Pinnow.[6] To 0.20 g. of 1,2,3-trimethylbenzimidazole chloride, dissolved in 1.5 ml. of distilled water, 0.4 g. of potassium hydroxide was added. A flocculent precipitate formed upon addition of the potassium hydroxide. The mixture was heated to boiling, cooled and filtered to give 0.15 g. (83%) of product, which after recrystallization from hot water melted sharply at 165° (cor.), which checked the value reported by previous investigators.

The melting points of the products obtained by both methods and of a mixture of the two products were determined simultaneously. All these samples melted at exactly the same temperature and in the same manner.

Summary

o-Phenylenediamine and malonic acid react to give *o*-phenylenemalonamide, a compound containing a seven-membered ring. Alkylation of this compound produced a N,N'-dimethyl derivative which underwent rearrangement on treatment with sulfuric acid to produce 1,2,3-trimethyl-2-hydroxy-2,3-dihydrobenzimidazole.

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[Contribution from Bacteriology Section, Iowa Agricultural Experiment Station, and Chemistry Department, Iowa State College, and Department of Bacteriology, Tuskegee Institute]

The Degradation of Heavy-Carbon Butyric Acid from the Butyl Alcohol Fermentation[1]

By H. G. Wood, R. W. Brown, C. H. Werkman and C. G. Stuckwisch

The present investigation was part of a study in which C^{13} compounds were used as tracers for determining the mechanism of the butyl alcohol fermentation. When $CH_3C^{13}OOH$ was added to a fermentation of corn mash by the butyl alcohol bacteria, butyl alcohol was formed which contained heavy carbon. This alcohol was separated from the other neutral products of the fermentation by oxidation to the corresponding acid and was isolated by the distillation procedure of Schicktanz, *et al.*[2]

For an understanding of the mechanism of formation of the butyl alcohol, it was necessary to know not only the position of the heavy carbon in the carbon chain but also the concentration of heavy carbon in each position. Such information is essential in deciding whether two molecules of acetic acid unite, for example, as follows

$$2CH_3C^{13}OOH \longrightarrow CH_3C^{13}OCH_2C^{13}OOH + H_2O$$

$$CH_3C^{13}OCH_2C^{13}OOH + 8H \longrightarrow CH_3C^{13}H_2CH_2C^{13}H_2OH + 2H_2O$$

or, whether the acetic acid unites with an intermediate compound formed from the cornstarch. In this latter case probably only one position in the molecule would contain a concentration of heavy carbon in excess of the normal, since that portion of the molecule arising from the starch would have a normal concentration of C^{13}. Moreover, both types of reactions might occur simultaneously. The relative concentration of C^{13} in the respective positions of the molecule would then be a measure of the relative rates of the two reactions and would also designate the parts of the molecule which arise preferentially from the acetic acid and from an intermediate formed from the starch carbon.

Because of the limited amount of heavy carbon compound available it was necessary to employ a degradation procedure that could be conducted on approximately two millimoles of butyric acid. With this quantity of material it is difficult to use a degradation involving a number of reactions with pure and dry compounds. For this reason a reaction which could be conducted in aqueous solution of the acid was sought and the oxidation of butyrate following the procedure of Allen and Witzemann[3] was accordingly investigated. These authors have found that, under proper conditions of oxidation by hydrogen peroxide, there are approximately the following yields of compounds from ammonium butyrate

Carbon dioxide	48%
Acetic acid	24%
Acetone	27%
Acetaldehyde (?)	8%

The aldehyde was not positively identified as acetaldehyde.

The oxidation offered possibility of giving the desired degradation because the acetone, if proved to arise from α, β and γ positions, could be used as a measure of the average C^{13} in these three positions, and by difference from the whole butyrate molecule the C^{13} in the carboxyl group could be calculated. Furthermore, by degradation of the acetone by the iodoform reaction, the C^{13} could be located in other positions.

$$CH_3C^{13}H_2CH_2C^{13}OOH \longrightarrow CH_3C^{13}OCH_3 + C^{13}O_2$$

$$CH_3C^{13}OCH_3 \xrightarrow{NaOI} CHI_3 + CH_3C^{13}OOH$$

It is evident from the above equations that the beta position of the butyric acid is equivalent to the carboxyl group of the acetic acid which is formed in the iodoform reaction. No further degradation is needed to determine the C^{13} in the carboxyl group of the acetic acid because the C^{13} of the iodoform has the same value as the methyl group of the acetic acid. C^{13} of the carboxyl carbon can, therefore, be calculated when the C^{13} of the whole molecule of acetic acid is known. The C^{13} value of the iodoform likewise will be a measure of the average value of both the α and γ positions of butyric acid since the

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[1] Journal Paper No. 1224 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 746.

[2] Schicktanz, Steeles and Blaisdell, *Ind. Eng. Chem., Anal. Ed.*, **12**, 320 (1940).

[3] Allen and Witzemann, This Journal, **63**, 1922 (1941).

OUTLINE FOR FRACTIONATION OF PRODUCTS OF OXIDATION OF AMMONIUM BUTYRATE BY HYDROGEN PEROXIDE

(1) 110 ml. of reaction mixture in a 200-ml. round-bottom flask: 80 ml. distilled in approximately one hour; receiving vessel is iced and furnished with an adapter which dips into 10 ml. of 1 *N* NaOH and 0.2 g. of MnO_2. Residue is cooled and 0.2 g. of MnO_2 and 2 ml. of 3 *N* NaOH added.

(2) Distillate of (1) is decanted from MnO_2 and distilled to two-thirds volume. Residue is combined with residue of (1).

(3) Distillate of (2) is oxidized in stoppered flask with 15 ml. of $K_2Cr_4O_7$[a] solution on steam-bath for fifteen minutes, then distilled to $^2/_3$ volume. Residue is combined with residue of (4).

(4) Distillate of (3) is neutralized to *p*H 8.0 and distilled to two-thirds volume. Distillate contains acetone.

Iodoform reaction is run on distillate of (4) and filtered.

Ppt. is oxidized (CHI_3 of acetone).

Filtrate is distilled to 25 ml. and then an additional 250 ml. of steam distillate is collected. Distillate is refluxed and aerated for two hours; Acids partitioned, then oxidized (CH_3COOH of acetone).

Residues of (3) and (4) are distilled to 15 ml. and then an additional 150 ml. of steam distillate is collected. Residue is discarded. Distillate is refluxed and aerated; acids are partitioned, then oxidized. (Acids from oxidation of neutral distillate.)

(5) Residue of (1) and residue of (2) are acidified with 3 ml. of 5 *N* H_2SO_4, then refluxed and aerated: CO_2 is collected (CO_2).

(6) Residue of (5) is distilled to 15 ml. and then an additional 150 ml. of steam distillate is collected.

Acids in distillate of 6 are partitioned then oxidized (volatile acids).

Residue of 6 is oxidized (residue of distillation).

[a] 134 grams of $K_2Cr_4O_7$ dissolved in 675 ml. of 10 *N* H_2SO_4 and diluted to one liter.

iodoform arises equally from either methyl group of the acetone.

The general procedure used in the oxidation and in the fractionation was designed especially to give pure acetone because the reliability of the estimation of C^{13} in different positions of the butyric acid is dependent upon an accurate measurement of this C^{13}. The acetone was separated from the volatile acids by distillation from an alkaline solution. It was next separated from other neutral volatile compounds by oxidation with acid dichromate and by alkaline distillation. This procedure separates the acetone from aldehydes and alcohols since these last two compounds are oxidized to acids. The acetone fraction was then subjected to the iodoform reaction and the acetone was determined quantitatively by determining the iodine consumed (Goodwin[4]). The iodoform was filtered off and dried over calcium chloride. The acetic acid was recovered from the filtrate by steam distillation and the fraction was shown to contain only acetic acid by the partition method of Osburn, *et al.*,[5] and by establishing the equivalence of the total carbon and the determined acetic acid. The combination of properties: volatility from alkaline solution, stability to dichromate oxidation and formation of only iodoform and acetic acid by the iodoform reaction, was considered proof that the isolated carbon was exclusively from the acetone.

The other products of the oxidation were determined quantitatively and their C^{13} content measured to obtain additional information on the mechanism of the oxidation. The total carbon was determined on each fraction as a check for presence of unidentified compounds in the fraction. Carbon determined usually was found to agree rather closely with the calculated values. The C^{13} was determined on the carbon dioxide obtained from the carbon determinations with a mass spectrometer constructed according to Nier.[6] The C^{13} values are presented as per cent. C^{13} in excess of the normal, *i. e.*, the concentration of C^{13} over and above the C^{13} complement (1.09%) which has been found for unenriched carbon. The per cent. C^{13} is given in moles of $C^{13} \times 100/$ moles of $C^{12} + C^{13}$. The mM. of excess C^{13} is calculated from the mM. of total carbon in the compound and the per cent. of excess of C^{13}.

Regarding details of the fractionation procedure of the outline the following are noted. The MnO_2 was added to the receiver in step 1 to break down the hydrogen peroxide in the distillate and thus prevent additional oxidation. Sodium hydroxide was added to trap the carbon dioxide. The residue of distillation 1 was treated similarly for the same reason. The volatile acid solution, prior to partitioning, was refluxed and aerated with carbon dioxide-free air to remove dissolved carbon dioxide. Prolonged refluxing was used on the distillate from the iodoform titration to remove hydrogen sulfide which was formed from thiosulfate. The total carbon determinations were made by the method of Osburn and Werkman[7] except for the oxidation of iodoform where the method of Friedemann and Kendall[8] was used.

Table I shows the results of two experiments in which $CH_3CH_2CH_2C^{13}OOH$ was oxidized with

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[4] Goodwin, THIS JOURNAL, **42**, 39 (1920).

[5] Osburn, Wood and Werkman, *Ind. Eng. Chem., Anal. Ed.*, **8**, 270 (1936).

[6] Nier, *Rev. Sci. Instruments*, **11**, 212 (1940).

[7] Osburn and Werkman, *Ind. Eng. Chem., Anal. Ed.*, **4**, 421 (1932).

[8] Friedemann and Kendall, *J. Biol. Chem.*, **82**, 45 (1929).

Table I

Products of the Oxidation of $CH_3CH_2CH_2C^{13}OOH$ with H_2O_2

No. 1: 30 ml. of 0.056 *M* butyric acid, 7.80 ml. of 0.203 *M* NH_4OH, 2.75 ml. of 30% H_2O_2, 70 ml. of H_2O. No. 2: 40 ml. of 0.056 *M* butyric acid, 13.40 ml. of 0.168 *M* NH_4OH, 2.75 ml. of 30% H_2O_2, 53.8 ml. of H_2O.

Fraction		mM.		C^{13} in excess of normal, %		C^{13} in excess of normal, mM.		Recovery of C^{13} of butyric acid, %		Recovery of Total carbon of butyric acid, %	
Expt. no. →		1	2	1	2	1	2	1	2	1	2
CO_2		1.08	2.75	3.96	3.16	0.043	0.087	50	76	16	31
Volatile acids	Acetic	0.31	0.35	0.81	0.11	.013	.001	15	1	24	8
	Butyric	.24	.01								
Neutral distillate (oxidized $K_2Cr_4O_7$)	Acetic	.17	.66	.12	.02	.001		1		10	17
	Propionic	.11	.07								
	Acetone	.30	.67	.00	.00	.000		0	0	13	22
Residue of distillation		1.21	.42	.96	.74	.012	.003	14	3	18	5
				Totals		.069	.092	80	80	81	83
Original butyric acid		1.68	2.24	1.28	1.28	.086	.115				

hydrogen peroxide. These experiments were for the purpose of proving that the acetone is formed exclusively from the α, β and γ carbons of the butyrate. The butyrate was synthesized from propylmagnesium bromide and $C^{13}O_2$. The products of the oxidation are acetic acid, acetic and propionic acids from the oxidation of the neutral distillate, acetone and unidentified non-volatile compounds in the residue of distillation. These products together with unoxidized butyrate account for somewhat more than 80% of the total carbon and of the excess C^{13} of the original butyrate. The acetic and propionic acids, obtained by oxidation of the neutral distillate, are probably derived from the corresponding aldehydes. Low recovery of carbon results from losses incurred in the rather extensive fractionation procedure. It has been observed by total carbon determination on fractions 2 and 6 of the outline that the carbon of the butyrate may be accounted for quantitatively.

Four conclusions may be drawn from the results shown in Table I:

First, there is no excess C^{13} *in the acetone which is formed by oxidation of* $CH_3CH_2CH_2C^{13}OOH$. It may, therefore, be concluded that the acetone arises exclusively from the α, β and γ positions of the butyric acid. Furthermore, it is a fairly safe assumption that there is no shifting of positions of carbon atoms within the carbon chains which arise from the oxidation. On this basis the carbon of the carbonyl group of the acetone is identical with the β carbon of the butyric acid; the methyl groups of the acetone represent the α and γ positions.

Second, there is no excess C^{13} *in the acetic acid which is formed in the oxidation of* $CH_3CH_2CH_2C^{13}OOH$. Any C^{13} that is present in the volatile acid fraction is accounted for by the presence of unoxidized C^{13}-butyric acid. If calculations are made on the basis that the butyric acid contains the original concentration of C^{13} (1.28% excess C^{13}) and the acetic acid no excess C^{13}, the calculated percentage for the volatile acids of expt. 1 is [(4 × 0.24 × 0.0128) 100 ÷ (2 × 0.31 + 4 × 0.24), *i. e.*, (mM. of C^{13} ÷ mM. of carbon) 100] 0.78%. The observed value is 0.81%. The calculated value for expt. 2 is 0.07%, the observed value 0.11%. The difference between the observed and calculated values is probably within the limits of experimental error. It may, therefore, be concluded that none of the acetic acid is formed by a 2- and 2-carbon cleavage of the 4-carbon chain. The evidence does not permit a conclusion as to the exact origin of the acetic acid, *i. e.*, whether or not the acetic acid is formed from only two positions (α, β or β, γ) of the butyrate or from three positions (α, β and β, γ).

The third conclusion is that the acetic and propionic acids from the dichromate oxidation of the neutral distillate are of the α, β and γ carbon origin. In the case of Expt. 1 there was a small amount of excess C^{13} in the fraction, which indicates a slight amount of carboxyl carbon was present in the fraction. However, in experiment 2, where more of the compounds are available for fractionation and the results are thus more reliable, the C^{13} was normal within experimental limits. The neutral products of the oxidation are probably for the most part acetaldehyde and a small amount of propionaldehyde.

The fourth conclusion is that the carboxyl carbon occurs only in the carbon dioxide and in unidentified compounds in the residue of the distillation. The carbon dioxide is formed both from the carboxyl carbon and from other positions in the molecule. This is evident since the carboxyl carbon contained 5.12% excess C^{13} whereas the carbon dioxide contained 3.96 and 3.16% excess C^{13}, respectively. On the basis of the C^{13} in the CO_2 the proportion of the carboxyl carbon (labelled carbon) to non-labelled normal carbon may be calculated.[9] Seventy-eight per cent. of the car-

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[9] Experiment 1 is used as an illustration. The excess C^{13} was 5.12% in the carboxyl positions. 1.08 mM. of carbon dioxide was produced which contained 3.96% excess C^{13}. 1.08 × 0.0396 = 0.043 mM. of excess C^{13} which was present in the carbon dioxide. The only source of labelled carbon is the carboxyl of the butyrate. The mM. of carboxyl carbon required to supply the excess C^{13} of the carbon dioxide is therefore 0.043 ÷ 0.0512 = 0.84 mM. (mM. of C^{13} ÷ per cent. = mM. of carbon). This labelled carbon (0.84 mM.) is 78% of the carbon dioxide (1.08 mM.).

TABLE II

PRODUCTS OF THE OXIDATION OF $CH_3C^{13}H_2CH_2C^{13}OOH$ WITH H_2O_2

No. 3: 90 ml. of acid soln.,[a] 7.5 ml. of 0.203 *M* NH_4OH, 2.75 ml. of 30% H_2O_2, 10 ml. of H_2O. No. 4: 90 ml. of acid soln.,[b] 15.3 ml. of 0.168 *M* NH_4OH, 2.75 ml. of 30% H_2O_2, 2.00 ml. of H_2O.

Fraction		mM.		C^{13} in excess of normal, %		C^{13} in excess of normal, mM.		Recovery of C^{13} of butyric acid, %		Recovery of total carbon of butyric acid, %	
Experiment no. →		3	4	3	4	3	4	3	4	3	4
CO_2		2.31	2.07	1.48	0.76	0.034	0.016	47	31	38	21
Volatile acids	Acetic	0.24	0.52	1.10	.46	.005	.007	7	14	8	15
	Butyric	.00	.13								
Neutral distillate (oxidized by $K_2Cr_4O_7$)	Acetic acid	.42	.54	1.03	.45	.010	.006	14	12	17	14
	Propionic acid	.06	.01								
	Acetone	.40	.64							20	19
	CHI_3[c]			0.00	.00	.000	.000	0	0		
	CH_3COOH[c]			1.10	.53	.009	.007	12	14		
Residue of distillation		.12	1.81	0.37	.38	.001	.007	1	14	2	18
				Totals		.059	.043	81	85	85	87
Original acid	Butyric	1.49	2.34	1.20	.51	.073	.051				
	Propionic	0.05	0.24								

[a] Acid isolated from a fermentation by *Cl. butylicum*. [b] Acid isolated from a fermentation by *Cl. acetobutylicum*. [c] Iodoform and acetic acid obtained from acetone by the iodoform reaction.

bon dioxide was formed from carboxyl carbon in Expt. 1 and 62% in Expt. 2. The remaining carbon dioxide was from other positions which are not determinable from present data.

With regard to the compound (or compounds) in the residue of distillation it seems probable that there is an acid present since there is carboxyl carbon involved as evidenced by the C^{13} concentration. From the concentration of C^{13} in the fraction it is estimated that there are from 4.5 to 6 carbons from the α, β and γ positions of the butyrate for each carbon from the carboxyl group. This fact may mean that there is a mixture of compounds in the fraction. On the other hand a single compound like oxalic acid may be formed and originate only in part from the carboxyl portion of the molecule. Such a case is the oxidation of $CH_3CH_2C^{13}OOH$ with alkaline permanganate).[10] The oxalic acid is formed from the α and β positions as well as from the α and carboxyl positions of the propionate.

After obtaining the results from the oxidation of $CH_3CH_2CH_2C^{13}OOH$, it was clear that the oxidation was suitable for the selective breakdown of butyrate. The oxidation was, therefore, conducted on the acid isolated from the fermentation solutions. The original acid solution which was used in this oxidation contained a small amount of propionic acid. This propionic acid was formed along with butyric acid when the butyl alcohol from the fermentation liquor was oxidized with dichromate. The subsequent fractionation of these acids by the distillation procedure of Schicktanz, *et al.*,[2] did not give complete separation of the butyric and propionic acids.

The results from the oxidation of this butyric acid, which corresponds to butyl alcohol isolated from fermentations to which $CH_3C^{13}OOH$ was added, are shown in Table II.

The following pertinent facts are evident from the results.

First: *The butyric acid (butyl alcohol from the fermentation) contains the* C^{13} *in the carboxyl and* β *positions of the molecule.* This is evident for, contrary to the results from the oxidation of $CH_3CH_2CH_2C^{13}OOH$, the acid from the fermentation yielded, in addition to CO_2, acetone which contained excess C^{13}. The presence of excess C^{13} in the acetone proves that the C^{13} was not exclusively in the carboxyl group of the acid. Degradation of the acetone by the iodoform reaction showed that the excess C^{13} from this portion of the butyric acid was in the β position. The CHI_3, which is equivalent to the methyl positions of the acetone or α and γ positions of the butyrate, contained no excess C^{13}, whereas the acetic acid which contains the carbonyl carbon of the acetone or β position of the butyrate contained all the excess C^{13} of the acetone.

Regarding the quantitative distribution of the excess C^{13} between the carbonyl and β positions of the butyrate, the C^{13} concentrations are shown to be identical or nearly so in the two positions. In the case of Expt. 3, in which the acid from the fermentation by *Clostridium butylicum* was used, the C^{13} is indicated to have been slightly more concentrated in the carboxyl position than in the β position. The excess C^{13} in the β position may be calculated from the C^{13} in the acetic acid of the acetone. (Average C^{13} of CH_3COOH = (C^{13} of CH_3 + C^{13} of COOH) ÷ 2.) The experimental figure for the average of the molecule was 1.10% and there was no excess C^{13} in the methyl position; therefore, the C^{13} of the carboxyl is calculated equal to 2.20 per cent. This is the experimental value for the C^{13} in the β position of the butyrate. With this value for the β position and that the α and γ positions contain no excess C^{13} and the average C^{13} of the whole mole-

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(10) Wood, Werkman, Hemingway, Nier and Stuckwisch, THIS JOURNAL, 63, 2140 (1941).

cule is 1.21,[11] the C^{13} in the carboxyl of the butyrate is calculated to be 2.64 $(0.00 + 2.20 + 0.00 + C^{13}$ of COOH$) \div 4 = 1.21$. In the case of Expt. 4 by similar calculation the β position is equal to 1.06, $(0.00 + C^{13}$ of COOH$) \div 2 = 0.53$. The C^{13} of the carboxyl of the butyrate is 1.02, $(0.00 + 1.06 + 0.00 + C^{13}$ of COOH$) \div 4 = 0.52$. These C^{13} concentrations (1.06 and 1.02) are, within experimental error, identical for the case of Expt. 4. To establish whether the indicated difference between the β and carboxyl positions of the butyric acid of Expt. 3 is real requires further investigation. An undetected impurity in the fraction may have caused a slight dilution of the C^{13}. At any rate, if there is a difference in the C^{13} concentrations of the positions, it is not large.

The significance of these results in interpreting mechanisms of the butyl alcohol fermentation will be considered in detail in a later publication. From the presented results it is evident, however, that the 2-carbon compound acetic acid is converted into butyl alcohol. There is no significant evidence of a preferential formation of one part of the butyl alcohol from starch carbon rather than from acetate carbon. This fact indicates that it is the acetate as such or derivative of the acetate which gives rise to the butyl alcohol. Davies[12] has recently obtained evidence which indicated the condensation might involve both pyruvate and acetate.

The establishment of the concentration and distribution of the C^{13} in the butyric acid permits consideration of the pertinent facts relative to the other products of the oxidation.

It is observed, Table II, that *the acetic acid which was formed from the* $CH_3C^{13}H_2CH_2C^{13}OOH$ *contained* C^{13} *in a concentration equivalent to that of the whole molecule.* This fact is in agreement with the previous findings with $CH_3CH_2CH_2C^{13}OOH$, *i. e.*, that the acetic acid is formed exclusively from α, β and γ carbons. It is evident that no matter how the oxidation of $CH_3C^{13}H_2CH_2C^{13}OOH$ occurs, whether by forming acetic acid from the α and β carbons or from the β and γ carbons or by both reactions, one of the carbons contains no excess C^{13} and the other contains excess C^{13}. Any of these reactions will, therefore, give acetate with the same average concentration of C^{13}. This concentration will be the same as that for the whole butyrate molecule, since there are two carbon atoms in the butyrate with no excess C^{13} and two carbons containing an equivalent of excess C^{13}. Unfortunately this oxidation gives no new information as to the exact origin of the acetic acid. In order to obtain this information it is necessary to oxidize a butyrate which is labelled at the α or γ carbons. With such a butyrate molecule it would be possible to decide whether the oxidation occurs, for example, as

$$CH_3CH_2C^{13}H_2COOH \longrightarrow CH_3COOH + C^{13}O_2 + CO_2\text{, or}$$

$$CH_3CH_2C^{13}H_2COOH \longrightarrow CH_3COC^{13}H_3 + CO_2$$

$$CH_3COC^{13}H_3 \longrightarrow CH_3COOH + C^{13}O_2 \text{ and}$$

$$CH_3COC^{13}H_3 \longrightarrow C^{13}H_3COOH + CO_2$$

If the acetic acid was formed by the first type of reaction, in which acetic acid arises exclusively from the β and γ carbons, it would be possible to determine the C^{13} concentration in each position of the molecule. The carboxyl, β and average values of the α and γ positions, could be determined by iodoform degradation of the acetone. The C^{13} in the acetic acid (β and γ carbons) being determined, the C^{13} in the γ carbon could be calculated, by use of the known C^{13} value of the β carbon (carbonyl of acetone). The γ carbon being thus determined, the α carbon could be calculated from the known value for the average of the γ and α carbons. (CHI_3 of acetone). Such information would be of interest not only as a means for selective determination of the C^{13} but also for interpreting the mechanism of the oxidation. Possible application of this knowledge toward an understanding of the mechanism of biological oxidation of fatty acids is of interest.[13]

It should be mentioned that the determination of the volatile acids by the partition method was not entirely satisfactory, perhaps because of the small amount of acid available. There was occasionally an indication of the presence of a small quantity of propionic acid. However, in the absence of a more accurate analysis this small value for propionic acid was disregarded. The presence of propionic acid may in part account for the finding of C^{13} values for the volatile acids which are somewhat lower than the C^{13} of the butyric acid.

The acetic acid from the dichromate oxidation of the neutral distillate contained C^{13} *approximately equivalent to that of the* $CH_3C^{13}H_2CH_2C^{13}OOH$. Calculation of the C^{13} concentration in the acetic acid by adjustment for the propionic acid, on the basis that the propionate contains only one carbon with excess C^{13}, gives 1.12 for the acetic acid and from the neutral distillate of experiment No. 3 and 0.48 for the Expt. 4 (*cf.* footnote 11 for type of calculation). In general the same considerations apply to the acetic acid of the neutral distillate as given above for the acetic acid of the

(11) The excess C^{13} in the original butyric acid of Experiment 3 is calculated to have been 1.21. This is the figure obtained when correction is made for dilution of the C^{13} of 1.49 mM. of the butyric acid by the presence of 0.05 mM. of propionic acid. The propionic acid, which was formed during the dichromate oxidation of the butyl alcohol, is assumed to have contained excess C^{13} only in the α position. The calculation is as follows: $(2 \times 1.49)X + (1 \times 0.05)X = (4 \times 1.49 + 3 \times 0.05)\ 1.20$, X = per cent. C^{13} in the labelled carbon, *i. e.*, the mM. of excess C^{13} in the labelled carbon of the compounds = mM. of excess C^{13} in the compounds as calculated from the average C^{13} of the total carbon of the compounds. $X = 2.42$, therefore, from the whole molecule the average is 1.21. A similar calculation for Experiment 4 gives the 0.52% C^{13} for the butyric acid. These calculations assume the C^{13} is equally concentrated in all labelled carbon, which is approximately correct as proved by the experimental results.

(12) Davies, *Biochem. J.*, **36**, 582 (1942).

(13) Witzemann, *J. Biol. Chem.*, **107**, 475 (1934).

volatile acid fraction. The observed concentration of C^{13} is in agreement with formation of the acetic acid (presumably acetaldehyde) from the α, β and γ positions and degradation of butyrate labelled at the α or γ position will be necessary to determine the exact origin of the aldehyde. The mechanism of formation of the aldehyde is of interest. Aldehyde may or may not have the same origin as the acetic acid of the volatile acid fraction.

The C^{13} *in the carbon dioxide from the oxidation of* $CH_3C^{13}H_2CH_2C^{13}OOH$ *is predominantly formed from the carboxyl carbon.* Conversely there is very little C^{13} in the carbon dioxide which is from the β position. Comparison of the oxidation of the two types of butyrate (Tables I and II) illustrate this point. As judged from the per cent. recovery of the total carbon in the respective products of the oxidation, Expt. 1 was comparable to Expt. 4 and the oxidation of Expt. 2 was similar to that in Expt. 3. Comparison of the calculated proportion of labelled to non-labelled carbon shows that about 78%[11] of the carbon dioxide carbon was labelled carbon in Expt. 1 and 73% in Expt. 4; in no. 2 the value is 62% as compared to 61% in no. 3. Thus in similar oxidations approximately the same proportion of labelled carbon was converted to carbon dioxide whether the carboxyl alone was labelled or whether both the carboxyl and β positions were labelled. This relationship indicates the β position is not oxidized in significant amount to carbon dioxide, and is in accord with indications that a very small amount of the butyrate undergoes complete oxidation.

The compound (or compounds) in the residue of distillation contain carbon from the β *and carboxyl positions.* The results from the oxidation of $CH_3CH_2CH_2C^{13}OOH$ proved that the carboxyl carbon is present in the fraction in a ratio of 1 to 4.5 and 6 of the α, β and γ carbons. In oxidation no. 4, in which there was a significant formation of the non-volatile compound, the ratio was 1 of labelled carbon to 1.6 of non-labelled carbon. This increase in the proportion of labelled carbons from the $CH_3C^{13}H_2CH_2C^{13}OOH$ as compared to the $CH_3CH_2CH_2C^{13}OOH$ is an indication that a substantial part of the β carbon is contained in the non-volatile fraction. The identity of the compound is not known. Apparently Allen and Witzemann[3] did not observe the formation of such a compound in their investigations. However Cahen and Hurtley[14] under different conditions have shown that 50% of butyric acid is converted to succinic acid with hydrogen peroxide.

Discussion

It should be emphasized that the results reported in this investigation apply only to the special conditions used for the oxidation. The conditions were chosen for optimum formation of acetone and were based on the experiments of Allen and Witzemann.[3] They obtained a substantial yield of acetone from butyrate when ammonium ion was used as a catalyst. It frequently has been observed that oxidations varying from almost complete conversion to carbon dioxide to oxidations such as studied here may be obtained with hydrogen peroxide. The *p*H, the catalyst, the concentration of hydrogen peroxide and temperature largely determine the type of oxidation.

In this investigation the ammonium ion was added in amounts equivalent to the butyric acid and the concentration of hydrogen peroxide was 0.75%. Even with standardized conditions there was considerable variation in the yield of the respective products, particularly in the non-volatile compounds of the residue of distillation.

The question of the mechanism of the oxidation of butyrate has been studied in some detail by Witzemann.[13] Possible intermediate compounds, α-crotonic, α-hydroxybutyric and β-hydroxybutyric acids have been oxidized and the products compared with those from butyric acid. Evidence is presented that there is both an α and β oxidation of butyrate. Acetic acid is presumed to arise by an α-oxidation, acetone by a β-oxidation.

If acetic acid is formed by α-oxidation, it is probable that it would be formed from the β and γ carbons of the butyrate, since it is unlikely that the oxidized α carbon would be reduced and form the methyl of the acetic acid or that the γ carbon would be split off as carbon dioxide. For a definite determination of the origin of the acetic acid it will be necessary to degrade the properly labelled acid.

That there is some α oxidation is indicated by the observed formation of a compound, tentatively identified as propionaldehyde. The compound is separated in the neutral distillate fraction and is oxidized by dichromate to propionic acid. Additional information is needed for final identification but it is reasonably certain that a 3-carbon compound is formed and that the α carbon will be oxidized in the formation of such a compound.

With regard to mechanism indicated in this paper, it should be clearly understood that the reactions are presented only to illustrate the types of cleavage that might occur in the carbon chain and not the actual intermediate compounds involved. The purpose of the investigation has been to determine the origin of the fragments from the carbon chain without concern as to the intermediates. When this information is fully in hand the possible intermediates may be considered with greater accuracy.

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Summary

The oxidation of $CH_3CH_2CH_2C^{13}OOH$ and $CH_3C^{13}H_2CH_2C^{13}OOH$ by hydrogen peroxide has been

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[14] Cahen and Hurtley, *Biochem. J.*, **11**, 164 (1917).

investigated with the purpose of obtaining a method for selective isolation of fragments of the carbon chain of butyrate. The products of the oxidation, carbon dioxide, acetic acid, acetone, acetaldehyde, propionaldehyde, and an unidentified non-volatile compound have been determined quantitatively and their C^{13} contents measured. Identification of the acetaldehyde and propionaldehyde is tentative.

From $CH_3CH_2CH_2C^{13}OOH$ the only products that contained excess C^{13} were carbon dioxide and the non-volatile fraction. The other products, therefore, are formed from the α, β and γ carbons of the butyric acid. From $CH_3C^{13}H_2CH_2C^{13}OOH$ each product was found to contain excess C^{13}. The excess C^{13} in the acetone was determined by iodoform degradation to be exclusively in the carbonyl group. Using this type of degradation the C^{13} in the carboxyl and β positions of the butyrate can be measured quantitatively. The average of the α and γ carbons is also obtained.

The exact origin of the acetic acid and acetaldehyde could not be definitely established. It will be necessary to oxidize butyrate which is labelled at either the α or γ position to obtain this information.

The $CH_3C^{13}H_2CH_2C^{13}OOH$ was isolated from butyl alcohol fermentations of corn mash to which $CH_3C^{13}OOH$ was added. The distribution of the C^{13} in the molecule supports the suggestion that butyl alcohol is formed by a condensation of acetic acid or its derivative.

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[Contribution No. 973 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology]

The Hydration of Unsaturated Compounds. XII. The Rate of Hydration of β,β-Dimethylacrolein and its Equilibrium with β-Hydroxyisovaleraldehyde[1]

By H. J. Lucas, W. T. Stewart and D. Pressman

The reaction involved in the hydration of β,β-dimethylacrolein is shown by Eq. 1

$$(CH_3)_2C{=}CHCHO + H_2O + H_3O^+ \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} (CH_3)_2C(OH)CH_2CHO + H_3O^+ \quad (1)$$

We have measured the rate constants, k_1 and k_{-1} of the hydration and dehydration reactions, respectively, and the equilibrium constant, K, at 20°, 25° and 35° in 0.5 and 1.0 N nitric acid solutions of various ionic strengths as regulated by potassium or sodium nitrate.

The hydration reaction was found to be first order with respect to the concentration of the hydronium ion since at constant ionic strength the initial slopes of the curves obtained by plotting $(1 - x)$ against t are proportional to the hydronium ion concentrations (Fig. 1). Here x is the fraction of the β,β-dimethylacrolein which has been hydrated at time t. The value of the slope is 0.014 at 1.04 N nitric acid and 0.007 at 0.52 N nitric acid and 0.52 N potassium nitrate.

The reversibility of the reaction was shown indirectly by permitting reaction mixtures to come to equilibrium at 15 or 25°. When the temperature was then raised to 35° the fraction of unsaturation became greater, approaching by dehydration the value of $(1 - \epsilon)$ determined by direct hydration at 35°.

The hydration and dehydration reactions are first order with respect to β,β-dimethylacrolein and β-hydroxyisovaleraldehyde, respectively. This is shown by the straight line character of the curves obtained by plotting $\log_{10} \epsilon/(\epsilon - x)$ against t (Fig. 2), which is the case for two first order reactions coming to equilibrium according to the integrated equation, Eq. 2

$$\log_{10} \epsilon/(\epsilon - x) = (k_1 + k_{-1})t/2.303 \quad (2)$$

The data of Table I, plotted in Figs. 1 and 2, are typical values.

Table I

Experimental Data of Hydration of β,β-Dimethylacrolein at 25° at Ionic Strength 1.04[a]

HNO₃, 1.04 N, $\epsilon = 0.28$		HNO₃, 0.521 N, $\epsilon = 0.28$	
Time, hr.	$(1 - x)$	Time, hr.	$(1 - x)$
0.58	0.991	1.74	0.989
1.08	.985	4.63	.968
1.48	.978	18.42	.897
1.95	.974	26.67	.867
2.40	.967	43.74	.814
2.85	.964	68.08	.766
3.35	.961	94.90	.732
4.00	.950	117.00	.718
4.68	.945	139.00	.710
5.17	.939	162.70	.701
5.83	.935	187.00	.698
13.63	.868	212.00	.695
47.68	.748	234.13	.689
101.87	.717	283.77	.689
147.57	.704	331.00	.687
217.57	.690		

[a] Regulated by potassium nitrate.

The determination of ϵ was complicated by the fact that there was a slow decrease in unsaturation even after equilibrium should have been reached (Fig. 1). On this account ϵ could not be deter-

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[1] Previous communication, XI, This Journal, 64, 1953 (1942).