METABOLISM OF ω-AMINO ACIDS

I. Fermentation of γ-Aminobutyric Acid by *Clostridium aminobutyricum* n. sp.[1]

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Received for publication August 24, 1959

A number of amino acids are known to undergo reductive deamination reactions; in the general case, the products are the corresponding fatty acid and ammonia (Stickland, 1934; Woods, 1936). In at least one instance, that of the reduction of glycine to acetate and ammonia by *Clostridium sticklandii*, useful energy in the form of adenosine triphosphate is made available to the organism as a result of catalysis of the overall reaction (Stadtman and Elliott, 1956). Related anaerobic bacteria such as *Clostridium sporogenes* and *Clostridium lentoputrescens* appear to share this ability (Stadtman, 1959).

In view of the fact that the mechanism of this reduction is as yet unknown, it was deemed desirable to search for microorganisms able to ferment related amino acids. Such organisms might provide more suitable biological systems for the elucidation of the mechanism of reductive deaminations.

The substrates chosen were two higher homologues of glycine, namely, γ-aminobutyrate and Δ-aminovalerate. Two different species of anaerobic bacteria were isolated by the enrichment culture technique,[3] specific for each one of these amino acids respectively, when furnished as the sole carbon and nitrogen source. The present paper describes the isolation and identification of the organism that grows on γ-aminobutyrate together with the stoichiometry of the fermentation that it catalyzes. Similar studies carried out with the organism isolated on Δ-aminovalerate are reported in the following paper (Hardman and Stadtman, 1960).

A preliminary report of this work has been made (Hardman *et al.*, 1958).

MATERIALS AND METHODS

Isolation and culture. The organism described here was isolated from North Carolina swamp mud by means of the anaerobic enrichment culture technique. The enrichment medium contained the following: γ-aminobutyrate, 0.5 per cent, as the sole carbon and nitrogen source; $MgCl_2 \cdot 6H_2O$, 0.02 per cent; $FeCl_3 \cdot 6H_2O$, 0.001 per cent; $CaCl_2 \cdot 2H_2O$, 0.001 per cent; $MnSO_4$, 0.0001 per cent; Na_2MoO_4, 0.0001 per cent, and methylene blue, 0.0002 per cent. The medium was made 0.05 M with respect to potassium phosphate buffer (pH 7.4). Before inoculation, anaerobiosis was achieved by the addition of sodium sulfide (final concentration, 0.03 per cent), and immediate stoppering. All incubations were carried out either at 31 or 37 C.

A pure culture of the organism on γ-aminobutyrate was isolated by means of agar shake cultures as described by Barker (1936).

Growth responses to varying cultural conditions were estimated either visually or turbidimetrically in a Klett-Summerson colorimeter with a no. 66 filter.

For growth experiments in which carbon dioxide was not determined, a pyrogallol-K_2CO_3 saturated cotton plug was used as an anaerobic seal. For fermentation balances, an oxsorbent seal (Burrell Corporation) was used to remove oxygen from the gas above the liquid in the culture vessel.

In preparing a medium in which carbonate or sugars were to be included, these ingredients were autoclaved separately as was the sodium sulfide and subsequently added to the medium

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

[2] This work has been presented to the Graduate School of Georgetown in partial fulfillment of the requirements for the M.S. degree in Chemistry.

[3] The initial isolation of the Δ-aminovalerate organism and the identification of the fatty acid end products were carried out by Dr. Jekisiel Szulmajster, Institut de Biologie Physico- Chemique, 13 Rue Pierre Curie, Paris.

after cooling. The carbonate was autoclaved with a few drops of 0.05 per cent phenol red present and titrated with sterile 2 N HCl before addition to the medium.

4-C^{14}-γ-Aminobutyrate was prepared from 2-C^{14}-glutamic acid (Tracer Laboratories, Inc.) by the glutamic acid decarboxylase (Umbreit and Gunsalus, 1945) from *Escherichia coli* and was isolated from the reaction mixture by means of paper chromatography (Benson *et al.*, 1950).

Analytical methods. Ammonia was determined by nesslerization after diffusion into 0.5 N H_2SO_4 in Conway microdiffusion vessels and the optical density was read in either a Coleman Jr. spectrophotometer at 410 mμ or a Klett-Summerson colorimeter with a no. 42 filter.

γ-Aminobutyrate was isolated from samples of the culture medium both before and after growth by paper chromatography (Benson *et al.*, 1950), and after elution from the paper, was determined quantitatively by the photometric ninhydrin method of Moore and Stein (1948). The other amino acid substrates tested were also chromatographed in the same solvent system and the spots detected by treatment with ninhydrin.

The volatile fatty acid products were separated from the medium, after acidification, by steam distillation in the apparatus described by Markham (1942). The individual fatty acids were separated by partition chromatography using a silica gel column with an internal indicator as described by Elsden (1946). The identity of the isolated fatty acids was established by Duclaux distillation and by paper chromatography (Kennedy and Barker, 1951). In the cases where C^{14} fatty acids were involved, the fractions obtained from Duclaux distillations were counted. The radioactivity in the fractions corresponded to the titration values. The sodium salts of the radioactive fatty acid products were degraded in a stepwise fashion to barium carbonate by the Schmidt reaction according to Phares (1951). Since the yield of barium carbonate from the methyl carbon of acetate was poor, the methylamine from this carbon was collected in HCl and determined as methylamine hydrochloride.

Carbon dioxide in the growth medium was distilled into alkali after acidification with 85 per cent lactic acid. It was precipitated as barium carbonate which was washed, weighed, and examined for radioactivity.

Methods for species identification. Deep agar colonies were observed in the medium described below that supports optimum growth supplemented with agar (Difco), 1.5 per cent. Surface colonies were observed on horse meat infusion broth plus 2 per cent agar (Stitt *et al.*, 1948) and 2 per cent glucose.

Fermentation reactions were determined in carbohydrate-phenol red media (Difco). Gas production was detected by means of Durham tubes.

Tests for proteolytic activity were carried out as follows: (a) gelatin liquefaction in horse meat infusion broth containing gelatin, 12.5 per cent; (b) action on litmus milk in litmus milk medium (*Manual of Microbiological Methods*, 1957); (c) hydrogen sulfide production in a medium containing proteose peptone, 2 per cent; agar 1.5 per cent; lead acetate, 0.05 per cent; glucose, 0.1 per cent; (d) blackening of brain medium, growth on egg-yolk medium (Stitt *et al.*, 1948); indole formation in tryptone medium (Difco). Indole was estimated with the reagent of Kovac.

Nitrate reduction was determined in peptone (Difco), 0.1 per cent, and KNO_3, 0.02 per cent, medium. Sulfanilic acid and α-napthylamine were used to estimate nitrite formation.

Hemolytic activity was determined on defibrinated rabbit blood (Stitt *et al.*, 1948).

EXPERIMENTAL RESULTS

I. Growth studies. Growth medium. The medium that supports optimum growth contained, in addition to the mineral components (see Materials and Methods), γ-aminobutyrate, 0.5 per cent; yeast extract (Difco), 0.3 per cent; and sodium carbonate, 0.2 per cent. Maximum growth usually occurred at 17 to 20 hr after inoculation.

Nutrition studies. There was no growth observed on γ-aminobutyrate alone unless yeast extract was added. Tryptone, peptone, malt extract, liver extract (Difco), vitamin-free casein hydrolyzate (Nutritional Biochemicals Corporation) and a vitamin mixture[4] did not stimulate growth nor could they be substituted for the yeast extract. The addition of nitrogen

[4] Vitamin mixture contains: thiamine, calcium pantothenate, nicotinic acid, *p*-aminobenzoic acid, pyridoxine, pyridoxamine, pyridoxal, biotin, and trace amounts of riboflavin and folic acid.

as ammonium sulfate (0.2 per cent) likewise provided no enhancement of growth.

In contrast to *C. aminovalericum* (Hardman and Stadtman, 1960), carbonate (0.2 per cent) was stimulatory for growth of the γ-aminobutyrate fermenting organism although it was not absolutely required.

This organism does not produce gas during the fermentation of γ-aminobutyrate. Vigorous gas evolution is observed during growth on a variety of sugars.

Optimum pH. Optimum growth was obtained at pH 7.4 to 7.7 with no observable growth occurring below pH 7.

Energy substrates. Although a number of carbohydrates are fermented (see section II), there is a high degree of amino acid substrate specificity exhibited by growing cultures of the γ-aminobutyrate organism. Glutamate, β-alanine, α-aminobutyrate, β-aminoisobutyrate, 2, 4-diaminobutyrate, Δ-aminovalerate, and ornithine do not support growth.

However, resting cells of this organism liberate ammonia from both 2,4-diaminobutyrate and glutamate as well as γ-aminobutyrate (see section IV).

II. Species identification. As determined by standard bacteriological tests, the following are the characteristics of the organism isolated on γ-aminobutyrate.

Cell morphology. Short rods measuring 0.4 to 0.8 μ by 1.2 to 1.7μ, occurring singly or in pairs, occasionally in short chains of 3 to 5 cells, very rarely in longer chains. Oval spores located subterminally to terminally, bulging the sporangia slightly. Actively motile. Gram-positive.

Glucose agar surface colonies (anaerobic). Round to irregular, convex, entire, smooth, white, shiny.

Deep agar colonies (anaerobic). Biconvex, 2 to 4 mm in diameter, entire, yellowish-white with translucent borders, becoming very viscous and gelatinous with age.

Carbohydrates. Acid and gas from glucose, arabinose, galactose, xylose, lactose, maltose, cellobiose, mannitol, and levulose. Sucrose, starch, dextrin, dulcitol, glycerol, and sorbitol are weakly fermented. Inulin, adonitol and pectin are not fermented.

Nitrates reduced to nitrites.

Hydrogen sulfide produced.

Indole produced.

Gelatin. Rapid liquefaction.

Litmus milk. Stormy fermentation, rapid coagulation with no digestion of clot, abundant gas formation within 24 hr.

Blood agar surface colonies (anaerobic). Abundant gas formation, colonies filamentous, no hemolysis.

Meat infusion broth. Abundant diffuse growth.

Potato slant (anaerobic). Same as growth on glucose agar.

Egg yolk medium-slant (anaerobic). No growth.

Brain medium. No growth.

Obligately anaerobic.

Grows well from 31 to 37 C.

Source. Originally isolated from North Carolina swamp mud.

III. Fermentation balance. Fermentation of 4-C^{14}-γ-aminobutyrate. One-hundred milliliters of the medium were prepared as described in section I except that no carbonate was added. 4-C^{14}-γ-Aminobutyrate was included. After inoculation, 10 ml were removed for zero time assays and

TABLE 1

Fermentation of 4-C^{14}-γ-aminobutyric acid

	Initial		Final		Δ
	μmoles/100 ml	*cpm/μmole*	*μmoles/100 ml*	*cpm/μmole*	*μmoles*
γ-Aminobutyrate	4800	47	0	0	−4800
Ammonia			4898	—	+4898
Butyrate			2350	55	+2350
Acetate			4940	26	+4940

Nitrogen recovery	102%
Carbon recovery	100%
Carbon-14 recovery	113%
Molar redox index	1.02%

the culture was incubated anaerobically until growth had ceased.

Table 1 shows that ammonia and butyric and acetic acids are formed from γ-aminobutyrate. Two moles of γ-aminobutyrate yield two moles of ammonia, one mole of butyrate, and two moles of acetate.

The molar redox index of 1.02 shows this to be a balanced oxidation reduction process.

The specific activity of the butyrate is comparable to that of the amino acid substrate whereas the specific activity of the acetate is one-half. This would be expected if the following reactions describe the over-all fermentation:

$$\begin{array}{l} CH_2CH_2CH_2COOH + 2H_2O \rightarrow \\ | \\ NH_2 \end{array} \qquad (1)$$

$$NH_3 + 2CH_3COOH + 2H^+$$

$$\begin{array}{l} CH_2CH_2CH_2COOH + 2H^+ \rightarrow \\ | \\ NH_2 \end{array} \qquad (2)$$

$$NH_3 + CH_3CH_2CH_2COOH$$

$$\begin{array}{l} \text{Sum: } 2\ CH_2CH_2\ CH_2COOH + 2H_2O \rightarrow \\ \quad\quad\ | \\ \quad\quad NH_2 \end{array} \qquad (3)$$

$$2NH_3 + 2CH_3COOH + CH_3CH_2CH_2COOH$$

It can be seen that, by reaction 1, the oxidation of 4-C^{14}-γ-aminobutyrate would give rise to a mole of labeled acetate from carbon atoms 3 and 4, and a mole of unlabeled acetate from carbons 1 and 2. The isolated radioactive acetate was decarboxylated and found to be unlabeled in the carboxyl carbon; hence carbon 4 of 4-C^{14}-γ-aminobutyrate becomes carbon 2 of acetate and this is consistent with a β-oxidation mechanism operative in reaction 1.

IV. Deamination of amino acids by cell suspensions. Experiments were carried out to determine whether resting cells of the γ-aminobutyrate organism exhibits the same high degree of substrate specificity for amino acids as do growing cultures. Lyophilized cells of the organism that had been grown as described under Materials and Methods were prepared and tested for their ability to ferment other related amino acids.

Substrate-dependent ammonia release was measured in samples containing 0.04 M potassium phosphate buffer, pH 7.4, 0.02 M amino acid, and 8 mg dried cells per ml. Incubations were carried out in a helium atmosphere for 2 hr at 31 C. There were 6.6 μmoles of ammonia liberated per ml, after correction for the blank, in the sample containing glutamate; the same amount was also formed from γ-aminobutyrate. From 2,4-diaminobutyrate there was twice as much ammonia produced. β-Alanine, α-aminobutyrate, β-aminobutyrate, β-aminoisobutyrate, and Δ-aminovalerate did not yield any ammonia.

It is not known whether the greater amount of ammonia liberated from 2,4-diaminobutyrate is reflection of a difference in rate of deamination of the mono- and diamino compounds or whether both amino groups were removed. Ammonia liberation from glutamate was undoubtedly effected by the very active diphosphopyridine nucleotide-dependent glutamic dehydrogenase which has subsequently been identified in sonic extracts of the organism. Endogenous materials in the dried cells could serve to reoxidize the reduced diphosphopyridine nucleotide.

To see if perhaps the inability to ferment homologues of γ-aminobutyrate was due to a specific transaminase that might be involved initially, the various amino acids were tested, at 0.02 M levels, in a crude cell-free extract of the γ-aminobutyrate organism for their ability to undergo transamination, forming glutamate from α-ketoglutarate (0.01 M). Samples of the reaction mixtures, after deproteinization with perchloric acid and neutralization with KOH, were chromatographed. Apparent transamination had occurred, as determined by the appearance of a glutamate spot on the paper, only when γ-aminobutyrate or Δ-aminovalerate was employed as substrate. Thus, the amino acid substrate specificity for growth of this organism apparently is not due to a specific transaminase; although the organism cannot grow on Δ-aminovalerate it can utilize it as a substrate for a transamination reaction. No glutamate was detected with 2,4-diaminobutyrate as the substrate nor was any α- or γ-aminobutyrate found in this sample. Again no detectable transamination occurred with β-alanine, α-aminobutyrate, β-aminobutyrate, or β-aminoisobutyrate.

DISCUSSION

The organism described here belongs to the genus *Clostridium* and the most closely related species on the basis of standard biochemical and bacteriological tests is *Clostridium tale*. Because of the incompleteness of the description of *C. tale*, it is difficult in some respects to differentiate

between the two species. They are similar in their physiological properties except for the slow action on milk by *C. tale*. However, due to its size, which is significantly different, and lack of motility, *C. tale* can readily be distinguished from the γ-aminobutyrate organism; the latter, therefore, is considered to be a distinctly different species. Since this organism appears to be highly specific with respect to its growth on γ-aminobutyrate, the name *Clostridium aminobutyricum* n. sp. is proposed.

Chemical balance studies together with isotope experiments, indicate that the fermentation of γ-aminobutyrate, like Δ-aminovalerate, involves a β-oxidation mechanism coupled with a reductive process; the latter yields the corresponding fatty acid in each case. This conclusion is borne out by more detailed studies, currently in progress, on the mechanism of γ-aminobutyrate decomposition by enzyme preparations of *C. aminobutyricum*.

SUMMARY

An organism has been isolated in pure culture from swamp mud by the anaerobic enrichment culture technique with γ-aminobutyrate as the sole organic substrate.

A study of the biochemical and morphological properties of this organism indicate that it is a previously unidentified *Clostridium*. In view of its extreme growth specificity for γ-aminobutyrate, the name, *Clostridium aminobutyricum*, n. sp., is proposed.

Chemical and isotope balance experiments show that two moles of γ-aminobutyrate are converted to two moles of ammonia, two moles of acetic acid, and one mole of butyrate in an oxidation-reduction reaction.

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