodium pyruvate-2-C^{14} (3.2 mC/mmole) and sodium formate-C^{14} (5.0 mC/mmole) were from the Radiochemical Centre, Amersham, England. *Analysis of cell constituents.* In the experiments with formate-C^{14}, the methanol-water cell residues were brought to a TCA† concentration of 5% (w/v) and kept at 90°C for 30 minutes(3). Upon centrifugation, the precipitate was washed with 5% (w/v) TCA, centrifuged and the supernatants collected (nucleic acid fraction). The sediment was the protein fraction. Three hundred μl samples of each fraction (soluble, nucleic acids or protein) were plated on aluminum planchets and counted for radioactivity as described previously(4). For identification of the tricarboxylic acids, these were previously separated by chromatography with *sec*-butanol-formic acid(5). The ketoacids were condensed with 2,4-dinitrophenylhydrazine; the hydrazones were chromatographed for 16 hours on Whatman No. 1 paper with the butanol-bicarbonate system and detected by the yellow color and fluorescence under ultraviolet light(6). Fumarase was measured spectrophotometrically at 240 mμ with L-malate as substrate(7). Aconitase was measured with citrate or *cis*-aconitate as substrates; *iso*citrate formation was measured by reduction of NADP followed spectrophotometrically, at 340 mμ(7,8). The extracts contained *iso*citrate dehydrogenase which was demonstrated spectrophotometrically, at 340 mμ, by the reduction of NADP with the respective substrate(7).

Results and discussion. Resting *Past. multocida* oxidized pyruvate and most of Krebs cycle intermediates with significant velocity (Table I). On the other hand, acetate and citrate were not apparently oxidized. The slower oxidation of the tricarboxylic acids recalls similar observations with *Past. pestis* (8,9) but the metabolic significance of this fact is limited since permeability factors could affect the over-all rate of oxidation of the tricarboxylic acids (and malate). On the other hand, the lack of oxidation of acetate (and acetaldehyde) seems a distinctive fea-

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† Abbreviations used are: TCA, trichloracetic acid; Tris-buffer, tris(hydroxymethyl)aminomethane buffer; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PMS, phenazine methosulfate; Qo_2, μl oxygen uptake/mg of cells (dry weight)/hr.

TABLE I. Oxidation of Krebs Cycle Intermediates by *Past. multocida*. Organism (mg): 22.2 (exp *A*) and 15.4 (exp *B*); 3 ml mM phosphate buffer pH 7.2 with 10 mM substrates. Incubation at 37° for 60 min.

Substrate	Q_{O_2}
Exp A	
Citrate	3.7
*dl-Iso*citrate	6.2
cis-Aconitate	4.5
α-Ketoglutarate	19.0
Succinate	26.0
Fumarate	13.0
L-Malate	5.0
Pyruvate	24.0
None	3.5
Exp B	
Acetaldehyde	3.4
Acetate	2.7
None	2.2

ture of *Past. multocida* because *Past. pestis* oxidizes acetate with velocity near that corresponding to glucose(8,9). Malonate a typical inhibitor of the Krebs cycle, in concentration 10 mM diminished oxygen uptake by 53% with succinate, 19% with *dl-iso*citrate and 12% with pyruvate (or fumarate) as substrates. On the other hand, malonate did not affect endogenous respiration (Table II).

The pathway of pyruvate oxidation was followed by C^{14} distribution after oxidation of pyruvate-2-C^{14} (Table III). Radioactivity was found in (*a*) the Krebs cycle intermediates, (*b*) amino acids and (*c*) glucose, pyruvic and lactic acids. The presence of lactic acid is consistent with pyruvate dismutation, a reaction described in *Past. pestis*(10). With proliferating cells, labeling of amino acids was comparatively higher than with resting cells. A close relationship can be established

TABLE II. Effect of Malonate on Substrate Oxidation by *Past. multocida*. Organism suspended in 3 ml mM phosphate buffer. Incubation at 37° for 60 min.

Organism (mg)	Substrate (10 mM)	Malonate None Q_{O_2}	Malonate 10 mM Q_{O_2}	Inhibition (%)
26.6	Succinate	29.0	13.5	53
26.6	None	3.4	4.0	–18
19.2	Pyruvate	20.0	17.5	12
19.2	*dl*-Isocitrate	6.2	5.0	19
19.2	None	3.0	3.2	–7
22.2	Fumarate	13.0	12.0	12
22.2	None	4.8	4.2	8

between several of the amino acids listed in Table III and the Krebs cycle intermediates. In fact, aspartate and threonine are derived from oxalacetate, and glutamate, ornithine and arginine from α-ketoglutarate. Alanine

TABLE III. Distribution (%) of C^{14} After Oxidation of Pyruvate-2-C^{14} by *Past. multocida*. Exp with proliferating cells. Reaction mixture contained organism (65 mg, initial; 78 mg, final) pyruvate-2-C^{14} (25.2 μmoles; 9.2 × 10^6 c.p.m.) and culture medium. Total vol: 22.4 ml. After incubation at 37° for 60 min the cells were separated by centrifugation, washed once in the centrifuge with 1 ml 0.85% NaCl and mixed with 1 ml distilled water and 9 ml of methanol for chromatography and radioautography. Exp with resting cells. 142 mg of cells; pyruvate-2-C^{14} (μmole): 112 (Exp *A*) or 202 (Exp *B*) (C^{14}: 4.8 × 10^6 c.p.m.); mM phosphate buffer, pH 7.2. Total vol: 10 ml. After incubation at 37° for 60 min the cell suspension was mixed with 9 vol of methanol for chromatography and radioautography.

Substance	C^{14} fixed (% of total in methanol-water soluble substances) Proliferating cells	Resting cells Exp A	Resting cells Exp B
Krebs-cycle intermediates			
Citric acid	39.0	29.6	30.0
*Iso*citric "	0	14.2	2.1
cis-Aconitic "	0	20.0	7.8
α-Ketoglutaric "	0	.3	2.4
Succinic "	0	.1	.2
Fumaric "	7.4	3.3	1.8
Malic "	0	.1	4.1
Amino acids			
Alanine	5.6	.4	1.4
Cystine	0	.2	0
Cysteic acid	0	11.6	10.6
Serine	2.1	.3	0
Glycine	1.6	.6	.7
Aspartic acid	0	.3	.7
Threonine	6.6	0	.7
Glutamic acid	2.5	3.2	2.4
Ornithine	0	.4	0
Lysine-arginine	5.3	.6	.4
Leucine	2.8	.2	.3
Histidine	5.3	0	.7
Tyrosine	0	.2	.4
Tryptophan-valine	6.0	0	0
Glycolysis intermediates			
Glucose	4.0	.2	.7
Lactic acid	0	2.7	4.7
Pyruvic "	0	.6	10.6

is formed from pyruvate and leucine and valine are synthesized from acetate-1-C^{14}. In the 3 experiments, the tricarboxylic acids (especially citric acid) contained most of the radioactivity incorporated into the soluble

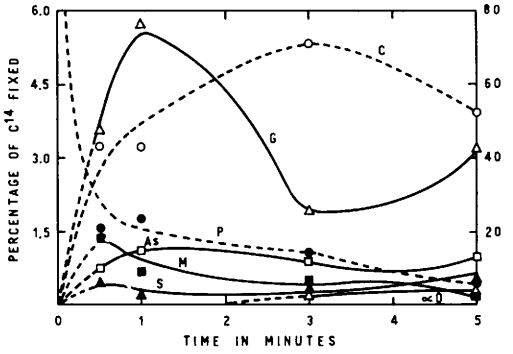

FIG. 1. Kinetics of C^{14} distribution after oxidation of pyruvate-2-C^{14} by resting *Past. multocida*. 142 mg of cells suspended in 10 ml of mM phosphate buffer, pH 7.2 containing pyruvate-2-C^{14} (12.2 μmole); C^{14}, 4.8 $\times$ 10^6 cpm. Incubation at 37°. P (●), pyruvic acid; C (○), citric, isocitric and *cis*-aconitic acids; G (△), glutamic acid; As (□), aspartic acid; S (▲), succinic acid; M (■), malic acid and αO (△), α-ketoglutaric acid. Lines P and C refer to right-hand ordinate and the other lines to left-hand ordinate.

cell fraction. Cysteic acid was the most radioactive amino acid (exp. with resting cells) and probably derives from cysteine (cystine) which is easily oxidized during paper chromatography.

Fig. 1 shows the kinetics of C^{14} distribution after oxidation of pyruvate-2-C^{14} by the resting organism. In the first minute of incubation there was a sharp diminution of radioactivity in pyruvic acid with a simultaneous increase in tricarboxylic acids and glutamic acid. Radioactivity in succinic, malic and aspartic acids increased subsequently, at the expense of glutamic acid. Malic and succinic acids had initial negative slopes but in all probability at shorter times of incubation the respective slopes should be positive, like the curve of aspartic acid. After the first 10 minutes of incubation the percentage distribution of C^{14} remained almost constant (plot not shown in Fig. 1). The kinetics is consistent with the operation of the Krebs cycle as terminal metabolic pathway, and this assumption was further supported by the demonstration with cell-free extracts of several enzymes essential for the cycle operation (Table IV). Thus, reduction of NADP with *iso*citrate as substrate evidenced *iso*citrate dehydrogenase (exp. *A*) and consequently, reduction of NADP with citrate and *cis*-aconitate showed the presence of aconitase (exp. *B*). Malic dehydrogenase activity was shown by the specific reduction of NAD with L-malate as substrate (exp. *C*), and fumarase activity by fumarate formation in presence of L-malate (exp. *D*). Succinic dehydrogenase was evidenced by succinate oxidation with disrupted cells and PMS as oxygen carrier (exp. *E*).

The enzymes which catalyze the operation of the Krebs cycle are frequently associated with the cytochrome system. On this basis cell suspensions of *Past. multocida* were subjected to differential spectrophotometry in the range of 400-600 mμ with a Beckman DU spectrophotometer. Suspensions were made of whole cells (7.0 mg/ml) or disrupted cells (3.0 mg/ml) in a mixture of 0.3 M phosphate buffer and glycerol (30% v/v). However,

TABLE IV. Enzymes Related to Krebs Cycle in *Past. multocida*. Spectrophotometric measurements. 3.0 ml of reaction mixture. Exp *A* and *B*: 32 mM glycylglycine buffer (pH 7.4), 0.33 mM $MnCl_2$ and cell-free extract (1.2 mg of protein). Exp *C*: 26 mM Tris buffer (pH 7.5), 10 mM KCN (neutralized), 0.33 mM $MnCl_2$ and cell-free extract (1.2 mg of protein). Exp *D*: 1.6 mM phosphate buffer (pH 7.2) and cell-free extract (80 μg of protein). Temp 30°. Manometric measurement (Exp *E*): 50 mM phosphate buffer, 1 mM KCN and 1.2 mg of disrupted cells. Final vol: 3 ml. Incubation for 60 min at 30°.

Additions	Enzyme activity $\left(\frac{\Delta A340 \times 10^3}{min}\right)$
Exp *A*: *iso*citrate dehydrogenase assay	
*dl-Iso*citrate + .06 mM NADP	332
.06 mM NADP	10
Exp *B*: Aconitase assay	
5 mM *cis*-Aconitate + .06 mM NADP	50
5 mM Citrate + .06 mM NADP	28
.06 mM NADP	6
5 mM Citrate	3
Exp *C*: malic dehydrogenase assay	
3.3 mM L-malate + .66 mM NAD	30
3.3 mM L-malate + .66 mM NADP	3
.66 mM NAD	8
	$\left(\frac{\Delta A240 \times 10^3}{min}\right)$
Exp *D*: fumarase assay	
8.3 mM L-malate	100
None	10
Exp *E*: succinic dehydrogenase assay	(Q_{O_2})
20 mM succinate + PMS (.06%)	29.0
20 mM succinate	18.7
None	15.4

TABLE V. Formate-C^{14} Oxidation by *Past. multocida*. 8.3 (Exp *A*) and 15.4 (Exp *B*) mg of organism suspended in 3 ml of mM phosphate buffer, pH 7.2; formate-C^{14}, 1.02 μmole (5.9×10^5 c.p.m.). 0.15 ml 3.3 N KOH in the center well of the Warburg flask. Incubation at 37° for 60 min. The CO_2 fixed in KOH solution was precipitated as $BaCO_3$ and counted for C^{14}.

		C^{14} fixed in cells (c.p.m./mg)				C^{14} in $BaCO_3$	
Exp	Additions	Total	Soluble fraction	Nucleic acid fraction	Protein fraction	(c.p.m.)	(c.p.m./mg)
A	10 mM Formate	1236	642	294	300	6780	1931
	5 mM Glucose	1993	1133	456	400	19000	3011
B	10 mM Pyruvate	539	473	138	20	9750	1544

after addition of solid dithionite, the absorbency of the reduced suspensions was not significantly different from that of the oxidized control which suggests that in *Past. multocida* the content of cytochromes must be small. In this connection it must be noted that in *Past. pestis* cytochrome oxidase could not be demonstrated(7).

In *Past. pestis* formate is produced from pyruvate by the phosphoroclastic reaction (10), and on this basis formate metabolism was investigated in *Past. multocida*. After incubation with formate-C^{14} (Table V) the cells incorporated C^{14} and produced radioactive CO_2. Glucose stimulated formate-C^{14} incorporation and oxidation while pyruvate had the opposite action. The C^{14} fixed by the cells was distributed in the soluble cell-fraction, the nucleic acids and the protein. Labeling of the nucleic acid fraction was considerable (25-23% of the total C^{14} fixed). In the soluble cell fraction (Table VI), C^{14} was found in the Krebs cycle intermediates and the related amino acids, particularly aspartic acid, glutamic acid and alanine. The serine group (glycine, serine, cystine and cysteic acid) fixed 17-13% of total C^{14} in this fraction. The low incorporation of C^{14} into the tricarboxylic acids constituted a striking difference with C^{14} distribution after oxidation of pyruvate-2-C^{14} (Table III) where the labeling of the tricarboxylic acids largely exceeded that of the serine group. Alanine was labeled also by formate-C^{14} in a significant proportion especially in the presence of glucose.

The evidence summarized above demonstrates the operation of the Krebs cycle as shown by (*a*) the labeling of the cycle intermediates with pyruvate-2-C^{14}, (*b*) the kinetics of C^{14} distribution in the cycle intermediates and (*c*) the presence of the Krebs cycle enzymes in cell-free extracts. If the cycle intermediates have the same specific activity (as in baker's yeast(11)) the size of the pools would differ largely, particularly with growing *Past. multocida* where some intermediates are apparently absent. The Krebs cycle is a source of energy and carbon skeletons for amino acids as shown by the labeling of alanine, aspartic acid, threonine, glutamic acid, ornithine and arginine from pyruvate-2-C^{14}. During growth, the biosynthetic function becomes more apparent since the relative incorporation of C^{14} into the cycle intermediates decreases with simultaneous increase in the amino acids. Besides the Krebs cycle, other metabolic reactions contribute in *Past. multocida* to the biosynthesis of amino acids. Thus, labeling of tyrosine (and eventually tryptophan) proves

TABLE VI. Distribution (%) of C^{14} After Incorporation of Formate-C^{14} by *Past. multocida*. Reaction mixture contained 31 mg of resting cells in 12 ml of mM phosphate buffer, pH 7.2. Formate-C^{14}, 20.4 μmole (1.18×10^7 c.p.m.). Glucose, 60 μmole (Exp *A*) or none (Exp *B*). Incubation at 37° for 60 min.

	C^{14} fixed (% of total in the methanol-water-soluble substances)	
Substances	Exp *A*	Exp *B*
Citric acid	2.1	6.4
Cis-Aconitic "	1.4	3.4
Succinic "	8.2	3.5
Fumaric "	7.1	2.0
Malic "	6.2	0
Aspartic "	2.4	3.3
Glutamic "	9.7	5.8
Alanine	12.1	1.4
Glycine	1.9	2.8
Serine	1.2	1.4
Cystine	6.1	5.7
Cysteic acid	8.5	3.5
Leucine	1.2	2.4
Tyrosine	0	1.8

the contribution of the pentose pathway(1) through heptose and shikimic acid(12); on the other hand, leucine (and eventually valine) should be synthesized from acetate-1-C^{14}.

Formate is extensively used by *Past. multocida* for synthesis of amino acids (Table VI) and purine bases(13). The relatively higher incorporation of C^{14} into the serine group (glycine-serine-cystine-cysteic acid) is consistent with formate fixation in serine as described in animal tissues(14). After transformation into pyruvic acid (serine dehydrase reaction), serine can label the Krebs cycle intermediates and related amino acids. However, the possibility of direct incorporation of formate into pyruvate(15) cannot be ruled out. The synthesis of cystine from a 3-carbon compound agrees well with the incorporation of sulfur by *Past. multocida*(16).

Summary. *Past. multocida* oxidizes pyruvate according to the Krebs cycle model. *Iso*-citrate dehydrogenase, aconitase, succinic dehydrogenase, fumarase and malic dehydrogenase were demonstrated with cell-free extracts or disrupted-cell preparations. The organism synthesizes aliphatic and aromatic amino acids from pyruvate carbon atoms. Formate carbon was incorporated into the serine-group of amino acids, alanine, the Krebs cycle intermediates and related amino acids, and nucleic acids.

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Development of Lactic Dehydrogenase Isozymes in the Chick Embryo.* (28960)

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Cells cultured *in vitro* undergo extensive ultrastructural(1) and histochemical(2,3) alterations. Among the histochemical alterations is the acquisition of a common electrophoretic pattern of lactic dehydrogenase (LDH) isozymes by organs which exhibited different patterns of isozymes prior to culture (3). In contrast, cells which have been grown on the chick chorioallantoic membrane (CAM) exhibit morphological characteristics not unlike those of the same cells cultivated *in vivo*(1). However, it has not been determined if the apparent morphological stability of CAM cultures is accompanied by stability of isozymes.

In the present study the LDH isozymes of

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