ANAEROBIC DEGRADATION OF CHOLINE

I. Fermentation of Choline by an Anaerobic, Cytochrome-Producing Bacterium, *Vibrio cholinicus* n. sp.[1]

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The aerobic metabolism in mammalian tissues of the onium compounds choline and betaine has been elucidated by several investigators (Du Vigneaud, 1952; Mackenzie, 1955; Stetten, 1941; Soloway and Stetten, 1953). Generally, aminoethanol is methylated in a stepwise fashion through its N-methyl- and N-dimethyl derivatives to choline. Oxidation of choline to betaine via betaine aldehyde labilizes the methyl groups; betaine then is progressively demethylated through dimethylglycine and sarcosine to glycine with the concomitant formation of "active 1-carbon fragments."

The anaerobic metabolism of these compounds and their partially demethylated derivatives has been largely neglected, however. By analogy to the high energy sulfonium compounds, for example S-adenosylmethionine (Cantoni, 1952), such quaternary amines as choline and betaine offer the possibility of realizing biologically useful energy from the transformation of an onium compound to a non-onium product as well as from oxido-reduction reactions on the carbon chain moieties of the molecules. Thus, new high energy compounds of general biochemical interest might be sought in systems capable of such fermentations. Further, the metabolism of compounds of this general class by anaerobic organisms might offer an opportunity to study anaerobic "1-carbon fragment" metabolism.

Microorganisms capable of utilizing an onium compound as the sole substrate for growth should be the biological material best suited to such studies. Accordingly, using the enrichment culture technique, a search was made for anaerobic bacteria able to grow on these compounds as single substrates. Pure cultures of several organisms were obtained on choline, N-dimethylaminoethanol, N-methylaminoethanol, betaine,

[1] A culture of this organism has been sent to the American Type Culture Collection.

dimethylglycine, and sarcosine. The present communication is concerned with the isolation and characterization of one of these organisms, isolated on choline, and with the over-all fermentation reaction which supports growth.

MATERIALS AND METHODS

Enrichment culture medium. The original enrichment cultures were made by inoculating, with an approximately 25-g sample of mud, 100 ml of a medium of the following composition: choline chloride, 0.5 per cent; KH_2PO_4, 0.272 per cent; NH_4Cl, 0.4 per cent; $MgCl_2 \cdot 6H_2O$, 0.02 per cent; $FeCl_3 \cdot 6H_2O$, 0.0001 per cent; $CaCl_2 \cdot 2H_2O$, 0.001 per cent; methylene blue, 0.0002 per cent; phenol red, 0.0003 per cent; and $Na_2S \cdot 9H_2O$, 0.05 per cent. In later cultures it was determined that sodium hydrosulfite, 0.0035 per cent, was tolerated by the organism and it was used thereafter in place of sulfide to remove oxygen from the culture medium prior to inoculation. The pH was adjusted to 6.8 to 6.9 with HCl. After the third transfer, $MnSO_4 \cdot 2H_2O$ and Na_2MoO_4 were added routinely at a concentration of 0.0001 per cent each. Growth responses were estimated either visually or turbidimetrically at 660 mμ with a Klett photoelectric colorimeter.

Analytical methods. Acetate was quantitated by titration of the steam distillate of acidified growth medium and identified by Duclaux distillation. Total steam volatile base was similarly measured by titration of the steam distillate from alkalinized growth medium. Ammonia was measured independently either by direct Nesslerization or by Nesslerization following microdiffusion into 0.5 N H_2SO_4 in Conway dishes. Since these methods gave identical results, the direct procedure was routinely used. The amount of organic steam volatile base was calculated by difference between total steam volatile base and ammonia.

Ethanol was allowed to diffuse into a standard solution of 0.05 N sodium dichromate in 10 N H_2SO_4 for 18 hr at room temperature in Conway dishes. The amount of dichromate consumed was estimated by oxidation of KI with the excess dichromate and titration of the iodine formed with thiosulfate. Carbon dioxide was measured by the Conway (1957) microdiffusion technique.

In some cases, choline was precipitated as the reineckate and quantitated by a modification of the method of Engel et al. (1954) developed by Dr. M. Horning (1958, personal communication). However, trimethylamine, a product of choline fermentation, also precipitates reinecke salt; hence, in the carbon balance studies, an accurately weighed amount of oven-dried choline was added initially and the culture incubated at 37 C for at least 10 days, to insure complete utilization of the substrate. By this time, choline was no longer detectable chromatographically under conditions where as little as 0.7 μmole per ml was readily detectable. An n-butanol-ethanol-acetic acid-water (8:2:3:1) solvent system was employed and color was developed with 0.2 per cent dipicrylamine in 50 per cent aqueous acetone (Augustinsson and Grahn, 1953). In fermentation media containing choline labeled with C^{14} in the 1,2-positions, choline was determined as acid-stable radioactivity (the only labeled products, acetate and ethanol, are volatilized when a sample is dried at acid pH).

Radioactivity was assayed with an end window Geiger-Muller tube; all data are corrected for background and self-absorption. Carbon-14-labeled choline was purchased from the Volk Radiochemical Company, Chicago, Illinois.

RESULTS

Isolation of pure cultures. The sources of the organisms were black muds from a small stagnant creek near Chapel Hill, North Carolina, and from a similar stream near Bethesda, Maryland. Gas production was prominent in the first two cultures as early as the first day, but in later cultures no gas production was observed. After three successive subcultures, using 10 per cent inocula, a medium sized, actively motile rod overwhelmingly predominated; the organisms were then passed several times through deep agar shake cultures, and a pure culture obtained. No difficulty was encountered in effecting isolation of the organism from the North Carlina mud, but

in the case of the Maryland organism, another bacterium persistently formed satellite colonies around the colonies of the choline-fermenting organism. The organism in the satellite colonies was found to grow luxuriantly on a mixture of acetate, ethanol, and trimethylamine, later shown to be the products of choline fermentation, and was effectively eliminated by a series of rapid transfers. Since both organisms carry out the same fermentation and are essentially identical morphologically, they are assumed to be different strains of the same species. The organism isolated from the North Carolina mud was used for the experiments reported here.

Nutritional characteristics. Of several compounds tested the organism prefers choline as a substrate for growth, although 1 per cent yeast extract (Difco) is nearly as effective; 1 per cent proteose peptone (Difco) or beef broth are somewhat less effective in supporting growth. Several sugars, alcohols, and amino acids, betaine, N-dimethylaminoethanol, N-methylaminoethanol, aminoethanol, and dimethylpropiothetin do not support growth. Although not absolutely essential for growth on choline, 0.1 to 0.2 per cent yeast extract (Difco) was markedly stimulatory and was routinely included in the growth medium. Similarly, carbon dioxide, though it was not incorporated into the reaction products and disappeared from the medium only in very small amounts, was found to stimulate growth and was included in the growth medium as $NaHCO_3$, 0.3 to 0.5 per cent. The optimum pH for growth was 6.6 to 6.9; the bicarbonate thus served also as a buffer. In large scale cultures it was later noted that when the bicarbonate was neutralized with sulfuric acid rather than hydrochloric acid a significant increment in growth occurred. Under such conditions appreciable amounts of hydrogen sulfide were formed.

Standard microbiological tests were performed under anaerobic conditions with the following results:

Action on carbohydrates determined in phenol red media (Difco), Durham tubes in place: No acid or gas from glucose, galactose, fructose, arabinose, xylose, maltose, sucrose, starch, dextrin, cellobiose, inulin, mannitol, glycerol, sorbitol, adonitol, dulcitol, or pectin.

Indole not produced in tryptone medium (Difco).

Nitrates not reduced to nitrite in 0.1 per cent

peptone medium containing 0.02 per cent potassium nitrate.

Gelatin not liquefied in horse meat infusion broth containing 12.5 per cent gelatin.

Hydrogen sulfide formed from sulfate in the choline medium described in Materials and Methods.

No growth in litmus milk.

No growth on defibrinated rabbit blood agar slants.

Egg yolk agar slants: no growth.

Potato slants: no growth.

Horse meat infusion broth: no growth.

Morphological characteristics. The organism is characterized by rather pronounced variability of its morphology depending on the age of the culture and the conditions under which it is grown. It is a small, slightly curved rod measuring about 0.5 by 2 to 4 μ, occurring usually singly, occasionally in pairs, and, in actively growing cultures, in spiral chains of up to 10 cells. It is actively motile, possessing a single polar flagellum. During the log phase of growth in the medium described above, it is routinely gram-negative, but in very old or very young cultures, it is definitely gram-positive and displays a markedly beaded appearance throughout the cytoplasm. In several old cultures, dark, round, sporelike bodies which are highly refractile have been seen; these are usually subterminal and slightly bulge the cells. However, such preparations when stained with malachite green revealed no spores. These structures have not been observed in any of the cultures grown in the presence of large amounts of sulfate. In deep agar cultures, in the medium described, colonies are brown, lens-shaped, and 0.5 to 3 mm in diameter. After the substrate is consumed in liquid cultures, the cells readily autolyze. Growth occurs only in the absence of oxygen.

Presence of a cytochrome pigment. Since extracts of the organism are frequently red in color, their spectral characteristics were examined over the visible range with the Cary recording spectrophotometer. Several sonic extracts were thus examined and all were found to contain a substance which, upon reduction with sodium hydrosulfite, revealed the typical absorption spectrum of a cytochrome c type pigment. Thus, in the reduced state the absorption maxima are at 418, 523, and 553 mμ, and in the oxidized form there is a single peak at 407 mμ. From the intensity of the peaks at 407 and 418 mμ it is calculated, assuming the same extinction as for beef heart cytochrome c, that the crude sonic extracts (50 mg protein per ml) contain about 0.015 μmole of cytochrome per ml.

Identification of products and stoichiometry of choline fermentation. Carbon balances were carried out using 100 ml growing cultures in the above medium with 0.1 per cent yeast extract and 0.3 to 0.5 per cent NaHCO$_3$. The presence or absence of the added CO$_2$ did not detectably alter the products of the reaction or the stoichiometry. After 4 or 5 days of active growth, the turbidity disappeared and the cultures were opened. Samples of growth medium before and after growth were analyzed for choline, steam volatile acid, steam volatile base, ammonia, alcohol, and carbon dioxide. Typical data are presented in table 1. Thus, for each mole of choline consumed, approximately 1 mole of steam volatile base (after correcting for the ammonia present), $\frac{1}{2}$ mole of steam volatile acid, and $\frac{1}{2}$ mole of alcohol were formed. The acid was shown to be acetate by Duclaux distillation. The alcohol was identified as ethanol by steam distillation of the mixture after oxidation with dichromate and determination of Duclaux constants on the distillate; these were identical with the constants obtained for pure acetic acid.

The picrate of the steam volatile base was prepared, recrystallized 3 times from boiling ethanol, and the melting point determined. Comparison with an authentic sample of trimethylamine

TABLE 1

*Carbon balance of choline fermentation by Vibrio cholinicus*

Compound	Initial	Final	Δ
Choline...............	3.50	0.31	−3.19
Acetate..............	0.15	1.74	+1.59
Ethanol..............	0.03	1.35	+1.32
Trimethylamine......	0	3.18	+3.18
Ammonia............	3.11	3.05	−0.06
Carbon dioxide.......	3.89	3.72	−0.17

Data are expressed as mmoles per 100 ml of culture medium. Initial and final values are those before and after growth, respectively, as described in the text. In this case, choline was measured as the reineckate after chromatographic separation from trimethylamine.

picrate (experimental, 215.5 to 216.5 C; authentic, 216 C; mixed, 215 to 216 C) showed that the fermentation product is trimethylamine.

Carbon balance studies on cultures grown in the absence of supplemental carbon dioxide were also carried out. The same products were identified and the stoichiometry was identical with that for cultures with added carbon dioxide. Furthermore, in experiments with $C^{14}O_2$, none of the isotope was found in acetate or trimethylamine and about 95 per cent was recovered from the culture medium as barium carbonate. The remainder was presumably incorporated into cell material.

The over-all reaction for the fermentation of choline by this organism can thus be written:

$$2(CH_3)_3N^+CH_2CH_2OH \; + \; H_2O \; \rightarrow$$

$$2(CH_3)_3\overset{+}{N}H \; + \; CH_3COOH \; + \; CH_3CH_2OH$$
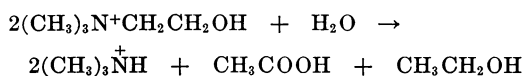
Further evidence in support of this formulation is derived from experiments using C^{14}-labeled choline; representative data are presented in table 2. Thus, starting with methyl-labeled choline, nearly all of the isotope appears in the trimethylamine, and the specific activity of the product is approximately that of the initial choline. No radioactivity is present in the acetate or ethanol. When the substrate is labeled in the side chain, however, all of the isotope is recovered in the acetate and ethanol and none is incorporated into the trimethylamine. The fact that the

TABLE 2

Fermentation of C^{14}-labeled choline by Vibrio cholinicus

Compound	Specific Activity	
	Choline-methyl-C^{14}	Choline-1,2-C^{14}
	cpm/μmole	*cpm/μmole*
Choline...................	77.5	1500
Acetate..................	0	1600
Ethanol..................	0	1030
Trimethylamine...........	63.5	<10
Carbon dioxide...........	0	<1

Values given for choline are those before growth; those for the other compounds were determined after growth had ceased. Each culture initially contained 3.5 mmoles of choline per 100 ml of culture medium. Conversion to products was complete and the stoichiometry agreed with that presented in table 1.

ethanol assay is not absolutely specific probably accounts for the low specific activity of the ethanol observed here; i. e., any neutral volatile compound capable of reducing dichromate is measured. In neither case is there any appreciable radioactivity recovered in the carbon dioxide.

DISCUSSION

The bacterial fermentation of choline described above is quite unlike the known pathways of choline metabolism in mammalian tissues. Clearly this is an energy-yielding reaction since the organism must derive all of its energy for growth from choline degradation when this is the only organic substrate in the growth medium. It is probable that the primary source of energy from this over-all process is the oxido-reduction reactions on the 2-carbon moiety of choline, although the possibility of some contribution from the "onium bond energy" cannot absolutely be excluded.

The presence of a cytochrome in an obligately anaerobic comma-shaped bacterium which appears to reduce sulfate is reminiscent of the sulfate-reducing vibrio, *Desulfovibrio desulfuricans*. But the organism described here is clearly distinguished from Desulfovibrio by virtue of its highly specific substrate requirements for growth. Reaction to the Gram stain is also different; cells in very young cultures of the choline organism are gram-positive, as are the numerous granules that are characteristically present in old cells. Also, *D. desulfuricans* does not develop in the choline medium unless supplemented with sulfate.

By the morphological and nutritional characteristics described, the choline organism corresponds to no previously described organism known to the authors, and it is thus considered to be a new species. It is tentatively classed in the genus *Vibrio*, and the proposed name is *Vibrio cholinicus* n. sp. It is clearly different from the two strictly anaerobic species of *Vibrio* described in *Bergey's Manual of Determinative Bacteriology* (7th ed.), *V. sputorum* and *V. niger*. The former is larger and grows well on blood media, whereas the latter decomposes a variety of carbohydrates; the choline organism shares none of these properties. If the sporelike bodies that are sometimes observed in cells of the organism prove to be true endospores, then it might properly be considered a member of the genus *Clostridium*.

Cytochrome pigments, previously considered

to be distributed only in aerobic forms of life, have recently been found to occur in two types of obligately anaerobic organisms: certain photo-autotrophs, e. g., *Chromatium* and *Chlorobium* (Vernon and Kamen, 1954; Newton and Kamen, 1956) and the sulfate-reducing *D. desulfuricans* (Postgate, 1954; Ishimoto *et al.*, 1954). In the latter organism, the studies of Postgate (1956) and of Ishimoto *et al.* (1958) have revealed that the cytochrome has a very low potential and functions in the over-all process of sulfate reduction. The organism described here is clearly related to *D. desulfuricans*, but the functional role of its cytochrome has not as yet been elucidated. The possibility exists that it functions in an electron transport reaction associated with sulfate reduction; it might also be involved as an electron carrier in the fermentation of choline. Studies of its function in this organism are being pursued.

SUMMARY

A new member of the genus *Vibrio* has been isolated from soil by the enrichment culture technique with choline as the sole carbon source. The morphological, nutritional, and physiological characteristics of the organism are described. In the over-all fermentation reaction, 1 mole of choline is converted to 1 mole of trimethylamine, and $\frac{1}{2}$ mole each of acetic acid and ethanol. Experiments with C^{14}-labeled choline reveal that trimethylamine is the only radioactive product when choline-methyl-C^{14} is fermented; when choline-1,2-C^{14} is the substrate, all of the isotope is recovered in the ethanol and acetate.

A striking characteristic of this organism, an obligate anaerobe, is its production of rather large amounts of a cytochrome-like pigment. Possible functions of this pigment are discussed.

The organism has been tentatively placed in the genus *Vibrio* by morphological and nutritional considerations, and the name *Vibrio cholinicus* n. sp. is proposed.

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