METABOLISM OF ω-AMINO ACIDS

II. Fermentation of Δ-Aminovaleric Acid by *Clostridium aminovalericum* n. sp.[1]

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Although Δ-aminovalerate is accumulated by a number of clostridia that effect a reductive ring cleavage of proline to form this amino acid (Stickland, 1935; Stadtman, 1956), its further metabolism has been little investigated. It serves as a substrate for transaminases in microorganisms and animal tissues (Roberts, 1954; Suda *et al.*, 1954). For reasons discussed in the preceding communication (Hardman and Stadtman, 1960), a search was made for an anaerobic organism able to utilize Δ-aminovalerate as the sole carbon and nitrogen source.

The present communication concerns the isolation and characterization of such a microorganism together with a description of the fermentation that it catalyses.[3]

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

MATERIALS AND METHODS

The analytical methods employed are identical with those in the accompanying paper describing *Clostridium aminobutyricum*. Also similar procedures for the isolation of the organism and species identification were used except that Δ-aminovalerate was employed as the amino acid substrate and that sewage sludge was the source of the organism.

Uniformly labeled Δ-aminovalerate was synthesized by the enzymatic reduction of uniformly labeled L-proline (Schwarz Laboratories, Inc.) (Stadtman, 1956). 2-C^{14}-Δ-Aminovalerate was prepared by the reduction of 2-C^{14}-DL-ornithine (Tracerlab, Inc.) in growing cultures of an unidentified ornithine-fermenting organism similar to *Clostridium sporogenes* (Woods, 1936). In each case, the labeled amino acid product was isolated from the reactants by means of paper chromatography in the butanol-water-propionic acid solvent system described by Benson *et al.* (1950).

EXPERIMENTAL RESULTS

I. Growth studies. Growth medium. The medium finally developed that supports optimum growth is as follows: Δ-aminovalerate, 0.5 per cent, or mannitol, 1.0 per cent, and Δ-aminovalerate, 0.05 per cent; yeast extract, 0.05 per cent; metal components, same as in the enrichment medium. With this medium, visible growth occurred usually within 2 days at 37 C and maximum growth by the third day.

Nutrition studies. There was no growth observed when the amino acid, together with the usual mineral medium, was supplied as the sole source of carbon and nitrogen. Upon the addition of yeast extract, 0.05 per cent, good growth was observed which could be further stimulated by the addition of mannitol, 1.0 per cent, or glucose, 1.0 per cent. Other sources of growth promoting factors such as tryptone, peptone, malt extract, liver extract (Difco), vitamin-free casein hydrolyzate (Nutritional Biochemicals Corporation) and a vitamin mixture[4] did not promote growth above that observed with yeast extract alone, nor could they be substituted for it. The addition of

[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 University in partial fulfillment of the requirements for the M.S. degree in Chemistry.

[3] The initial isolation of this 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.

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

ammonium sulfate or of carbonate did not stimulate growth.

Although carbon dioxide is evolved when the organism is grown on the mannitol medium, the organism does not produce gas from Δ-aminovalerate alone.

Optimum pH. The optimum range of pH for growth is pH 7.4 to 7.7, with no growth occurring below pH 7.0.

Energy substrates. Although many carbohydrates can serve as the sole carbon source (see section II), there is a high degree of specificity as regards the amino acid substrate. Higher and lower homologues of Δ-aminovalerate such as β-alanine, γ-aminobutyrate, norvaline, ornithine, and ε-aminocaproate (all concentrations, 0.5 per cent) when substituted for Δ-aminovalerate, could not support growth.

II. Species identification. Standard bacteriological tests were performed to determine the morphological and physiological characteristics of a pure culture of the organism fermenting Δ-aminovalerate with the following results.

Cell morphology. Short rods measuring 0.4 to 0.6 μ by 0.8 to 1.2 μ, occurring singly and in pairs, rarely in chains of 3 to 5 cells. Oval spores are located terminally bulging the sporangia extremely. Occasionally beady, gram-positive staining material in old (gram-negative) cells. Actively motile. Gram-positive in young cultures, becoming gram-negative.

Glucose agar surface colonies (anaerobic). Growth abundant, spreading, clear, glistening.

Deep agar colonies (anaerobic). Biconvex, 2 to 4 mm in diameter, entire, yellowish-white with translucent borders, becoming very ropy with age. Occasionally filamentous outgrowths in old cultures.

Carbohydrates. Acid and gas from glucose, arabinose, xylose, maltose, starch, dextrin, cellobiose, mannitol, and levulose. Slower growth with slight acid and no gas from sucrose and galactose. Lactose, inulin, glycerol, sorbitol, adonitol, dulcitol, and pectin are not fermented.

Nitrates not reduced.

Hydrogen sulfide produced.

Indole not produced.

Gelatin. No liquefaction.

Litmus mild. Litmus decolorized, no coagulation.

Blood agar surface colonies (anaerobic). No growth.

Meat infusion broth. Abundant diffuse growth.

Potato slant (anaerobic). No growth.

Egg yolk slant (anaerobic). No growth.

Brain medium. No growth.

Obligately anaerobic.

Grows well from 31 to 37 C.

Source. Originally isolated from sewage sludge.

III. Fermentation balances and tracer experiments. Fermentation of C^{14}-Δ-aminovalerate. One-hundred milliliters of the growth medium were prepared, as described in section I, containing Δ-aminovalerate, 0.5 per cent. Mannitol was omitted. To this the reducing agent was added, the medium was inoculated from an active culture, and 10 ml were withdrawn for zero

TABLE 1

Fermentation of C^{14}-Δ-aminovaleric acid

	Initial			Final			Δ
		Total activity	Specific activity		Total activity	Specific activity	
	μmoles/100 ml	*cpm/100 ml*	*cpm/μc-atom*	*μmoles/100 ml*	*cpm/100 ml*	*cpm/μc-atom*	*μmoles*
Δ-Aminovalerate	2960	30,950	2.08	330	3000	1.85	−2863
Ammonia	33	—	—	2840	—	—	+2000
Acetate				1400	4850	1.78	+1407
Propionate				1470	8240	1.86	+1470
Valerate				1285	10,350	1.62	+1285

Nitrogen recovery	107%
Carbon recovery	103%
Carbon-14 recovery	85.5%
Molar redox index	0.987

TABLE 2

Fermentation of 2-C^{14}-Δ-aminovaleric acid

	Initial			Final			Δ
		Total activity	Specific activity		Total activity	Specific activity	
	μmoles/100 ml	*cpm/100 ml*	*cpm/μmole*	*μmoles/100 ml*	*cpm/100 ml*	*cpm/μmole*	*μmoles*
Δ-Aminovalerate	3720	108,000	29.3	129	2810	21.8	−3591
Ammonia	44	—	—	3310	—	—	+3266
Acetate				1830	25,800	14.1	+1830
Propionate				1590	1735	1·09	+1590
Valerate				1320	32,000	24.2	+1320

Nitrogen recovery	91%
Carbon recovery	83.7%
Carbon-14 recovery	58.3%
Molar redox index	1.29

time determinations. The culture was incubated anaerobically at 31 C until growth had ceased.

Analysis of the growth medium showed that acetic, propionic, and valeric acids and ammonia are the products (table 1). No carbon dioxide was formed and there was a quantitative recovery of carbon and nitrogen. The fatty acid products contained essentially all of the carbon-14. It can be seen that the stoichiometry is such that for every two moles of Δ-aminovalerate consumed, there are formed two moles of ammonia and one mole each of valeric, propionic and acetic acid.

The molar redox index is 0.987 indicating that this is a balanced oxidation-reduction process and that all of the products are detected.

Fermentation of 2-C^{14}-Δ-aminovalerate. The objective of this experiment was to determine the point of cleavage of the Δ-aminovalerate, or one of its metabolites in the oxidative step that gives rise to acetate and propionate. The medium was identical with that used for the first fermentation except that 2-C^{14}-Δ-aminovalerate was added instead of uniformly labeled-C^{14}-Δ-aminovalerate. Carbon 2 of the amino acid was found exclusively in acetate and valerate; virtually no carbon-14 was found in the propionate (table 2). This result would be expected if cleavage occurs between carbons 2 and 3. The acetate was subsequently degraded and the radioactivity was found solely in the methyl carbon. Thus the carbon chain of Δ-aminovalerate apparently undergoes a β-oxidation to give rise to acetate from carbon atoms 1 and 2 and propionate.

TABLE 3

Fermentation of Δ-aminovalerate and 1-C^{14}-acetate

	Specific Activity		
	Undegraded acid	—COOH*	C—C—C—C—†
	cpm/μmole	*cpm/μmole*	*cpm/μmole*
Valerate	56.7	35	0.15
Propionate	3.5	—	
Acetate	530		

* Determined as $BaCO_3$.

† Determined as butylamine·HCl.

Fermentation of Δ-aminovalerate and 1-C^{14}-acetate. When unlabeled Δ-aminovalerate was fermented in the presence of 1-C^{14}-acetate, only about 10 per cent of the radioactivity of the acetate was found in the valerate, the reduced product (table 3). Thus, under conditions of growth, there is only slight reversibility of the oxidative part of the reaction. Upon degradation of the valerate, the carbon-14 was found almost exclusively in the carboxyl carbon as would be expected if incorporation is achieved by a reversal of the degradative process. The reason for lack of similarity in the specific activity of the undegraded acid and the carboxyl carbon, which was counted as $BaC^{14}O_3$, is not known. Some contamination of the $C^{14}O_2$ from the carboxyl carbon with unlabeled CO_2 may have occurred during the degradation procedure.

DISCUSSION

Since this organism is an obligately anaerobic, gram-positive, spore-producing rod-shaped bacterium it belongs to the genus *Clostridium*. A comparison of the morphological and biochemical characteristics of this organism with those of other clostridia as described in *Bergey's Manual of Determinative Bacteriology* (7th edition) clearly indicates that it is a previously unidentified species. Its marked amino acid substrate specificity for growth has been selected as the character for naming this organism; it is proposed to call it *Clostridium aminovalericum* n. sp.

The data indicate that the fermentation of Δ-aminovalerate may be described by the following reactions:

$$\underset{\displaystyle NH_2}{\underset{|}{CH_2}}CH_2CH_2CH_2COOH + 2H_2O \rightarrow NH_3 + CH_3CH_2COOH + CH_3COOH + 2H^+ \qquad (1)$$

$$\underset{\displaystyle NH_2}{\underset{|}{CH_2}}CH_2CH_2CH_2COOH + 2H^+ \rightarrow NH_3 + CH_3CH_2CH_2CH_2COOH \qquad (2)$$

$$\text{Sum: } 2\ \underset{\displaystyle NH_2}{\underset{|}{CH_2}}CH_2CH_2CH_2COOH + 2H_2O \rightarrow 2NH_3 + CH_3CH_2COOH + CH_3COOH + CH_3CH_2CH_2CH_2COOH \qquad (3)$$

The over-all fermentation (reaction 3) carried out by this organism is analogous to the Stickland reaction in that it is a coupled oxidation reduction process. However, in this case, only one, rather than two different amino acids, is fermented. One mole of Δ-aminovalerate is oxidized by a process involving a β-oxidation to one mole each of ammonia, propionate and acetate (reaction 1); the other mole of the amino acid is reduced to ammonia and valeric acid (reaction 2).

As in other similar amino acid fermentations, the organism must obviously derive its energy for growth from Δ-aminovalerate degradation when this is the only organic substrate for growth. At least part of this energy may result from the oxido-reduction reactions involved in the formation of the reduced product, valerate, and the oxidized products, acetate, and propionate.

SUMMARY

An organism belonging to the genus *Clostridium* has been isolated from sewage sludge by the anaerobic enrichment culture technique; it is capable of utilizing Δ-aminovalerate as its sole energy source.

A consideration of the nutritional requirements together with the morphological and physiological characteristics of the isolate, suggest it to be a new species. The name, *Clostridium aminovalericum* n. sp., is proposed.

The over-all reaction catalyzed by this organism is a coupled oxidation reduction process in which two moles of Δ-aminovalerate are converted to two moles of ammonia and one mole each of valeric, propionic, and acetic acids.

REFERENCES

Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Hass, V. A., and Stepka, W. 1950 The path of carbon in photosynthesis. V. Paper chromatography and radioautography of the products. J. Am. Chem. Soc., **72**, 1710–1718.

Hardman, J. K. and Stadtman, T. C. 1960 Metabolism of ω-amino acids. I. Fermentation of γ-aminobutyric acid by *Clostridium aminobutyricum* n. sp. J. Bacteriol., **79**, 544–548.

Hardman, J. K., Stadtman, T. C., and Szulmajster, J. 1958 Bcterial fermentation of Δ-aminovaleric acid. Bacterial. Proc., **1958**, 120.

Roberts, E. 1954 Studies of transamination. Arch. Biochem. Biophys., **48**, 395–401.

Stadtman, T. C. 1956 Studies on the enzymic reduction of amino acids: A proline reductase of an amino acid-fermenting *Clostridium*, strain HF. Biochem. J., **62**, 614–621.

Stickland, L. H. 1935 Studies in the metabolism of the strict anaerobes (genus *Clostridium*). II. The reduction of proline by *C. sporogenes*. Biochem. J., **29**, 288–290.

Suda, M., Kamahora, T., and Hagihira, H. 1954 Metabolism of L-lysine by bacterial enzymes. Med. J. Osaka Univ., **5**, 119-126.

Woods, D. D. 1936 Studies in the metabolism of the strict anaerobes (genus *Clostridium*). V. Further experiments on the coupled reactions between pairs of amino acids induced by *C. sporogenes*. Biochem. J., **30**, 1934–1946.