SOME METABOLIC ACTIVITIES OF *VIBRIO FETUS* OF BOVINE ORIGIN[1]

E. M. KIGGINS AND W. N. PLASTRIDGE

Department of Animal Diseases, University of Connecticut, Storrs, Connecticut

Received for publication August 26, 1957

Little is known concerning the basic nutritional requirements of *Vibrio fetus*, an organism now recognized to be an important economic cause of sterility in cattle. Attempts to grow the organism in synthetic media have failed, although it will grow in a variety of rather complex media. In an effort to elucidate the nutritional requirements, a study of the oxidative metabolism employing the Warburg respirometer was undertaken. The results are reported here.

MATERIALS AND METHODS

Cultures. Two cultures of *V. fetus* which were isolated from aborted bovine fetuses were used; a laboratory strain which had been maintained in thiol medium (Difco) for seven years, and a recently isolated strain. The organisms were catalase positive, reduced nitrates, did not produce hydrogen sulfide, and grew optimally in an atmosphere of 5 per cent oxygen, 10 per cent carbon dioxide and 85 per cent nitrogen (Kiggins and Plastridge, 1956).

Preparation of cellular suspensions. Cells for the manometric studies were prepared by inoculating the surface of thiol agar (thiol medium with 2.5 per cent added agar) in Kolle flasks with a 48-hr-old thiol culture and incubating the flasks at 37 C in 5 per cent oxygen, 10 per cent carbon dioxide and 85 per cent nitrogen for 24 hr. The cells were suspended in 0.2 M Tris buffer, tris-(hydroxymethyl)aminomethane, pH 7.0, centrifuged, and resuspended in Tris buffer so that 1 ml contained approximately 1 mg of total nitrogen. The nitrogen determinations were made on cells suspended in Sorensen's phosphate buffer (pH 7.0). A loopful of the cell suspension was streaked on the surface of a blood agar plate to check for contamination.

Preparation of cell free extract. Crude extracts were prepared by subjecting a suspension of cells, adjusted so that 1 ml contained approximately 1 mg of total nitrogen, to sonic oscillation in a Raytheon 10 kc sonic oscillator for 10 min. This crude extract was used as such in the Warburg flasks and was not supplemented.

Manometric methods. Conventional manometric techniques (Umbreit *et al.*, 1951) were employed using a Warburg apparatus at 30 C. Substrates were used in quantities of either 10 or 100 μmoles. All flasks contained 1 ml of buffer (pH 7.0) and 1 ml of cell suspension in the main compartment, 1 ml of substrate in the side arm and 0.2 ml of 20 per cent KOH in the center well. The atmosphere was either air or 5 per cent oxygen and 95 per cent nitrogen. The oxygen uptake with each substrate was tested at least three times. Therefore, the figures in the results represent an average.

Determination of citric acid. Citric acid accumulation was determined by the method of Ettinger *et al.* (1952). To large Thunberg tubes was added 100 μmoles each of acetate and oxalacetate, 10 μmoles $MgCl_2$, 1 μmole adenosine triphosphate, 1 μg coenzyme A (CO A, obtained from Nutritional Biochemical Corporation), 1 ml of cell suspension, and enough buffer (pH 8.0) to bring the volume to 25 ml. The tubes were incubated for 24 hr at 37 C in an atmosphere of nitrogen. Due attention was given to the unknown decomposition product of oxalacetate, believed to interfere with the determination of citric acid (Umbarger, 1954).

A blank consisting of the above components but substituting water for the acetate and oxalacetate solutions was negative for citrate.

RESULTS

Oxidation of common carbohydrates. There was no oxygen uptake with any of the common carbohydrates, either with whole cells or crude extracts, in an atmosphere of either air or 5 per cent oxygen and 95 per cent nitrogen, or with the substrates

[1] Supported in part by funds provided by the Research and Marketing Act of 1946 for the regional research project NE-1, Sterility in Dairy Cattle; and Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

Based in part on a thesis submitted by the senior author to the Graduate School of the University of Connecticut in partial fulfillment of the requirements for the Ph.D. degree.

at a concentration of 10 or 100 μmoles. The compounds used were: adonitol, D-arabinose, galactose, inositol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. Also, there was no oxygen uptake with glycerol (1 or 5 per cent).

Oxidation of glycolytic compounds. Whole cells or crude extracts were added to Warburg flasks containing 10 and 100 μmoles of the following substrates: glucose, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose, fructose 1,6-diphosphate, phosphoglyceric acid, sodium pyruvate and ribose-5-phosphate. The atmosphere was either air or 5 per cent oxygen and 95 per cent nitrogen. With the exception of sodium pyruvate, there was no significant oxygen uptake exceeding the endogenous. The $Q_{O_2}^{(N)}$ values with sodium pyruvate for the laboratory strain were 274 (whole cells) and 190 (crude extract). For the recently isolated strain, the $Q_{O_2}^{(N)}$ values were 218 (whole cells) and 66 (crude extract).

Oxidation of compounds of the tricarboxylic acid cycle. There was rapid oxygen uptake with all members of the tricarboxylic acid cycle which were tested when a crude extract was employed (table 1). Extracts of the old isolate showed a significantly higher oxygen uptake than extracts of the recent isolate. However, with whole cells, there was only slight or no oxygen uptake with citrate, *cis*-aconitate, isocitrate and α-ketoglutarate. There was little difference in the oxidative activities of whole cells of the old and recent isolate.

The incubation of 1 ml of crude extract (about 1 mg nitrogen) with 100 μmoles each of acetate and oxalacetate resulted in an average accumulation of 17 μmoles of citric acid for the old isolate and 19 μmoles for the recently isolated strain.

Oxidation of amino acids. Of the 23 amino acids tested, there was oxygen uptake only with glutamic acid, glutamine, aspartic acid, asparagine, proline, and cysteine (table 2). The $Q_{O_2}^{(N)}$ values were relatively high with all of these amino acids and relatively low with cysteine. There was little difference in the oxidative activities of the two strains. There was no oxygen uptake with whole cells or crude extracts of either strain with L-cystine, hydroxy-L-proline, L-leucine, DL-isoleucine, DL-valine, DL-threonine, DL-ornithine (mono HCl), L-arginine, glycine, DL-methionine, DL-serine, DL-alanine, L-tyrosine, L-histidine, L-phenylalanine, L-lysine (mono HCl) and L-tryptophan.

To determine if any of the amino acids were inhibitory, the amino acids that were not oxidized were added to Warburg flasks containing either glutamic acid, glutamine, aspartic acid,

TABLE 1

Respiratory metabolism of whole cells and crude extracts of Vibrio fetus with the tricarboxylic acid cycle compounds

Substrate*	Old Isolate				Recent Isolate			
	Whole cells		Crude extract		Whole cells		Crude extract	
	Q_{O_2} (N)†	Total μL O_2	Q_{O_2} (N)†	Total μL O_2	Q_{O_2} (N)†	Total μL O_2	Q_{O_2} (N)†	Total μL O_2
Citrate	4	6	197	336	6	10	27	34
cis-Aconitate	4	6	116	204	0	0	40	60
Isocitrate	5	7	89	105	0	0	56	70
α-Ketoglutarate	23	30	196	251	0	0	107	237
Succinate	299	310	189	283	410	420	155	218
Fumarate	300	309	190	285	411	425	156	220
Malate	330	335	200	364	442	446	166	243
Oxalacetate	120	128	165	206	80	85	40	65

* All flasks contained 10 μmoles of substrate. Gas phase air, temperature 30 C. Total time of incubation, 3 hr.

† Endogenous subtracted. The magnitude of the endogenous oxygen uptake per hr per mg N for the old isolate was 15 μL (whole cells) and 30 μL (crude extract). For the recently isolated strain the endogenous oxygen uptake was 20 μL (whole cells) and 28 μL (crude extract).

TABLE 2

Oxidative dissimilation of amino acids by whole cells and crude extracts of Vibrio fetus

Substrate*	Old Isolate				Recent Isolate			
	Whole cells		Crude extract		Whole cells		Crude extract	
	Q_{O_2} (N)†	Total μL O_2	Q_{O_2} (N)†	Total μL O_2	Q_{O_2} (N)†	Total μL O_2	Q_{O_2} (N)†	Total μL O_2
L-Glutamic acid	451	808	449	895	350	485	300	405
Glutamine	79	380	88	419	70	210	72	168
L-Aspartic acid	327	479	350	472	275	310	131	156
L-Asparagine	341	460	315	495	280	306	138	174
L-Proline	610	850	530	897	196	384	137	326
L-Cysteine	69	155	47	151	53	89	48	73

* All flasks contained 10 μmoles of substrate. Gas phase air, temperature 30 C. Total time of incubation, 3 hr.

† Endogenous subtracted. The magnitude of the endogenous oxygen uptake per hr per mg N for the old isolate was 14 μL (whole cells) and 28 μL (crude extract). For the recently isolated strain the endogeneous oxygen uptake was 18 μL (whole cells) and 25 μL (crude extract).

asparagine, proline, or cysteine. In no instance was there a decrease or an increase in the oxidative activity.

Oxidation of fatty acids. The following fatty acids were supplemented with 1 μg of CO A and added to Warburg flasks: acetate, arachidic acid, butyric acid, capric acid, caproic acid, caprylic acid, lauric acid, linoleic acid, oleic acid, palmitic acid, and stearic acid. These were supplied in 10 and 100 μmole quantities. The fatty acid was employed as the free acid and as the sodium salt. Most of the acids were insoluble in water and therefore an emulsion was prepared by subjecting the fatty acid-water mixture to 30 min of sonic oscillation (10 kc).

The only fatty acid that displayed any oxygen uptake was acetate, with or without added CO A. The $Q_{O_2}{}^{(N)}$ values for the old isolate were 130 (crude extract) and 101 (whole cells). For the recently isolated strain the $Q_{O_2}{}^{(N)}$ values were 211 (whole cells) and 73 (crude extract).

DISCUSSION

V. fetus does not seem to possess the ability to oxidize any of the common carbohydrates. This substantiates the findings of Smith and Taylor (1919) and Plastridge and Williams (1943) who found no indication of acid or gas formation from a number of carbohydrates. Further, *V. fetus* does not seem to possess the enzymes of the Embden-Meyerhof glycolytic schema. There was no oxygen uptake with any of the members of this schema that were tested, with the exception of pyruvate. Although other types of anaerobic respiration exist, these results, along with the finding of Kiggins and Plastridge (1956) that *V. fetus* will not grow under anaerobic conditions, suggests that the organism does not respire anaerobically.

All of the intermediates of the tricarboxylic acid cycle tested were oxidized by *V. fetus*. The $Q_{O_2}{}^{(N)}$ values were relatively high for fumarate, succinate and malate. Citrate, *cis*-aconitate, isocitrate and α-ketoglutarate exhibited little or no oxygen uptake with whole cells. However when crude extracts were used, there was a significant oxygen uptake indicating that these four compounds were not permeable to the intact cell. Acetate condensed with oxalacetate to form citrate. These findings indicate an active tricarboxylic acid cycle in *V. fetus*.

Six of the 23 amino acids tested were oxidized. All of these can readily enter the tricarboxylic acid cycle with the possible exception of proline. However, there is evidence that proline can be converted to glutamic acid (Vogel and Davis, 1952; and Strecker, 1957).

From the results presented here it would appear that *V. fetus* can obtain energy only from compounds either of the tricarboxylic acid cycle or compounds which can be easily introduced into the cycle.

It was found by Kiggins and Plastridge (1956)

that *V. fetus* would not grow in air. Consequently, at the beginning of this study an atmosphere of 5 per cent oxygen and 95 per cent nitrogen was used. When air was substituted, in every instance, the oxygen uptake was greater. Therefore, it would appear that the growth of *V. fetus* is dependent on some metabolic activity other than oxidation of the compounds tested in this report.

SUMMARY

Investigation of the metabolic activities of *Vibrio fetus* using a Warburg apparatus showed that the common carbohydrates were not oxidized, and further, with the exception of sodium pyruvate oxidation the Embden-Meyerhof glycolytic schema was not operative. However, all the compounds of the tricarboxylic acid cycle were oxidized. Of the 23 amino acids tried, there was oxygen uptake only with glutamic acid, aspartic acid, glutamine, asparagine, proline and cysteine. Acetate was the only fatty acid tested which was oxidized.

REFERENCES

ETTINGER, R. H., GOLDBAUM, L. F. AND SMITH, L. H. 1952 A simplified photometric method for the determination of citric acid in biological fluids. J. Biol. Chem., **199**, 531–536.

KIGGINS, E. M. AND PLASTRIDGE, W. N. 1956 Effect of gaseous environment on growth and catalase content of *Vibrio fetus* cultures of bovine origin. J. Bacteriol., **72**, 397–400.

PLASTRIDGE, W. N. AND WILLIAMS, L. F. 1943 Observations on *Vibrio foetus* infection in cattle. J. Am. Vet. Med. Assoc., **102**, 89–95.

SMITH, T. AND TAYLOR, M. S. 1919 Some morphological and biological characters of the spirilla (*Vibrio fetus*, n. sp.) associated with disease of the fetal membranes in cattle. J. Exptl. Med., **30**, 299–312.

STRECKER, H. J. 1957 The interconversion of glutamic acid and proline. I. The formation of Δ′pyrroline-5-carboxylic acid from glutamic acid in *Escherichia coli*. J. Biol. Chem., **225**, 825–834.

UMBREIT, W. W., BURRIS, R. H., AND STAUFFER, J. F. 1951. In *Manometric techniques and tissue metabolism*, 2nd ed. Burgess Publishing Co., Minneapolis.

UMBARGER, H. E. 1954 The influence of the environment on acetate metabolism in *Escherichia coli*. J. Bacteriol., **68**, 140–145.

VOGEL, H. J. AND DAVIS, B. D. 1952 Glutamic γ-semialdehyde and Δ′pyrroline-5-carboxylic acid, intermediates in the biosynthesis of proline. J. Am. Chem. Soc., **74**, 109–112.