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*THE SYNTHESIS OF BUTYRIC AND CAPROIC ACIDS FROM
ETHANOL AND ACETIC ACID BY CLOSTRIDIUM KLUYVERI*

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Unpublished investigations in this laboratory have shown that *Clostridium kluuyveri* can metabolize acetic acid and ethanol under anaerobic conditions producing butyric and caproic acids in accordance with equations (1) and (2).



The relative yields of the two acids are determined by the relative amounts of acetic acid and ethanol available. If acetic acid is present in excess, a considerable amount of butyric acid is formed, while if ethanol is in excess, caproic acid is the main product. These relations suggest that butyric acid may be an intermediate in the synthesis of caproic acid from acetic acid. This is supported by the observation that ethanol and butyric acid can be converted to caproic acid according to equation (3).



In the present investigation we have obtained positive proof for the conversion of acetic acid to caproic acid via butyric acid by studying the action of *Cl. kluuyveri* on media containing synthetic fatty acids labeled with the long-lived radioactive carbon isotope C^{14} . In addition we have obtained various types of evidence which help to elucidate the mechanism of fatty acid synthesis from C_2 molecules.

Experimental Methods.—Carboxyl-labeled acetic, butyric and caproic acids were prepared from C^*O_2 and methyl iodide, propyl bromide and amyl bromide, respectively, by the Grignard synthesis.¹

The general technique of estimating the C^{14} content of carbon dioxide and fatty acids has already been described elsewhere.² The barium salts

of the fatty acids dried at 100°C. were used in the radio-assay. The individual acids were separated from mixtures by the distillation procedure of Schickanz, *et al.*³ The purity of each acid was established by Duclaux distillation. The methods used in locating C¹⁴ in the individual carbon atoms of acetic and butyric acids have been described previously.^{2, 4, 5} The occurrence of C¹⁴ in the carboxyl group of caproic acid was determined by decarboxylating the barium salt. Control experiments with synthetic caproic acid showed that this method yields 52 per cent of the carboxyl carbon as barium carbonate.

The bacteria were grown in media of the following composition in g. per 100 ml.: ethanol 0.3–0.8, sodium salt of labeled acetic or butyric acid 0.3–0.6, yeast autolysate 0.3, pH 7.2, phosphate buffer *M*/40, (NH₄)₂SO₄ 0.1, MgSO₄·7H₂O 0.01, FeSO₄·7H₂O 0.0001, sodium thioglycollate 0.05,

TABLE 1
THE FERMENTATION OF CH₃CH₂OH AND CH₃C*OOH BY *Cl. kluveri*

Experiment 1			
COMPOUND	MM	CTS./MIN./MM	TOTAL CTS./MIN.
Before growth			
Ethanol	ca. 1.1
Acetic acid	0.830	18,900 ± 300	15,700
Carbon dioxide	ca. 0.2
After growth			
Acetic acid	0.395	7330 ± 150	2895
Butyric acid	0.196	18,200 ± 300	3570
Caproic acid	0.257	24,500 ± 500	6300
Carbon dioxide	0.185	26 ± 50	5
Ethanol	0.217	260 ± 50	74
Non-volatile cpds.	193
Percentage recovery of C ¹⁴			83

traces of salts of Ca, Mn and Mo, distilled water. In some experiments sodium carbonate (0.025 g. per 100 ml.) was added as a sterile solution after autoclaving the medium. Oxygen was removed by means of either a pyrogallol-potassium carbonate or an Oxsorbent seal. The cultures were incubated at 32–35° until growth ceased before being analyzed.

Results.—When *Cl. kluveri* was allowed to grow in a medium containing approximately equivalent amounts of ethanol and synthetic carboxyl-labeled sodium acetate, radioactive butyric and caproic acids were formed (table 1). The isotope was proved to be present in these acids, rather than in some associated compound, by establishing the constancy of the specific activities of the barium salts prepared from different fractions of a Duclaux distillation (table 2). A little C¹⁴ was also found in ethanol and unidentified non-volatile compounds, but no significant amount was present in carbon dioxide. It should be noted that there is no net production of

carbon dioxide in this fermentation. The recovery of C^{14} in all forms after growth was about 83 per cent of that initially added as acetic acid.

A particularly significant fact revealed by this experiment is the decrease in the molar activity of acetic acid during the fermentation. At the beginning of the experiment the activity expressed in cts./min./mM was 18.9×10^3 while at the end the value was 7.3×10^3 . The simplest and most likely explanation for this reduction in activity is the oxidation of ethanol to inactive acetic acid or some closely related compound in equilibrium with it. This would cause a dilution of the labeled acetic acid.

TABLE 2

SPECIFIC ACTIVITIES OF BARIUM SALTS OF BUTYRIC AND CAPROIC ACIDS PREPARED FROM
DUCLAUX DISTILLATIONS

Experiment 1 (total volume = 110 ml.)

FRACTION	CTS./MIN./MG. OF Ba SALT
Butyric acid	
0-20 ml.	47.5
20-40 ml.	47.6
40-110 ml.	46.4
Caproic acid	
0-20 ml.	63.7
20-110 ml.	62.2

Another possible explanation for the decrease in activity of acetic acid is that it is in chemical and isotopic equilibrium with the higher fatty acids formed from it and ethanol. This was tested by two experiments in one of which butyric acid labeled in the carboxyl group was added at the beginning of the fermentation; in the second experiment butyric acid labeled on all four carbon atoms was used. In the experiment with carboxyl-labeled butyric acid (table 4), no C^{14} could be detected in the final acetic acid, thus indicating that no exchange occurred between the carboxyl groups of butyric acid and acetic acid. In the second experiment with completely labeled butyric acid, a little C^{14} was recovered in the final acetic acid but it was less than 9 per cent of the amount to be expected if butyric and acetic acids were in isotopic equilibrium, and even this small effect may have been due to a slight contamination of the original butyric acid by labeled acetic acid. An exchange of C^{14} between acetic acid and the higher fatty acids can therefore be excluded as an explanation for the observed decrease in activity of acetic acid.

A third possible explanation for this effect is an exchange of C^{14} between ethanol and acetic acid as a result of reactions involving acetaldehyde or a similar compound as an intermediate. If such a reaction occurs, the residual ethanol should have the same molar activity as the acetic acid. Actually, the activity of the alcohol is of a lower order of magnitude

(table 1). Therefore an exchange of C^{14} between acetic acid and ethanol is not consistent with the observed results.

Whatever the actual mechanism of the dilution of the active acetic acid, the magnitude of the effect is about what would be expected if all the ethanol is oxidized to acetic acid while the latter is being converted to higher fatty acids. To calculate the expected decrease of acetic acid activity on this basis, let us assume that (1) the fermentation proceeds in two successive steps represented by equations (1) and (3), and that during the fermentation all the ethanol is first converted to acetic acid or some compound in isotopic equilibrium with it. In step 1, the formation of butyric acid from ethanol and acetic acid, let x = the amount of C^{14} present in acetic acid at any time, x_0 = the initial C^{14} , x_1 = the C^{14} at the end of step 1, V = the amount of acetic acid present at any time, V_0 = the initial acetic acid, and V_f = the final acetic acid. Now during step 1, when a small amount (ΔV) of alcohol is oxidized to inactive acetic acid, the removal of active acetic acid by conversion to butyric acid will be equal to $2\Delta V$. The loss of C^{14} from acetic acid is therefore

$$-\Delta x = -2\Delta V/V - \Delta V \cdot x. \quad (4)$$

In the limit as ΔV approaches zero

$$dx/x = 2dV/V. \quad (4a)$$

Integrating between the limits of V_0 and V_f , and x_0 and x_1 and changing to \log_{10} we get

$$\log x_1/x_0 = 2 \log V_f/V_0 \quad (5)$$

or

$$x_1 = x_0(V_f/V_0)^2. \quad (5a)$$

In step 2 (equation 3), the conversion of alcohol and butyric acid to caproic acid, there is no change in the total quantity of acetic acid; V_f is therefore a constant. C^{14} will nevertheless be lost from acetic acid if, as is assumed, it is an intermediate in the utilization of ethanol. When a small amount of alcohol (ΔA) is used, the loss of C^{14} from acetic acid is

$$-\Delta x = \Delta A/(V_f + \Delta A) \cdot x. \quad (6)$$

In the limit

$$-dx/x = dA/V_f. \quad (6a)$$

Integrating between the limits x_1 and x_f , and 0 and A_f , where A_f is the total amount of alcohol used in step 2

$$\ln x_1/x_f = 2.3 \log x_1/x_f = A_f/V_f \quad (7)$$

or

$$\log x_f = \log x_1 - A_f/2.3V_f \quad (7a)$$

When this method of calculation is applied to the data of table 1 where $x_0 = 15,600$ cts./min., $V_0 = 0.830$ mM, $V_f = 0.395$ mM and $A_f = 0.257$ mM, it is found that $x_1 = 3540$ cts./min., and $x_f = 1840$ cts./min. The calculated molar activity of acetic acid at the end of the fermentation is therefore 1840 cts./min./ 0.395 mM = 4660 cts./min./mM. This value is of the same order of magnitude though somewhat smaller than the observed molar activity of 7330 cts./min./mM. The discrepancy may be due to the fact that butyric and caproic acids are formed simultaneously rather than successively during part of the fermentation; this would raise the calculated activity of the final acetic acid and bring it into closer agreement with the observed value. The general conclusion to be drawn from a comparison of the calculated and observed decrease in the molar activity of acetic acid is that the results support the view that alcohol is oxidized to acetic acid or a closely related compound which is then condensed to butyric and caproic acids.

The following information was obtained concerning the distribution of C^{14} in the fatty acids at the end of the fermentation.

TABLE 3
DISTRIBUTION OF C^{14} IN BUTYRIC ACID
Experiment 2

CARBON ATOM	CTS./MIN./MM	PER CENT OF TOTAL RECOVERED
Carboxyl	1680 ± 50	54 ± 2
Alpha and gamma (average)	28 ± 50	1 ± 2
Beta	1395 ± 30	45 ± 2

The final like the initial acetic acid contained C^{14} only in the carboxyl group. The actual results were 7400 ± 200 cts./min./mM of carboxyl carbon and 0 ± 30 cts./min./mM of methyl carbon.

Data on the distribution of C^{14} in butyric acid are presented in table 3. The butyric acid was obtained from a second experiment identical with experiment 1. The data show that C^{14} is present in the carboxyl and beta positions but not in the alpha and gamma positions. The quantity of C^{14} in the carboxyl and beta carbon atoms is almost equal, though there appears to be a small but significantly greater amount in the carboxyl carbon. The smaller C^{14} content of the beta carbon may result from the fact that the beta (and gamma) carbon atoms are derived from an oxidation product of ethanol which is not in complete isotopic equilibrium with the preformed acetic acid.

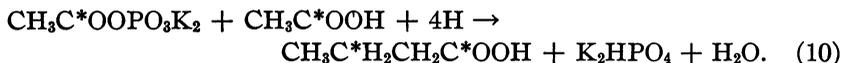
No method was available for determining the complete distribution of C^{14} in caproic acid. The presence and amount of C^{14} in the carboxyl group could, however, be determined by decarboxylation of the barium salt. We found 4150 cts./min./mM in the barium carbonate obtained by the

decarboxylation of barium caproate containing 11,600 cts./min./mM. Therefore, 35.7 per cent or slightly more than one-third of the C¹⁴ was present in the carboxyl group. The data are consistent with the view that the C¹⁴ is equally distributed among three of the six carbon atoms (probably those in the carboxyl, beta and delta positions), but this is not certain.

The results of experiment 1 (table 1), considered in conjunction with other information now available, lead us to postulate that the following reactions may be involved in the formation of butyric and caproic acids from acetic acid and ethanol.



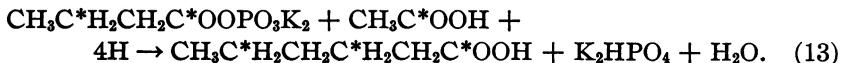
In the presence of synthetic CH₃C*OOH, the forward reaction (9) would cause a dilution of the active acetic acid and the reverse reaction would introduce C¹⁴ into the carboxyl group of acetyl phosphate. Although there is at present no direct evidence to indicate the occurrence of acetyl phosphate in this fermentation, the compound is almost certainly formed by *Cl. butylicum*,⁶ an organism which also produces butyric acid (and butyl alcohol) from acetic acid.⁵ Moreover the chemical properties of acetyl phosphate are consistent with its being an intermediate in fatty acid synthesis.^{7, 8} The labeled acetyl phosphate and the labeled acetic acid are assumed to condense to yield a product that is reduced to butyric acid. This reaction is not reversible.



A similar type of condensation and reduction involving butyric acid and acetyl phosphate could lead to the formation of caproic acid labeled in the carboxyl, beta and delta positions.

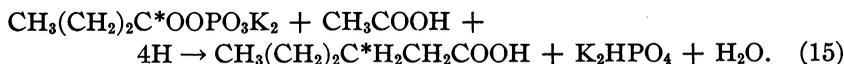


Reaction (11) represents only one of two possible ways in which caproic acid could be formed. Instead of there being a direct addition of the acetyl group to the terminal methyl group of butyric acid as is indicated above, the high energy phosphate group could be first transferred to butyric acid to give butyryl phosphate which could then condense with the methyl group of acetic acid.



Evidence has been presented by Koepsell, *et al.*,⁶ that a transphosphorylation between acetylphosphate and butyric acid occurs under the influence of an enzyme preparation obtained from *Cl. butylicum*. With either mechanism the isotope distribution of the resulting caproic acid would be the same under the conditions of experiment 1.

It is possible, however, to distinguish between these two types of condensation by carrying out a fermentation of ordinary ethanol and synthetic butyric acid labeled in the carboxyl group. The position of the C¹⁴ in the resulting caproic acid must depend on the way in which the condensation occurs. This is illustrated in equations (14) and (15) where the inactive acetyl phosphate and acetic acid are assumed to originate from the oxida-



tion of ethanol. It can be seen that a condensation of acetyl phosphate and labeled butyric acid would yield carboxyl-labeled caproic acid, while a condensation of labeled butyryl phosphate and ordinary acetic acid would yield caproic acid labeled in the beta position.

TABLE 4
THE FERMENTATION OF CH₃CH₂OH AND SYNTHETIC CH₃CH₂CH₂C*OOH BY
Cl. kluveri
Experiment 3

COMPOUND	MM/10 ML.	CTS./MIN./MM	TOTAL CTS./MIN.
Before growth			
Acetic acid	0.100	0	0
Butyric acid	0.384	17,200 ± 500	6610
Ethanol	ca. 1.7	0	0
After growth			
Acetic acid	0.101	0 ± 100	0
Butyric acid	0.05	18,000 ± 10,000	955
Caproic acid	0.427	12,600 ± 500	5400
Ethanol	0.306	250 ± 50	75

The results of an experiment designed to distinguish between these possible condensation mechanisms are given in table 4. Alcohol was added in excess to favor a high conversion of butyric acid to caproic acid.

It can be seen in table 4 that most (81.7 per cent) of the C¹⁴ originally present in butyric acid was converted into caproic acid. The molar activity of the caproic acid (12,600 ± 500 cts./min./mM) is somewhat less than that of the original butyric acid (17,200 ± 500 cts./min./mM) due to the fact that part of the caproic acid was completely built up from in-

active ethanol and acetic acid. This is evident from the fact that the quantity of caproic acid formed (0.427 mM) was greater than the butyric acid originally added (0.384 mM). When this dilution is taken into account, the calculated molar activity of the final caproic acid (17,200 cts./min./mM \times 0.384 mM/0.427 = 13,400 cts./min./mM) agrees fairly well with its observed molar activity (12,600 cts./min./mM).

The caproic acid formed in this experiment contained little or no C^{14} in the carboxyl group. The barium carbonate obtained by decarboxylation from two samples of barium caproate showing activities of 845 ± 20 and 657 ± 15 cts./min., were found to give only 6 ± 3 and 5 ± 3 cts./min., respectively. It will be recalled that synthetic carboxyl-labeled barium caproate yields 52 per cent of its C^{14} as barium carbonate.

The absence of C^{14} from the carboxyl group of caproic acid proves that a condensation of the type shown in equations (11) and (14) does not occur. The results are, however, consistent with the mechanism shown in equations (13) and (15), involving a condensation of butyryl phosphate with the methyl group of acetic acid. The evidence in favor of this type of condensation would be stronger if the C^{14} had been proved to be present only in the beta position. This has not yet been accomplished, but further work on this problem is in progress.

There are a few other points concerning experiment 3 (table 4) that should be mentioned.

The complete absence of activity in acetic acid definitely proves that the conversion of acetic to butyric acid is not appreciably reversible at least so far as the carboxyl carbon atom is concerned. Evidence has already been presented above against any considerable exchange of carbon between acetic acid and the alpha, beta or gamma carbon atoms of butyric.

A small but definite amount of activity was found in the neutral volatile compounds, reported as ethanol. It is possible that this activity is actually present in ethanol, though this would be difficult to reconcile with the fact that there is no activity in acetic acid which seems to be an intermediate between butyric acid and ethanol. Another possibility is that the activity attributed to ethanol is actually present in an associated compound such as butyl or hexyl alcohol. Since the molar activities of the C_4 and C_6 compounds are relatively very high, a small (1-2 per cent) contamination of the ethanol would account for the observed activity. With the small amount of alcohol and the low activity involved in the present experiment, the contamination theory could not be proved or disproved. However, it may be mentioned in this connection that the characteristic odor of *Cl. kluyveri* cultures suggests the presence of a higher alcohol or ester.

Summary.—1. When *Cl. kluyveri* is grown anaerobically in a medium containing ordinary ethanol and synthetic acetic acid labeled in the carboxyl group with C^{14} , labeled butyric and caproic acids are formed.

2. The butyric acid so formed has C¹⁴ almost equally distributed between the carboxyl and beta positions. The alpha and gamma positions are inactive.

3. The caproic acid has one-third of its C¹⁴ in the carboxyl group; probably the beta and delta positions are also labeled, though this has not been proved.

4. No active carbon dioxide is formed from carboxyl-labeled acetic acid. This indicates that carbon dioxide is not an intermediate in these reactions.

5. The C¹⁴ content of the residual acetic acid is much lower than that of the initial acetic acid. This evidently results from the oxidation of ethanol to acetic acid or a related compound in isotopic equilibrium with it.

6. When *Cl. kluveri* is grown with ordinary ethanol and synthetic carboxyl-labeled butyric acid, C¹⁴ is found in caproic acid but not in acetic acid.

7. The active caproic acid so formed contains almost no activity in its carboxyl group.

8. The above facts are consistent with the view that the formation of butyric acid involves a condensation between acetic acid or a reactive derivative thereof, such as acetylphosphate, formed by the anaerobic oxidation of ethanol, and a second molecule of acetic acid. The condensation product is then reduced to butyric acid. Caproic acid formation involves a condensation of the carboxyl group of butyric acid or some related C₄ compound, like butyrylphosphate, with the methyl group of acetic acid.

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