TRACER STUDIES ON ORNITHINE, LYSINE, AND FORMATE METABOLISM IN AN AMINO ACID FERMENTING CLOSTRIDIUM

THRESSA C. STADTMAN AND F. H. WHITE, JR.

National Institutes of Health, Bethesda, Maryland

Received for publication November 19, 1953

A clostridium, strain HF, has been shown to catalyze a coupled oxidation-reduction reaction between either ornithine and proline or ornithine and lysine (Stadtman, 1954). Formate is decomposed when added to the amino acid fermentations and under certain conditions markedly stimulates growth. In these fermentations the ornithine appears to be oxidized chiefly to acetate, carbon dioxide, and ammonia. Proline, at least in part, plays the role of oxidant and is reduced to δ-amino valeric acid whereas lysine undergoes much more extensive degradation to butyrate, acetate, and ammonia. Radioactive products derived from 2-C^{14}-ornithine, 2-C^{14}-lysine, and C^{14}-formate have been isolated and degraded in order to gain information concerning the nature of the reactions involved in these fermentations. From such experiments it is possible to assess more accurately the exact role of each substrate in the oxidation-reduction processes.

MATERIALS AND METHODS

Materials. The clostridium, strain HF, employed has been described previously (Stadtman, 1954). The basal mineral medium supplemented with 0.15 per cent L-arginine hydrochloride, 0.15 per cent L-lysine hydrochloride, and 0.2 per cent yeast extract (Difco) was employed for large scale (20 liter) cultures. Cells were harvested from these cultures in a Sharples supercentrifuge when maximum turbidity was attained (about 48 hours at 37 C). The cell pastes were washed thoroughly with 0.03 per cent $Na_2S \cdot 9H_2O$ and dried *in vacuo* over $CaCl_2$. The average yield of dried cells obtained under these conditions was 150 mg per liter.

2-C^{14}-DL-ornithine, 2-C^{14}-DL-lysine, and C^{14}-formate were obtained from Tracerlab, Inc., Boston, Massachusetts.

Analytical procedures. The amino acids in the culture media were separated by paper chromatography (Stadtman, 1954). Ornithine and proline were estimated by the Chinard ninhydrin procedure (1952); lysine and δ-amino valeric acid were determined by the Moore-Stein ninhydrin method (1948). In some cases (where resolution on chromatograms was poor), total ornithine plus lysine was determined by the Moore-Stein method, and ornithine by the Chinard procedure. The lysine could be calculated then by difference (after correcting for its slight color yield in the Chinard procedure). For critical specific activity measurements, the ornithine and lysine samples were rechromatographed to obtain pure fractions.

Fatty acids were steam distilled and then separated chromatographically on silica gel columns by a modification of the method of Ramsey and Patterson (1948). The purity of the isolated acids was established by Duclaux distillations.

The sodium salts of the radioactive fatty acids, after dilution with suitable amounts of carriers, were degraded in a stepwise manner to barium carbonate by the Schmidt reaction (Phares, 1951). Van Slyke-Folch wet oxidations were performed for total radioactivity measurements on the fatty acid salts (1940).

Formate was oxidized to carbon dioxide with HgO (Friedemann, 1938) and recovered as barium carbonate. Carbon dioxide was measured manometrically (Peters and Van Slyke, 1932). Ammonia was distilled into boric acid and titrated (Ma and Zuazaga, 1942).

EXPERIMENTAL RESULTS

Ornithine plus lysine fermentations. Two 100 ml cultures of the clostridium were prepared containing 0.2 per cent DL-ornithine hydrochloride, 0.2 per cent L-lysine hydrochloride, and 0.1 per cent sodium formate as substrates. To one of these were added 1.66 μM of 2-C^{14}-DL-ornithine (1 μC of C^{14}), and to the other were added 2.74 μM of 2-C^{14}-DL-lysine (1 μC of C^{14}). The cultures were incubated anaerobically ("oxsorbent" seals) at 33 C until growth and fermentation had ceased (5 days). The products were isolated and examined for radioactivity. In table 1 are shown the

TABLE 1

Fermentation of 2-C^{14}-DL-ornithine and L-lysine

COMPOUND	INITIAL		FINAL*		DIFFERENCE
	μM/100 ml	Specific activity cts/min/μM	μM/100 ml	Specific activity cts/min/μM	μM/100 ml
Ornithine.....	1,080	324	296	382	−784
Lysine.....	1,075	0	246	0	−829
HCOONa.....	1,580	0	379	2.4	−1,201
NH_3.....	1,000		4,300		+3,300
CO_2.....	0		1,067	2.6	+1,067
Valerate(?)...	0		180	0	+180
Butyrate.....	0		1,175	37.5	+1,175
Propionate....	0		149	0	+149
Acetate.....	0		1,650	38.1	+1,650

* Carbon recovery = 102 per cent; nitrogen recovery = 102 per cent; C^{14} recovery = 64 per cent; redox index = 108 per cent.

TABLE 2

Fermentation of 2-C^{14}-DL-lysine and DL-ornithine

COMPOUND	INITIAL		FINAL*		DIFFERENCE
	μM/100 ml	Specific activity cts/min/μM	μM/100 ml	Specific activity cts/min/μM	μM/100 ml
Ornithine.....	1,080	0	325	0	−755
Lysine.....	1,075	359	298	360	−777
HCOONa.....	1,580	0	390	1.3	−1,190
NH_3.....	1,000		4,200		+3,200
CO_2.....	0		1,023	3.3	+1,023
Valerate(?)...	0		155	28	+155
Butyrate.....	0		1,105	113.5	+1,105
Propionate....	0		74	0	+74
Acetate.....	0		1,803	34	+1,803

* Carbon recovery = 103 per cent; nitrogen recovery = 102 per cent; C^{14} recovery = 78.5 per cent; redox index = 110.5.

amounts and specific activities of the compounds obtained from the culture containing labeled ornithine. It is seen that carbon 2 of ornithine appears chiefly in acetate and butyrate. A small amount, about 1.5 per cent, was converted to carbon dioxide and formate. The comparable specific activities of the latter compounds bear out earlier observations that carbon dioxide carbon and formate carbon equilibrate in the clostridium HF system.

No labeled amino acid products were found [δ-amino valeric acid or alanine (Stadtman, 1954)], and all of the added carbon and nitrogen was recovered. The low C^{14} recovery in the labeled ornithine fermentation is very likely due to errors in specific activity measurements on the fatty acid salts.

Aliquots of the labeled ornithine and labeled lysine culture media were adjusted to pH 11.0 and steam distilled in order to recover volatile amines since the odor of the cultures suggested the presence of such compounds. The distillates were trapped in dilute sulfuric acid and the amine salts examined for radioactivity after removal of excess sulfate with barium. The amount of C^{14} found in these fractions accounted for only 0.15 to 0.2 per cent of the labeled ornithine and the labeled lysine fermented. It is likely that these small amounts of isotope were present in traces of tetramethylenediamine (putresine) or pentamethylenediamine (cadaverine), which are decarboxylation products of ornithine and lysine, respectively.

The products isolated from the culture containing 2-C^{14}-lysine are listed in table 2. Here, as in the case of labeled ornithine, the C^{14} was found mainly in acetate and butyrate. About 1.4 per cent of carbon 2 of lysine was converted to carbon dioxide and formate. Unlike the labeled ornithine fermentation, a small amount of a higher fatty acid that migrated on silica gel chromatograms, like valeric acid, contained C^{14}. This suggests that lysine may be converted to a slight extent to its corresponding α-keto acid, which by decarboxylation and deamination could yield carboxyl-labeled valeric acid. In both fermentations small amounts of unlabeled propionate accumulated.

The pure C^{14}-acetate and C^{14}-butyrate samples isolated from the labeled ornithine and lysine cultures were diluted with carrier acetate and butyrate and degraded by the Schmidt reaction (Phares, 1951).

The results of these degradations show that carboxyl-labeled acetate and 1,3-labeled butyrate are formed from 2-C^{14}-ornithine, whereas 2-C^{14}-lysine gives rise to methyl-labeled acetate and 2,4-labeled butyrate (table 3).

The labeled butyrate derived from the labeled ornithine fermentation appears to be formed almost entirely by condensation of C_2 fragments since the isotope is distributed approximately equally between positions 1 and 3. If all the

TABLE 3

Distribution of isotope in acetate and butyrate derived from 2-C^{14}-ornithine and 2-C^{14}-lysine

ORIGIN OF FATTY ACID	ACETATE		BUTYRATE			
	—COOH carbon 1	—CH_3 carbon 2	—COOH carbon 1	—CH_2— carbon 2	—CH_2— carbon 3	—CH_3 carbon 4
2-C^{14}-ornithine fermentation	51.0	1.5	20.7	3.3	26.4	0.95
2-C^{14}-lysine fermentation	0.6	10.9	0.3	89.7	0.3	18.4
2-C^{14}-lysine + H_2; dried cells			2.0	16.0	0.3	3.8*
2-C^{14}-lysine + arginine; dried cells	0.2	23.8	0.4	21.4	0.7	

The isotope distribution in the fatty acids is expressed as cts per min per mg $BaCO_3$. Recoveries were checked by total wet combustions on each acid.

* This value was calculated from the specific activity of carbons 3 + 4 determined as sodium acetate and carbon 3 determined as $BaCO_3$.

butyrate is formed by condensation of equilibrated C_2 pieces, then the specific activity of the butyrate should be twice that of the acetate. However, the molar specific activities of the acetate and butyrate derived from labeled ornithine were found to be equal (table 1). Therefore, some nonlabeled butyrate must have been derived from lysine without intermediary conversion to C_2 fragments or by condensation of unlabeled C_2 pieces not in equilibrium with the labeled C_2 fragment from ornithine. This conclusion is further strengthened by the finding that the specific activity of the labeled butyrate derived from labeled lysine is 3.3 times that of the acetate on a molar basis (table 2). Since the acetate is labeled in one carbon and the butyrate in only two (table 3), the specific activity of the butyrate can be only twice that of the acetate if it is synthesized from C_2 fragments in equilibrium with acetate. The distribution of isotope in the butyrate derived from labeled lysine is such as to suggest that 80 per cent of the labeled molecules is 2-C^{14}-butyrate and 20 per cent is formed by a condensation of labeled C_2 pieces.

Decomposition of 2-C^{14}-lysine by dried cells. Dried cells of clostridium, strain HF, as well as growing cultures, were found to catalyze the conversion of lysine to acetic and butyric acids. In table 4 the relative rates of conversion of carbon 2 of lysine to fatty acids are shown. It is seen that the addition of arginine markedly stimulates the rate of lysine decomposition whereas the addition of ornithine somewhat depresses the rate. The steam volatile acid fractions from each reaction mixture were examined for C^{14} containing fatty acids by chromatography of each sample after the addition of carrier acetic, propionic, butyric, valeric, and caproic acids. The reisolated propionic, valeric, and caproic acids contained no radioactivity. Only the acetic and butyric acids were labeled. The sample incubated in hydrogen contained a higher ratio of C^{14}-labeled butyrate to C^{14}-labeled acetate than did the other reaction mixtures. Degradation of these labeled products (table 3) showed the distribution of isotope to be comparable to that found in the acetic and butyric acids isolated from the 2-C^{14}-lysine fermentation.

Fermentation of 2-C^{14}-ornithine plus proline. Since trace amounts of δ-amino valeric acid were

TABLE 4

*Decomposition of 2-C^{14}-DL-lysine by dried clostridium, strain HF, cells**

SUBSTRATES	GAS PHASE	PER CENT OF ADDED C^{14} RECOVERED† IN ACETATE + BUTYRATE
10 μM L-lysine + 10 μM L-arginine	Helium	77.6
10 μM L-lysine + 10 μM DL-ornithine	Helium	18.8
10 μM L-lysine	Helium	40.6
10 μM L-lysine	H_2	28.6

* Each reaction mixture contained 50 mg dried cells in 0.05 M pH 7.4 K phosphate in a total volume of 2.0 ml. Incubation was at 38 C for 135 min.

† A boiled control was included for each reaction mixture; no C^{14} was found in the steam volatile acid fractions from these samples. In both reaction mixtures and boiled controls the residual C^{14} was recovered in undecomposed lysine.

detected in reaction mixtures containing arginine or ornithine incubated in H_2 with dried cells of clostridium, strain HF (Stadtman, 1954), there existed the possibility that some of the δ-amino valerate formed in the ornithine (or arginine) plus proline fermentation may have arisen by a reductive deamination of ornithine. Fermentation balances, which showed that much of the δ-amino valerate must have been formed from proline, did not exclude the possibility that some ornithine may have been reduced to δ-amino valerate as well as oxidized to acetate and CO_2. In order to assess more exactly the role of ornithine in the fermentation, clostridium, strain HF, was grown on a mixture of C^{14}-labeled ornithine and unlabeled proline. After growth had ceased, δ-amino valerate together with residual ornithine and proline was isolated from the medium and examined for radioactivity. The amounts of these compounds and their specific activities are shown in table 5.

It is seen that ornithine serves to a very limited extent as oxidant in the fermentation since, of the amount that disappeared, only 6 per cent was reduced to δ-amino valerate. The small amount of C^{14} present in the residual proline suggests that the reduction of proline to δ-amino valerate may be somewhat reversible under these conditions. Alternatively, ornithine (1.3 per cent) may have been converted to proline by way of α-keto-δ-amino valeric and Δ^1-pyrroline-5-carboxylic acids (Vogel and Davis, 1952).

Ornithine plus proline plus $HC^{14}OONa$ fermentation. As previously mentioned, the addition of formate under certain cultural conditions stimulates growth rather markedly. The role of this compound in the amino acid fermentations is not apparent from over-all carbon balances, particularly in view of the fact that under certain conditions (when thioglycolate instead of sulfide is used as the reducing agent) formate inhibits rather than stimulates the rate of growth. In order to clarify this point the metabolism of C^{14}-formate in the presence of ornithine and proline was investigated in media made anaerobic with (1) sodium sulfide and (2) sodium thioglycolate. The 100 ml cultures each contained 0.2 per cent DL-ornithine hydrochloride, 0.1 per cent L-proline, and 0.1 per cent sodium formate (1 μC C^{14}). After the fermentations were complete, aliquots of the media were steam distilled and examined for radioactivity both before and after removal of residual labeled formate. About 50 per cent of the formate was unfermented in the culture containing thioglycolate whereas it had been decomposed completely in the culture containing sulfide. In the latter it was found that labeled acetate, containing 60 per cent of the C^{14} added initially as formate, had accumulated. This alone does not give a true picture of the extent of acetate synthesis from one-carbon fragments since the formate carbon is diluted by CO_2 carbon in the system by an exchange mechanism. In the thioglycolate culture, however, no C^{14} was found in the acetate. Thus, the presence of 0.05 per cent sodium thioglycolate in the medium inhibits the synthesis of acetate from formate.

TABLE 5

The extent of conversion of ornithine to δ-amino valeric acid and proline

COMPOUND	μM*	SPECIFIC ACTIVITY CTS/MIN/μM	PER CENT OF ADDED C^{14}
Ornithine	10.3	306	0.9
Proline	273	16.4	1.3
δ-Amino valeric acid	502	40.6	5.8

Initially, 1,080 μM of 2-C^{14}-DL-ornithine (324 cts/min/μM) and about 870 μM of L-proline were present in the 100 ml culture.

* μM per 100 ml culture medium at the end of the fermentation.

TABLE 6

Isotope distribution in acetate derived from C^{14}-formate

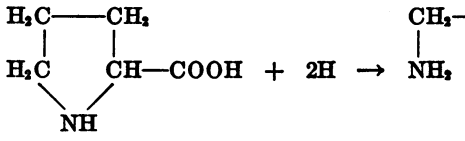
	CTS/MIN/MG $BaCO_3$	PER CENT OF TOTAL
Carbon 1 (—COOH)	211	41
Carbon 2 ($—CH_3$)	271	51
Carbons 1 + 2	257	100

Degradation of the labeled acetate isolated from the sulfide culture showed that both the methyl and carboxyl carbons were derived from formate (table 6). The ratio of activities of these two carbons (methyl to carboxyl) is 1.2.

DISCUSSION

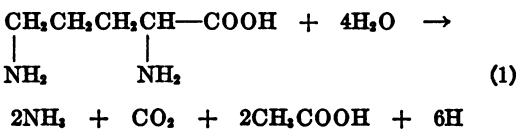
Evidence obtained from fermentations of isotopic ornithine supports the view that this amino acid undergoes oxidative degradation almost exclusively when metabolized by

clostridium, strain HF. The usual reduction product, δ-amino valerate, is not detected among the products of the ornithine plus lysine fermentation. In the ornithine plus proline fermentation, where δ-amino valerate is formed, not more than 6 per cent is derived from ornithine; the remainder (94 per cent) is formed by the reduction of proline. Thus, although the enzymes necessary



(4)

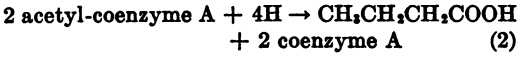
for the reductive deamination of ornithine are present in the organism, the reaction does not occur to any appreciable extent under normal growth conditions. A complete reductive deamination of ornithine to valerate seems to be excluded since no isotope was found in the valerate isolated from the 2-C^{14}-ornithine plus lysine fermentation. The over-all decomposition of ornithine to acetate, ammonia, and carbon dioxide can be described by equation 1.



(1)

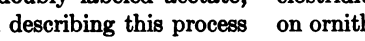
Since 2-C^{14}-ornithine gives rise to 1-C^{14}-acetate, it appears likely that the process involves a preliminary conversion to the α-keto acid followed by decarboxylation and subsequent oxidation of the 4-carbon residue to two moles of acetate (or acetyl derivatives, namely acetyl-coenzyme A).

As regards reductive processes in the fermentations whereby the 6 hydrogens from each ornithine molecule are removed, there are at least three possibilities. The doubly labeled butyrate formed must arise from condensation and consequent reduction of two labeled 2-carbon fragments. Thus, 2 acetyl-coenzyme A molecules may condense to form butyrate thereby using 4 hydrogens per mole of butyrate formed as indicated in reaction 2.



(2)

When H_2S is the reducing agent, labeled formate is converted to doubly labeled acetate, and the over-all equation describing this process shows that 4 hydrogens would be used per mole of acetate synthesized (equation 3).



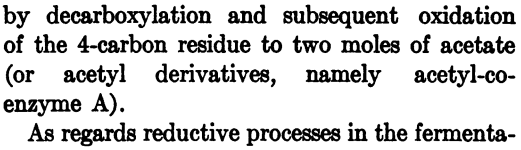
(3)

In the ornithine plus proline fermentation there is a third reductive process which is the conversion of proline to δ-amino valerate (equation 4).

If proline were the sole oxidant in the ornithine plus proline fermentation, then three moles would have to be reduced for every mole of ornithine oxidized. Actually, this ratio seldom obtains because the other reductive processes occur simultaneously.

In addition there is still another process, the evolution of hydrogen gas, that may serve to remove a small amount of the hydrogen formed by oxidation of ornithine.

Whereas proline appears to serve mainly as an electron acceptor in the ornithine-proline fermentation, a similar role of lysine in the ornithine-lysine fermentation seems to be contraindicated. Results of the experiments with 2-C^{14}-lysine indicate that this substance is fermented almost exclusively to ammonia, acetate, and butyrate (reaction 5).



(5)

Since this conversion does not involve a net oxidation or reduction, it is evident that the lysine does not serve primarily as an electron acceptor. Unless acetate derived from lysine serves as a "primer", its essential role as a source of acetyl derivatives seems improbable since C_2 fragments that recombine to form butyrate also are formed from ornithine. There remains the possibility, however, that some intermediate in the breakdown of lysine is required for growth of the organism and that this compound can be obtained also from proline but not to any appreciable extent from ornithine. Otherwise it would seem that clostridium, strain HF, should be able to grow on ornithine and formate alone.

The decomposition of lysine by clostridium, strain HF, to acetate, butyrate, and ammonia offers interesting possibilities for a study of the intermediary metabolism of lysine. The formation of methyl-labeled acetate from 2-C^{14}-lysine suggests that, to a certain extent, a process is involved whereby a C_2 fragment is split from the carboxyl end of the molecule and is converted to acetate. This is in agreement with the finding by Strassman and Weinhouse (1952) that, in yeast, carbons 1 and 2 of acetate give rise to carbons 1 and 2 of lysine.

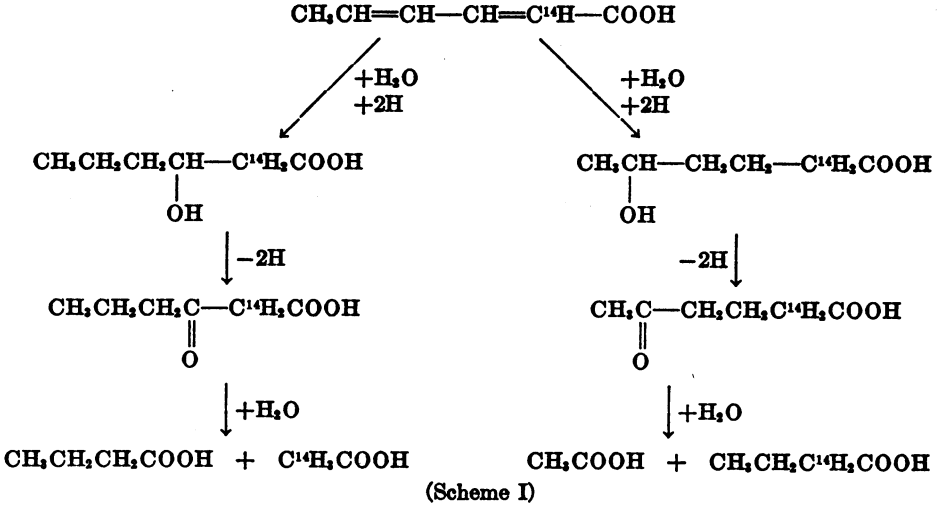
The distribution of isotope in butyrate derived from 2-C^{14}-lysine (predominantly 2-C^{14}-butyrate) suggests that an alternative and quantitatively significant process occurs involving the removal of carbons 5 and 6 of the lysine molecule, perhaps as a C_2 fragment, and conversion of carbons 1–4 to butyrate. As already pointed out, the data do not exclude an asymmetric synthesis of butyrate from C_2 intermediates similar to the asymmetric acetoacetate synthesis originally studied by Crandall and Gurin (1949). This mechanism, however, seems less likely in the clostridium, strain HF, system, for it would require a much higher degree of asymmetry than is usually encountered in fatty acid synthesis. In addition, it is difficult to see why symmetrically labeled butyrate should be formed from C_2 pieces derived from labeled ornithine but not from C_2 pieces derived from labeled lysine in these fermentations.

In view of the metabolic relationship of α-amino adipic acid, α-amino ϵ-hydroxy caproic acid and lysine in the neurospora system (Mitchell and Houlahan, 1948; Good *et al.*, 1950; Windsor, 1951), it is possible that these compounds may be intermediates in the degradation of lysine by clostridium, strain HF.

There exists also a precedent for suggesting that the various hexenoic acid intermediates formed by removal of one or both amino groups to give ammonia may occur in lysine decomposition since urocanic acid is formed in this manner from histidine (Tabor *et al.*, 1952). The formation of fumaric acid from aspartic acid originally described by Quastel and Woolf (1926) also is believed to occur by this mechanism although a more complex series of reactions involving an intermediate such as arginosuccinate (Ratner *et al.*, 1953) may actually be involved.

If the conjugated 2,4-hexadienoic acid (sorbic acid) were formed from lysine by removal of ammonia, and either double bond could be hydrated or reduced, then one could obtain 2-hydroxy caproic or 5-hydroxy caproic acid. Oxidation of these hydroxy acids to the corresponding keto acids and cleavage would then yield butyric and acetic acids derived from various portions of the lysine molecules in conformance with the present isotope data (scheme I).

To test this hypothesis, experiments were carried out in which labeled lysine was decomposed by dried cells in the presence of carrier sorbate. It was found that only a small amount of C^{14} (less than one per cent of that in the acetic and butyric acids formed) appeared in the reisolated sorbate. Whereas these data exclude



(Scheme I)

free sorbic acid as an intermediate in the process, they do not exclude the postulated pathway since the real intermediate might be an activated derivative of sorbic acid (namely sorbyl-coenzyme A) not in rapid equilibrium with free sorbic acid.

A similar situation has been shown to occur in *Clostridium kluyveri* where vinyl acetyl-coenzyme A, an intermediate in butyrate synthesis, is not in equilibrium with free vinyl acetic acid (Stadtman and Barker, 1949).

Examination of the acetic and butyric acids derived from 6-C^{14}-lysine should furnish information as to whether any of the above mentioned compounds can be seriously considered as intermediates in the pathway of lysine degradation by clostridium, strain HF. Moreover, the recent observation (unpublished) that cell-free extracts of the clostridium catalyze the conversion of lysine to fatty acids should simplify the elucidation of individual reactions of the over-all process.

SUMMARY

A clostridium, strain HF, was found to decompose 2-C^{14}-ornithine chiefly by an oxidative pathway to ammonia, carbon dioxide, and 1-C^{14}-acetate. A small amount of 1,3-C^{14}-butyrate is formed. Only about 6 per cent of the ornithine is reduced to δ-amino valeric acid.

2-C^{14}-lysine is converted to ammonia, 2-C^{14}-acetate, and butyrate which is labeled predominantly in carbon 2.

In the presence of sulfide, but not thioglycolate, C^{14}-formate is converted to doubly labeled acetate.

REFERENCES

CHINARD, F. P. 1952 Photometric estimation of proline and ornithine. J. Biol. Chem., **199**, 91–95.

CRANDALL, D. I., AND GURIN, S. 1949 Studies of acetoacetate formation with labeled carbon. I. Experiments with pyruvate, acetate, and fatty acids in washed liver homogenates. J. Biol. Chem., **181**, 829–843.

FRIEDEMANN, T. E. 1938 The identification and quantitative determination of volatile alcohols and acids. J. Biol. Chem., **123**, 161–184.

GOOD, N., HEILBRONNER, R., AND MITCHELL, H. K. 1950 ϵ-Hydroxynorleucine as a substitute for lysine for *Neurospora*. Arch. Biochem., **28**, 464–465.

MA, T. S., AND ZUAZAGA, G. 1942 Micro-Kjeldahl determination of nitrogen. Ind. Eng. Chem. Anal. Ed., **14**, 280–282.

MITCHELL, H. K., AND HOULAHAN, M. B. 1948 An intermediate in the biosynthesis of lysine in neurospora. J. Biol. Chem., **174**, 883–887.

MOORE, S., AND STEIN, W. H. 1948 Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem., **176**, 367–388.

PETERS, J. P., AND VAN SLYKE, D. D. 1932 Methods, p. 290. In *Quantitative clinical chemistry*. Vol. II. Williams & Wilkins Co., Baltimore, Maryland.

PHARES, E. F. 1951 Degradation of labeled propionic and acetic acids. Arch. Biochem. and Biophys., **33**, 173–178.

QUASTEL, J. H., AND WOOLF, B. 1926 The equilibrium between L-aspartic acid, fumaric acid and ammonia in the presence of resting bacteria. Biochem. J. (London), **20**, 545–555.

RAMSEY, L. L., AND PATTERSON, W. I. 1948 Separation and determination of the straight-chain saturated fatty acids C_6 to C_{10} by partition chromatography. J. Assoc. Offic. Agr. Chemists, **31**, 139–150.

RATNER, S., ANSLOW, W. P., AND PETRACK, B. 1953 Biosynthesis of urea. VI. Enzymatic cleavage of arginosuccinic acid to arginine and fumaric acid. J. Biol. Chem., **204**, 115–125.

STADTMAN, E. R., AND BARKER, H. A. 1949 Fatty acid synthesis by enzyme preparations of *Clostridium kluyveri*. V. A consideration of postulated 4-carbon intermediates in butyrate synthesis. J. Biol. Chem., **181**, 221–235.

STADTMAN, T. C. 1954 On the metabolism of an amino acid fermenting *Clostridium*. J. Bact., **67**, 314–320.

STRASSMAN, M., AND WEINHOUSE, S. 1952 Lysine biosynthesis in *Torulopsis utilis*. J. Am. Chem. Soc., **74**, 3457–3458.

TABOR, H., MEHLER, A. H., HAYAISHI, O., AND WHITE, J. 1952 Urocanic acid as an intermediate in the enzymatic conversion of histidine to glutamic and formic acids. J. Biol. Chem., **196**, 121–128.

VAN SLYKE, D. D., AND FOLCH, J. 1940 Manometric carbon determination. J. Biol. Chem., **136**, 509–541.

VOGEL, H. J., AND DAVIS, B. D. 1952 Glutamic γ-semialdehyde and Δ^1-pyrroline-5-carboxylic acid, intermediates in the biosynthesis of proline. J. Am. Chem. Soc., **74**, 109–112.

WINDSOR, E. 1951 α-Amino adipic acid as a precursor to lysine in *Neurospora*. J. Biol. Chem., **192**, 607–609.