# ON THE METABOLISM OF AN AMINO ACID FERMENTING CLOSTRIDIUM

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Two anaerobic microorganisms were isolated by the enrichment culture technique from a sodium formate-mineral salts medium inoculated with black mud from the San Francisco Bay. One of these, a motile coccus designated *Methanococcus vannielii*, carries out a methane fermentation of formate (Stadtman and Barker, 1951). The second organism that was almost equally numerous in the enrichment cultures is a clostridium that utilizes the Stickland reaction, a coupled oxido-reduction reaction between amino acid pairs. The addition of formate causes marked stimulation of growth of the clostridium under certain conditions. This observation suggested that the organism might be suitable biological material for studies on formate intermediary metabolism. Moreover, tracer experiments which are described in another communication showed that considerable amounts of formate carbon appear in alanine and the branched chain amino acids, valine, leucine, or isoleucine.

## METHODS

Growth responses to variations in the culture media were determined turbidimetrically at 540 mμ using a Coleman Junior spectrophotometer.

Qualitative and semiquantitative estimations of amino acid utilization were made on 0.02 ml aliquots of the culture media applied directly to Whatman no. 1 filter paper and chromatographed in 80 per cent phenol, 80 per cent pyridine, 70 per cent propanol, or the butanol-propionic acid-water solvent system of Benson *et al.* (1950). The amino acid spots were detected by treatment with ninhydrin. Complex media, before and after growth, were subjected to two dimensional chromatography whereas a single solvent system usually sufficed for examination of the media containing only a pair of amino acids. When a preliminary desalting step was necessary to achieve clean separations, "duolite C-3" or "dowex-50" columns in the hydrogen cycle were employed. When culture filtrates which had been evaporated to small volumes were passed over such columns, all cations including amino acids which have a net positive charge at acid pH (1.0 to 2.0) were retained whereas neutral compounds and other acids passed through the resins. The amino acids were eluted quantitatively from the columns with 1 N $NH_4OH$ and freed from excess ammonia by evaporation to dryness. Alanine and δ-amino valeric acid were estimated by the Moore and Stein photometric ninhydrin method (1948) after their isolation by means of paper chromatography. Proline and ornithine were estimated by the ninhydrin procedure of Chinard (1952). Arginine was determined in aliquots of the unfractionated culture media using a modification of the Sakaguchi method (Macpherson, 1942). Citrulline was also determined directly on aliquots of the culture media by the method of Archibald (1944).

Ammonia was distilled into boric acid and titrated with HCl (Ma and Zuazaga, 1942). Carbon dioxide was measured manometrically (Peters and Van Slyke, 1932).

Fatty acids were separated from the growth media by steam distillation in the apparatus described by Markham (1942) and were isolated by partition chromatography on silica gel columns (Elsden, 1946). Their purity was established by Duclaux distillation. Formic acid was estimated manometrically after oxidation to $CO_2$ (Pickett *et al.*, 1944).

*Morphology.* A clostridium, strain HF, is a gram positive, slender rod 1 to 4 μ in length occurring singly, in pairs, and occasionally in short chains. It is very actively motile; the type of flagellation has not been determined. Spores, which bulge the cells slightly, are located centrally or subterminally. Spore production is not abundant even in old cultures. Deep agar colonies are 1 to 2 mm in size, lens shaped, becoming lobate and soft.

## EXPERIMENTAL RESULTS

*Energy substrates.* The isolation of a pure culture of clostridium, strain HF, is described in an

earlier paper (Stadtman and Barker, 1951) where the organism is referred to as a rod shaped bacterium able to grow slightly in a formate-mineral medium supplemented with 0.1 per cent yeast extract (Difco) A small amount of gas consisting of hydrogen and $CO_2$ was evolved during growth in this medium. Formate, when added as the sole carbon source, did not support growth. The marked stimulation of growth by the addition of high levels of yeast extract (up to 5 per cent) suggested that one or more components of this material might be serving as the energy source for the clostridium. Acid hydrolyzed, vitamin-free casein (Nutritional Biochemical Corp.), when added to 0.1 per cent yeast extract, also supported good growth whereas a variety of other substrates such as glucose, lactate, pyruvate, acetate, glutamate, purine, and pyrimidine containing materials and combinations of amino acids known to undergo the Stickland reaction did not stimulate growth over and above the low level supported by 0.1 per cent yeast extract. Two dimensional paper chromatograms of the casein hydrolyzate media before and after growth revealed that serine, threonine, proline, lysine, and possibly some glycine and arginine disappeared during growth. A synthetic mixture of these amino acids substituted equally well for the casein hydrolyzate as determined by turbidimetric measurements. Lysine disappeared completely and most of the arginine and proline was utilized as judged by relative intensities of spots on the chromatograms after treatment with ninhydrin. Utilization of serine, threonine, and glycine was less marked. Accordingly, these six amino acids were tested in various combinations, and it was found that arginine plus proline or arginine plus lysine were effective as substrates. Alone or in other combinations, they did not support growth. Subsequently, it was found that either citrulline or ornithine would replace arginine.

*Growth factor requirements.* In addition to the amino acid pairs utilized for growth there was still a supplemental requirement for yeast extract or tryptone. Various mixtures of all of the usual vitamin supplements did not contain the missing component. Extracts of Fleischmann's 20–40 yeast, whole liver, *Clostridium kluyveri*, and *Methanococcus vannielii*, as well as liver extract (Difco), Seagram's distillers' solubles, and a commercial penicillin autolysate contained insignificant amounts of the required factor or were inhibitory. However, a liver fraction sold under the trade name of "reticulogen" (Eli Lilly & Co., Indianapolis, Ind.) was richer in the factor, on a dry weight basis, than was yeast extract (Difco). The stimulation was not due to the lipoic acid, citrovorum factor, or $B_{12}$ present in the reticulogen since these components were inactive when tested singly or in combination. A more readily available source of the active material proved to be the 70 per cent ethanol soluble material from liver fraction L (Wilson Laboratories, Chicago, Ill.) (Sprince and Woolley, 1944). Fractionation of the liver preparations by various methods yielded information concerning the general properties of the material required for growth of the clostridium on arginine plus proline. It was found to behave as a cation on duolite C-3 or dowex 50 resin columns in the $H^+$ cycle and was eluted with 1 M ammonium hydroxide. Treatment of the cation fraction with acid washed "norite A", a charcoal especially suited for adsorption of nucleotides, resulted in only slight loss of activity although the 245 mμ and 260 mμ absorbing materials were largely removed. Hydrolysis in 6 N HCl at 121 C for 16 hours resulted in almost complete loss of activity.

Chromatographic fractionation of reticulogen on Whatman no. 3 filter paper in 80 per cent phenol or in butanol-propionic acid-water resulted in the separation of a number of ninhydrin reactive bands. These were cut out, eluted with water, and tested for activity. Table 1 shows the distribution of activity on chromatograms of a reticulogen sample run in the two solvent systems. In both cases the entire chromatograms were sectioned into zones and tested. The unfractionated material was employed at a 0.1 per cent (dry weight) level in the test medium, and each chromatographic fraction was tested at a concentration corresponding to that at which it is present when 0.1 per cent (dry wt) of the unfractionated extract is used. In both solvent systems the fractions with significant activity contained ninhydrin reactive material. In view of these properties of the growth stimulatory material, it seemed entirely possible that it was peptidic in nature. Accordingly, mild acid hydrolyzates of insulin, prepared as for "strepogenin" standards (Kodicek and Mistry, 1952), were tested for activity. Addition of the hydrolyzed material (5 mg per 10 ml medium) allowed growth comparable to the unfractionated liver preparations whereas unhydrolyzed insulin

TABLE 1

*Growth responses to addition of reticulogen fractions*

| SUPPLEMENT | DENSITY OF CULTURES AT 540 mμ |
|---|---|
| None | 0.01 |
| Unfractionated "reticulogen" | 0.144 |
| Phenol fractions | |
| $R_f$ = 0.23 | 0.01 |
| $R_f$ = 0.34 | 0.01 |
| $R_f$ = 0.59 | 0.02 |
| $R_f$ = 0.82 | 0.058 |
| $R_f$ = 0.97 | 0.029 |
| Recombined fractions | 0.085 |
| BOH-HP fractions | |
| $R_f$ = 0.06 | 0.01 |
| $R_f$ = 0.15 | 0.02 |
| $R_f$ = 0.28 | 0.036 |
| $R_f$ = 0.42 | 0.041 |
| $R_f$ = 0.51 | 0.045 |
| $R_f$ = 0.61 | 0.063 |
| $R_f$ = 0.73 | 0.036 |
| $R_f$ = 0.85 | 0.015 |
| $R_f$ = 0.95 | 0.013 |
| Recombined fractions | 0.128 |

(5 mg or 10 mg per ml medium) did not support growth. Lower levels of the insulin hydrolyzate were correspondingly less active. The active material appears to be readily dialyzable at neutral pH since 70 per cent of the activity was lost during dialysis against distilled water for 3 hours at 2 C.

The ability of the insulin hydrolyzate to substitute for the liver fractions in supplying material needed for growth of the clostridium on arginine plus proline offers strong evidence in favor of the view that a peptide requirement is involved rather than some, as yet, unknown vitamin or coenzyme. The activity of several distinct ninhydrin reactive bands from the butanol-propionic acid chromatograms of the liver fractions suggests that more than one peptide satisfies this requirement. The identity of the peptide or peptides has not been investigated.

*Effect of formate on growth.* Addition of sodium formate (0.1 per cent) increased the rate of growth on amino acid pairs when 0.03 per cent $Na_2S \cdot 9H_2O$ was used as the reducing agent in the medium. Thioglycolate (0.05 per cent), although slightly inhibitory, was employed as the reducing agent in most experiments. However, in its presence, the addition of formate to the medium caused a marked delay in the initiation of growth.

*Effect of pH on growth.* The effect of the initial pH of the medium on growth of the clostridium was determined in proline-arginine-mineral media containing 0.04 M phosphate adjusted to pH 5.5, 6.6, 7.0, 7.5, and 8.0. Sodium thioglycolate was employed as the reducing agent, and 0.05 per cent sodium formate was present in the medium. In the absence of added $CO_2$ ("oxsorbent" anaerobic seals) the optimum range for growth is pH 7.5 to 8.0. In the series of media containing $CO_2$ (pyrogallol-$K_2CO_3$ anaerobic seals) growth was about 10 to 20 per cent reduced at all pH levels and was considerably poorer at pH 8.0 than at 7.0 or 7.5, the optimum range under these conditions. No growth occurred at pH 5.5 in either series, and at pH 6.6 the maximum turbidity was only 50 to 60 per cent that attained at the higher pH levels.

*Growth medium components.* On the basis of the results of the above studies a suitable medium for cultivation of the clostridium contains the following ingredients in grams per 100 ml: $NH_4Cl$, 0.05; $CaCl_2 \cdot 2H_2O$, 0.001; $MgSO_4 \cdot 7H_2O$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.001; $MnSO_4 \cdot 4H_2O$, 0.0001; $Na_2MoO_4 \cdot 2H_2O$, 0.0001; $K_2HPO_4$-$KH_2PO_4$, 0.04 M, pH 7.5; L-arginine-HCl, 2.0; L-proline, 1.0, or L-lysine-HCl, 2.0; HCOONa, 0.1; $Na_2S \cdot 9H_2O$, 0.03, or sodium thioglycolate, 0.05; phenol red indicator, 0.0003; methylene blue, 0.0002; yeast extract (Difco) or "reticulogen", 0.1; and distilled or tap water. Agar may be used as desired. If thioglycolate is used as a reducing agent, the formate should be omitted from the medium. Sodium sulfide is sterilized separately as a one per cent solution and added to the medium prior to inoculation. The final pH of the medium is 7.5. An "oxsorbent" seal is used since the organism grows only under strictly anaerobic conditions. Cultures are incubated at 30 to 37 C. Maximum growth is attained usually in 36 to 40 hours.

*Amino acids serving as hydrogen acceptors.* The amino acids that had been found to decrease in amount or disappear completely during growth on the casein hydrolyzate-yeast extract media were tested for their ability to serve as hydrogen acceptors. Dried cells (50 mg) of the clostridium or cell-free extracts (36 mg protein) prepared by grinding with alumina (Aluminum Corp. of America, A-301) were incubated in $H_2$ at 37 C for

3 hours with 10 μM of the test amino acid and 0.05 M pH 7.4 potassium phosphate buffer in a total volume of 2.0 ml. A boiled control was included for each amino acid. The reaction mixtures were deproteinized by the addition of ethanol to 80 per cent and examined for ninhydrin reactive products after chromatographic separation on Whatman no. 3 filter paper in 80 per cent phenol and in the butanol-propionic acid-water system. L-Proline, DL-serine, and DL-threonine completely disappeared; intense spots identified by cochromatography as δ-amino valeric acid, α-alanine, and α-amino butyric acid, respectively, appeared. In the absence of these added substrates the reduced products were found in only trace amounts or not at all. A small amount of a material identified chromatographically as glycine was formed also in the samples containing serine and threonine but not in any of the other samples. L-Arginine was decomposed giving rise to citrulline, ornithine, and a trace of δ-amino valeric acid. The first two compounds could have been formed by the so-called arginine dihydrolase series of reactions (Oginsky and Gehrig, 1952), and the latter compound could be the product of a reductive deamination of ornithine (Woods, 1936). $CO_2$ was evolved in the samples incubated with arginine, serine, and threonine. Little, if any, hydrogen uptake was noted in the reaction mixtures. Hence the added substrates either underwent dismutations or were reduced at the expense of oxidizable materials in the dried cells and undialyzed extracts. In support of the latter possibility is the observation that the dried cells, which are able to utilize oxygen as the terminal electron acceptor, showed considerable endogenous $O_2$ uptake.

*Fermentation balances.* Typical results of the arginine plus proline fermentations are presented in table 2. The analyses were performed on one liter cultures which had been incubated until growth had ceased. It is seen that δ-amino valeric acid was produced in amounts equivalent to 90 per cent or more of the proline that disappeared. Although this product also can be derived from ornithine by a reductive deamination at the α-position, it is clear that in these fermentations such a reaction could account for only a part of the δ-amino valeric acid found.

TABLE 2

*Fermentation balances*

| | A | | | B | | | C | | | D | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial | Final | Δ | Initial | Final | Δ | Initial | Final | Δ | Initial | Final | Δ |
| L-Arginine | 1,008 | 37 | −971 | 943 | 41 | −902 | 955 | 32 | −923 | 1,023 | 33 | −990 |
| L-Proline | 910 | 0 | −910 | 910 | 0 | −910 | 960 | 0 | −960 | 910 | 0 | −910 |
| HCOONa | 0 | 0 | | 1,470 | 0 | −1,470 | 0 | 0 | | 1,470 | 0 | −1,470 |
| Citrulline | | 253 | | | 301 | | | 290 | | | 162 | |
| Ornithine | | 312 | | | 421 | | | 117 | | | 40 | |
| Alanine | | 92 | | | 90 | | | 197 | | | 244 | |
| δ-$NH_2$valeric | | 963 | | | 829 | | | 867 | | | 851 | |
| $CO_2$ | | 1,141 | | | 1,418 | | | 953 | | | 1,757 | |
| $NH_3$ | 860 | 3,150 | +2,290 | 910 | 2,850 | +1,940 | 910 | 3,450 | +2,540 | 880 | 3,630 | +2,750 |
| Valeric and/or caproic | | 33 | | | 34 | | | | | | trace? | |
| Butyric | | ca 8 | | | ca 9 | | | ca 20 | | | ca 11 | |
| Propionic | | ca 8 | | | ca 6 | | | ca 12 | | | ca 3 | |
| Acetic | | 353 | | | 371 | | | 860 | | | 1,046 | |
| C recovery | | 99% | | | 94% | | | 96.5% | | | 84% | |
| N recovery | | 98.5% | | | 102% | | | 101% | | | 90.7% | |
| Redox index | | 102 | | | 94 | | | 103 | | | 111 | |

Results are given in μM per 100 ml medium.

Cultures A and B contained 0.05 per cent sodium thioglycolate; cultures C and D, 0.03 per cent $Na_2S \cdot 9H_2O$.

Approximately 90 mg per 100 ml of liver solids ("reticulogen") were present in these media.

The amounts of arginine that disappeared in samples A, B, and C, exclusive of that recovered as citrulline and ornithine, if converted entirely to δ-amino valeric acid via ornithine would still account for less than 50 per cent of the δ-amino valeric acid found. The only other source is proline. Hence, proline must serve, at least partially, as oxidant in the fermentation. Whether or not part of the δ-amino valeric acid also arises from ornithine (i.e., whether ornithine serves to a certain extent as oxidant) can be established by examination of the δ-amino valeric acid formed when $C^{14}$ labeled ornithine and proline are fermented.

The δ-amino valeric acid was isolated for purposes of identification in 200 to 300 mg amounts by paper chromatography of the cation fraction of the growth medium in the butanol-propionic acid-water system. Since the hydrochloride of the free amino acid is not satisfactory for characterization of the compound, the 2,4-dinitrophenol derivative was prepared (Porter, 1950). The melting point (169 to 172 C), infrared spectrum, and $R_f$ (0.68) in tertiary amyl alcohol-phthalate buffer, pH 5.0 (Blackburn and Lowther, 1951), of the derivative were identical with the derivative of authentic δ-amino valeric acid (Eastman).

The arginine was converted to a number of compounds, the oxidized products being chiefly acetic acid and carbon dioxide.

Keto acids accumulated in only trace amounts in the growing cultures; these were detected as their 2,4-dinitrophenyl hydrazones.

The formate added to samples B and D disappeared during the fermentations, but only in sample D was there an approximately equivalent increase in carbon dioxide yield. Although in sample B the fate of the 1,470 μM of added formate is not apparent from the carbon balance, it is possible that this is merely a reflection of the small percentage contribution (13 to 14) of the formate to the total carbon in the system. From isotope studies it is known that formate carbon appears in $CO_2$ and in alanine (Stadtman, *unpublished data*) when the clostridium is grown on yeast extract and $C^{14}$ formate. More exact information as to its role in the fermentation of arginine or ornithine and proline awaits further isotope studies.

Another ninhydrin reactive material that was found to accumulate to a considerable extent during growth on arginine and proline proved to be α-alanine. This was identified by chromatography with authentic α-alanine in a variety of solvent systems and also by elementary analysis and infrared spectra of the crystalline compound. To obtain enough material for analysis, the cation fraction from 1.5 liters of growth medium was chromatographed on Whatman no. 3 filter paper in the butanol-propionic acid-water system. The alanine bands were cut out and eluted with water. The eluate was evaporated, decolorized with charcoal, and the product crystallized from methanol-water in the cold. About 80 mg of a white crystalline compound were obtained. The infrared spectrum of this material, after recrystallization, was identical with that of authentic L-α-alanine. The compound contained 40.08 per cent C, 7.68 per cent H, and 15.59 per cent N. Calculated values for the empirical formula, $C_3H_7O_2N$, are 40.44 per cent C, 7.87 per cent H, and 15.73 per cent N.

When L-lysine instead of proline was added to the growth medium as the hydrogen acceptor, a small amount of α-amino caproic acid (norleucine) appeared but no ε-amino caproic acid accumulated. The possibility that lysine undergoes a complete reductive deamination to caproic acid was eliminated by examination of the fatty acid fraction which showed that no caproic acid accumulated. Instead, considerable amounts of butyric and acetic acids were found. It seems likely that the butyrate is derived chiefly from the lysine since only trace amounts of butyrate are found when arginine and proline are fermented. Thus, the role of lysine in the fermentation is complex and appears to involve processes other than reductive deamination to the corresponding fatty acid.

## DISCUSSION

The biochemical activities of a clostridium, strain HF, described above place it in the large group of nonsaccharolytic anaerobes in the genus *Clostridium*. In common with a number of these proteolytic bacteria, it catalyzes a "Stickland" reaction. *Clostridium sporogenes*, the organism studied extensively by Stickland (1935) and by Woods (1936), utilizes ornithine as a hydrogen acceptor and converts it to δ-amino valeric acid. Dried cells of clostridium, strain HF, form trace amounts of δ-amino valeric acid from ornithine when incubated with molecular hydrogen and also oxidize ornithine with molecular oxygen. However, growth of the organism does not occur

on ornithine alone. Either proline or lysine must also be added to the medium. The role of ornithine in the fermentations appears, at least in part, to be that of a hydrogen donor since none of the reduced product, δ-amino valeric acid, is found when ornithine plus lysine are fermented. The δ-amino valeric acid produced in the arginine (or ornithine) plus proline fermentation may arise in part from ornithine but at least 50 per cent must be formed by a reduction of proline. Hence, it seems likely that ornithine serves as a hydrogen donor and proline and lysine as hydrogen acceptors in these fermentations. Whether they also undergo dismutations to a certain extent can be determined by fermentation of labeled substrates.

Arginine and citrulline replace ornithine in the fermentations by virtue of the fact that they are converted to ornithine, presumably by the arginine dihydrolase series of reactions. Woods (1936) found that arginine as well as ornithine is reduced by *C. sporogenes*, and since three moles of ammonia per mole of arginine were released, it is likely that the actual hydrogen acceptor is ornithine.

Both clostridium, strain HF, and *C. sporogenes* reduce L-proline to δ-amino valeric acid. Lysine, however, is not utilized by *C. sporogenes* (Stickland, 1934).

The amino acid pair, alanine and glycine, which is utilized by *C. sporogenes* and a variety of other clostridia (Clifton, 1940; Nisman *et al.*, 1948) is not fermented by clostridium, strain HF.

DL-Serine and DL-threonine are reduced readily by dried cells and cell-free extracts of clostridium, strain HF, to alanine and α-amino butyric acid, respectively. The formation of another ninhydrin reactive material identified as glycine from these β-hydroxyamino acids suggests that the bacterial extracts may contain the glycine forming enzyme system studied by Vilenkina (1952) in rat and guinea pig liver extracts. The possible involvement of pyridoxal as coenzyme in the process is suggested by recent studies of Metzler *et al.* (1953) showing that a nonenzymatic cleavage of hydroxyamino acids to glycine and an aldehyde is catalyzed by pyridoxal and metal salts.

## SUMMARY

The energy metabolism and nutritional requirements of a clostridium, strain HF, have been investigated. A "Stickland" reaction between ornithine and proline or lysine is catalyzed by the organism. Arginine and citrulline are converted to ornithine and thus can replace the latter as a substrate for growth.

Fermentation balances for the decomposition of arginine plus proline in the presence and absence of added formate are shown.

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