The latter reaction is accelerated by soluble salts of barium, as well as by a number of nitrates (see Table III). The nitrate ion appears ineffective in the absence of barium, since 2-aminoquinoline is not formed by the action of sodium amide and sodium nitrate on quinoline (reaction mixture hydrolyzed with water).

**The Action of Salts on Quinoline in Liquid Ammonia.**—The observation of Chichibabin[2] to the effect that potassium anilide, $C_6H_5NHK$, reacts with pyridine under an inert hydrocarbon to give 2-anilinopyridine, suggests that salts containing very weak anions other than $NH_2^-$ might react with quinoline in liquid ammonia in the approximate sense of equations (2) and (3). With the exception of potassium ammonobarate, $Ba{=}NK{\cdot}2NH_3$, none of the salts examined reacted with quinoline in the expected manner. It is suggested that potassium ammonobarate is very slightly dissociated in liquid ammonia into potassium amide and barium amide, the latter of which reacts with quinoline in accordance with equation (1).

**Catalysis in the Formation of 2-Aminoquinoline.**—From the results of Table III, it is evident that barium thiocyanate[11] and barium nitrate exert the greatest catalytic effect, the reaction of equation (1) being as complete in one day at 20° as in a week or two with no catalyst. The slightly soluble barium bromide is without catalytic activity. The activity of the nitrates may in part be due to a decrease in the size of the crystals of the sparingly soluble reaction product, which therefore has less tendency to form an adherent coating over the barium amide.

The catalytic action of barium nitrate and barium thiocyanate is not due to the formation of an ammonia soluble ammonobasic salt, since the addition of a small quantity of potassium amide to a liquid ammonia solution of either of the above salts gives an immediate and permanent precipitate of barium amide.

(11) A solution of barium thiocyanate alone in liquid ammonia does not attack quinoline (Table II).

## Summary

1. The alkali amides in liquid ammonia, *n*-butylamine or triethylamine, convert quinoline to thick liquids or resinous substances. In the presence of metallic mercury, 5–11% of the theoretical amount of 2-aminoquinoline is formed.

2. 2-Aminoquinoline and hydrogen are formed in good yield by the action of barium amide on quinoline in liquid ammonia at room temperatures. Strontium amide attacks quinoline to a very slight extent, but calcium amide is unreactive.

3. The reaction between barium amide and quinoline is markedly accelerated by barium nitrate, barium thiocyanate, lithium nitrate and strontium nitrate. Sparingly soluble salts of barium have very little effect on the rate of the reaction. Thiocyanates of the alkali metals are not positive catalysts.

4. Of a number of salts containing weak anions, potassium ammonobarate is the only one that reacts with quinoline to form hydrogen and 2-aminoquinoline. It is suggested that potassium ammonobarate is slightly dissociated in liquid ammonia into the amides of potassium and barium.

STANFORD UNIV., CALIF. RECEIVED MARCH 28, 1934

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# The Methane Fermentation of Organic Acids and Carbohydrates[1,2]

BY D. TARVIN AND A. M. BUSWELL

Previous papers[3,4] from this Laboratory have reported investigations of the degradation of fatty acids, carbohydrates and other aliphatic compounds. Practically all of these were converted to carbon dioxide and methane with complete recovery of the carbon fed as gas. Carbon dioxide and hydrogen were shown to be converted into methane and water by bacteria and a general equation was formulated for the decomposition of compounds containing carbon, hydrogen and oxygen.

Production of fatty acids as intermediates in the fermentation had been noted by various workers[3,4,5] and an alpha oxidation was believed to be indicated in fatty acid decomposition, in contrast to Knoop's theory of beta oxidation in

(1) An abstract of a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate School of the University of Illinois, 1933.

(2) Presented before the Division of Biological Chemistry at the 86th meeting of the American Chemical Society, Chicago, Illinois, September 10–15, 1933.

(3) Neave and Buswell, THIS JOURNAL, **52**, 3308 (1930).

(4) Symons and Buswell, *ibid.*, **55**, 2028 (1933).

(5) Fischer, Lieske and Winzer, *Biochem. Z.*, **245**, 2 (1932).

the animal body.[6] It was thought that possibly the manner of anaerobic bacterial oxidation of fatty acids might be established by application of Knoop's method of feeding phenyl-substituted fatty acids and determining the resulting substances. Accordingly, an attempt to apply this method was made in the present study. Various types of aliphatic acids and carbohydrates were also fermented in an attempt to determine the nature of their degradation.

## Experimental

Continuous fermentations were carried on in apparatus similar to that developed by Neave and Buswell.[8] Inoculum consisted of liquor from a sludge digestion tank, filtered through asbestos and diluted with an equal volume of tap water. Asbestos was added to the liquor to serve as a resting-place for bacteria.[7] Where acids were fed, the sodium salt of the desired acid was fed at the beginning of fermentation to raise the buffer capacity of the liquor, then the free acid was fed. 1070 feedings were made of 36 different substrates. All gas evolved was measured and 624 gas analyses were made. Control determinations such as volatile acids, lactic acid, $P_H$, alkalinity and dissolved carbon dioxide were made on the liquor during the fermentations and at the ends of runs. The asbestos used was analyzed before and after fermentation for volatile matter and organic nitrogen. 738 analytical determinations were made on the fermentation liquor and asbestos, including 149 volatile acid determinations. From the data obtained, carbon balances were calculated and are summarized in Table I.

Attempts were made to isolate and identify intermediates suspected of playing important roles in the degradation of the various substrates fed. Where continuous fermentations were carried on in four-liter flasks liquor was withdrawn at intervals, examined and the accumulated residues saved for identification purposes. Because of the limited quantity of liquor which could be withdrawn at one time from a four-liter flask without affecting the fermentation, only small amounts of material were obtainable for examination as to intermediates. In order to obtain more workable quantities of material, a 20-liter bottle was used for production of fatty acids from dextrose. The concentration of acids in the bottle was built up by overfeeding of dextrose,[8] then ten liters of liquor was withdrawn and used for identification of intermediates. For identification of volatile acids produced during fermentation, sodium salts were concentrated and concentration followed by Duclaux distillation constant determinations, application of color tests and a specific test for formic acid. In the case of acids produced from dextrose, the boiling point, specific gravity, refractive index and neutral equivalent were determined and derivatives prepared according to the method of Drake and Bronitsky, using *p*-phenylphenacyl bromide.[9] Lactic acid was determined

TABLE I

SUMMARY OF FERMENTATIONS OF ACIDS

Nos. 3, 7, 8, 17 at 55°; No. 9 at 25–30°; all others at 32–34°

| Acid | Grams fed | Inoculum volatile matter, g. | Duration, days | Grams carbon in substrate fed | Grams carbon accounted for as $CO_2$ | $CH_4$ | Acids | Protoplasm | Total carbon accounted for, % | Ratio $CO_2$:$CH_4$ Actual | Theoretical |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Formic | 125.5 | 3.55 | 118 | 32.70 | 23.70 | 8.10 | | 0.45 | 98.6 | 2.92:1.00 | 3.00:1.00 |
| Acetic I | 186.0 | 18.00 | 118 | 74.40 | 36.90 | 39.30 | 2.90 | | 103.1 | 0.94:1.00 | 1.00:1.00 |
| Acetic II | 118.7 | 5.91 | 75 | 47.48 | 23.10 | 23.60 | 2.50[a] | .50 | 99.5 | .98:1.00 | 1.00:1.00 |
| Propionic | 72.0 | 3.19 | 128 | 35.00 | 14.40 | 21.00 | 0.30 | .60 | 100.0 | .69:1.00 | 0.71:1.00 |
| Butyric | 168.0 | 10.66 | 125 | 92.10 | 31.15 | 53.05 | 9.85 | 1.40 | 101.4 | .59:1.00 | .60:1.00 |
| Isobutyric | 84.8 | 2.58 | 122 | 46.65 | 16.41 | 28.25 | 0.26 | 1.15 | 98.7 | .58:1.00 | .60:1.00 |
| Valeric | 53.3 | 2.56 | 100 | 31.60 | 9.10 | 17.90 | 1.54 | 1.79 | 96.0 | .51:1.00 | .54:1.00 |
| Stearic | 12.4 | 3.40 | 180 | 9.45 | 2.47 | 7.08 | | 0.25 | 103.0 | .35:1.00 | .38:1.00 |
| Benzoic I | 45.5 | 5.50 | 160 | 31.20 | 13.58 | 16.20 | 0.34 | .66 | 98.6 | .84:1.00 | .87:1.00 |
| Benzoic II | 45.9 | 3.94 | 130 | 31.56 | 13.82 | 16.52 | | .98 | 99.3 | .84:1.00 | .87:1.00 |
| Phenylacetic | 31.3 | 2.61 | 144 | 22.50 | 8.16 | 11.38 | | .64 | 91.5 | .72:1.00 | .78:1.00 |
| Hydrocinnamic | 34.9 | 2.58 | 135 | 25.04 | 9.07 | 13.70 | | 1.40 | 96.4 | .66:1.00 | .71:1.00 |
| Cinnamic | 49.0 | 3.46 | 120 | 35.75 | 15.30 | 21.15 | | 0.42 | 103.0 | .72:1.00 | .86:1.00 |
| Lactic | 85.7 | 2.61 | 125 | 34.30 | 16.30 | 16.50 | 0.20 | 1.35 | 100.00 | .99:1.00 | 1.00:1.00 |
| Oxalic | 90.6 | 2.55 | 145 | 24.15 | 19.53 | 3.20 | | 0.32 | 95.5 | 6.10:1.00 | 7.00:1.00 |
| Succinic | 112.4 | 2.93 | 130 | 45.85 | 25.00 | 18.95 | | .90 | 100.0 | 9.20:7.00 | 9.00:7.00 |
| Malic | 126.9 | 3.72 | 125 | 45.40 | 27.10 | 15.65 | | .94 | 96.5 | 10.40:6.00 | 10.00:6.00 |
| Tartaric | 128.5 | 3.87 | 133 | 41.10 | 27.40 | 12.72 | | 1.00 | 97.6 | 11.75:5.00 | 11.00:5.00 |
| Alanine | 19.9 | 1.15 | 300 | 7.53 | 3.02 | 3.06 | .005 | 0.275 | 83.2 | 0.99:1.00 | 1.00:1.00 |
| Tyrosine | 15.0 | 1.15 | 300 | 8.95 | 2.45 | 3.01 | .38[b] | .42 | 70.0 | .81:1.00 | 0.89:1.00 |

[a] Inoculum carbon gasified. [b] Phenol.

(6) Dakin, "Oxidations and Reductions in the Animal Body," 2d ed., 1922.

(7) Breden and Buswell, *J. Bact.*, **25**, 69 (1933).

(8) Buswell, Boruff and Wiesman, *Ind. Eng. Chem.*, **24**, 1423 (1932).

(9) Drake and Bronitsky, THIS JOURNAL, **52**, 3715 (1930).

where it occurred.[10] Phenol was identified in tyrosine fermentation by means of its tribromo derivative and was quantitatively determined at intervals in tyrosine fermentation by Baylis' modification of the Gibbs method.[11] A summary of intermediates found in the various fermentations and manner of identification of each is given in Table II.

**Phenyl-substituted Fatty Acids.**—A surprisingly complete destruction of benzoic, phenylacetic, hydrocinnamic, and cinnamic acids was observed. The over-all results are in accordance with Symons' formula.[4] No anaerobic decomposition of these acids has been reported previously.

As stated above, it was hoped that the manner of oxidation of fatty acids in the methane fermentation could be

TABLE II

INTERMEDIATE STUDIES

| Substrate | Substances indicated | Manner of identification: Gas ratio | Duclaux constants | Color test | Specific test | Derivative |
|---|---|---|---|---|---|---|
| Propionic acid | Acetic acid | | | + | | |
| | Formic acid | | | + | + | |
| Isobutyric acid | Lower fatty acids | | + | | | |
| Benzoic acid | Formic acid | | + | | + | |
| Phenylacetic acid | Acetic acid | | + | | | |
| | Formic acid | | + | | | |
| Hydrocinnamic acid | Acetic acid | | + | | | |
| | Formic acid | | + | | | |
| Lactic acid | Propionic acid | + | + | + | | |
| Succinic acid | Propionic acid | | + | + | | |
| Malic acid | Propionic acid | | + | + | | |
| Tartaric acid | Propionic acid | | + | + | | |
| Pyruvic acid | Acetic acid | | + | + | | |
| | Formic acid | | + | + | + | |
| | Acetaldehyde | | | | + | |
| Alanine | Ammonia | Distillation and titration | | | + | |
| Tyrosine | Ammonia | Distillation and titration | | | + | |
| | Phenol | | | + | + | + |
| Dextrose | Butyric acid | + | + | + | | + |
| | Propionic acid | | + | + | | + |
| | Acetic acid | | + | + | | + |
| | Formic acid | | | | + | |
| | Lactic acid | | | | + | |
| Ethyl alcohol | Propionic acid | | + | | | |
| | Acetic acid | | + | | | |
| | Acetaldehyde | | | | + | |
| Milk waste | Propionic acid | | + | | | |
| | Acetic acid | | + | | | |
| | Formic acid | | | | + | |
| | Ester | | | | | |

The types of compounds investigated in the various fermentations are given below.

**Fatty Acids.**—The following were fermented: formic, acetic, propionic, butyric, isobutyric, valeric and stearic. The results obtained check those predicted by the formula given by Neave and Buswell[3] with good agreement between actual and theoretical data. This good agreement may in part be accounted for by the small weights of volatile matter present in the inoculum used in the various fermentations (Table I). Since the inoculum volatile matter represents only a small percentage of the total weight of material fed, any error attributable to decomposition of inoculum is negligible.

The intermediates produced in the decomposition of fatty acids were lower fatty acids.

(10) Friedmann, Cotonio and Shaffer, *J. Biol. Chem.*, **78**, 335 (1927).

(11) Baylis, *J. Am. Water Works Association*, **19**, 597 (1928).

established by application to the problem of Knoop's method of feeding phenyl-substituted fatty acids and determining the resulting substances.[6] However, complete destruction and gasification of the ring and attendant side chains of the acids fed rendered the proposed method useless. No aromatic derivatives of the acids fed could be isolated and the only intermediates produced in the case of benzoic, phenylacetic and hydrocinnamic acids appeared to be lower fatty acids.

**Effect of Substituent Groups on the Benzene Ring on Fermentability.**—It appears that benzoic acid, *o*-phthalic acid, salicylic acid and phenol can be decomposed. Phenol was found to be produced from tyrosine. Of 450 mg. of phenol present after feeding tyrosine, 150 mg. or 33⅓% was decomposed in sixteen days. No other intermediates were found.

Benzyl alcohol was slightly attacked but benzaldehyde, benzene, bromobenzene, toluene and aniline were not attacked.

**Hydroxy Acids.**—The bacterial reduction of lactic to propionic acid and subsequent disappearance of the latter was observed. The reduction of lactic acid is similar to that observed in malic and tartaric acids and dextrose. Malic and tartaric acids were reduced and decarboxylated with production of propionic acid.

A study of the maximum fermentation rate of acetic acid and a similar study for succinic, malic and tartaric acids revealed that decreasing rates of decomposition were in the following order: tartaric, malic, acetic, succinic.

**Keto Acid.**—The pyruvic acid was vacuum-distilled a few hours before feeding. Tests made after feeding pyruvic acid showed lactic and propionic acids to be absent from the fermentation liquor. After a few hours acetic and formic acids were found, indicating the true hydrolysis of an alpha-keto acid.

**Formic and Oxalic Acids.**—These acids were found to be decarboxylated, a portion of the resulting carbon dioxide and all of the hydrogen being converted quantitatively to methane and water.

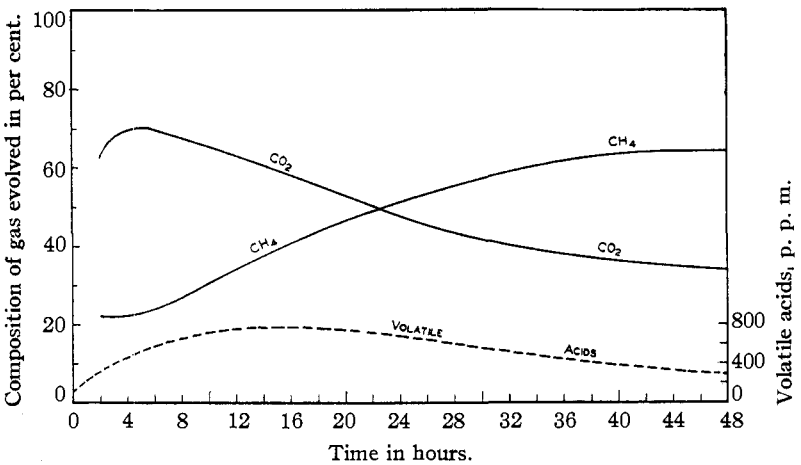


Fig. 1.—Fermentation of dextrose.

**Amino Acids.**—These were deaminated with increases in the ammonia nitrogen content of the liquor of 2120 mg. and 640 mg. for alanine and tyrosine, respectively. Heavy feedings of tyrosine produced 450 mg. of phenol.

**Carbohydrates.**—If the nature of a fermenting substrate changes, the change in composition of gas evolved during the fermentation should reflect the nature of that change. For example, the over-all ratio of carbon dioxide to methane obtained in the fermentation of formic acid is 3:1, for acetic it is 1:1 and for butyric it is 3:5. It is apparent that if dextrose is converted to a fatty acid, the nature of that acid should be revealed in the composition of gas evolved during its subsequent decomposition. Accordingly, attempts were made to determine the mechanism of carbohydrate fermentation by investigation of change of composition of the gas produced during fermentation. For this purpose, the 20-liter fermentation bottle was used and 50 g. of substrate fed at one time. Gas evolved was analyzed every two hours until fermentation subsided. Analyses of the liquor were also made at intervals; these included dissolved carbon dioxide, volatile acids, and $P_H$.

The results obtained with dextrose are shown in the graph. A nearly constant 3:1 ratio of carbon dioxide to methane was observed during the first six hours of fermentation. Following this, there was a steady downward drift of the carbon dioxide curve and a corresponding upward drift of the methane curve. At thirty-six hours the curves again approached the horizontal and an approximate 3:5 ratio of carbon dioxide to methane continued during the last twelve hours. These ratios would appear to indicate production and decomposition of butyric acid in accordance with

$$2C_6H_{12}O_6 = 2C_3H_7COOH + 3CO_2 + CH_4 + 2H_2O$$

$$2C_3H_7COOH + 2H_2O = 3CO_2 + 5CH_4$$

$$2C_6H_{12}O_6 = 6CO_2 + 6CH_4$$

Butyric acid had been previously identified in dextrose fermentation, together with propionic and acetic (Table II), but no evidence was obtained as to which was produced first.

The above postulated reactions do not agree completely with the facts observed. A comparison on the weight basis, from data obtained, shows that during the first six hours of fermentation 9.70 g. of butyric acid was produced, together with 13.90 g. of carbon dioxide and 1.41 g. of methane, whereas the theoretical would be 7.30 g. of carbon dioxide and 0.88 g. of methane. Therefore, it appears that some other intermediate such as a non-volatile substance must be produced, either in conjunction with or separate from butyric acid. The exact substance cannot be stated at present, but the observed 3:1 ratio of carbon dioxide to methane at the beginning of fermentation theoretically may be correlated with production of either succinic or levulinic acid, as well as butyric.

The other carbohydrates studied, *i. e.*, levulose, sucrose and starch, produced methane and carbon dioxide curves similar to the one for dextrose. High carbon dioxide and low methane production at the beginning of fermentation were reversed at the end. Volatile acids were produced as intermediates but calculations of these as butyric acid do not correlate directly with the weights of carbon dioxide and methane produced at the beginning of fermentation. Starch showed a twelve hour lag phase before beginning of fermentation which may possibly indicate hydrolysis during that time.

As stated above, the methane and carbon dioxide curves for the mono-, di-, and polysaccharides are quite similar. However, the curves for hydrogen are somewhat different and the average amounts of hydrogen produced from 50 g. each of the various carbohydrates may be significant. These are indicated in the accompanying table.

A survey of the above data indicates that the monosaccharides produce less hydrogen than the disaccharide and polysaccharide. In contrast to the carbohydrates, no hydrogen was produced from any of the lower fatty

| | Total fermentation time, hours | Total hydrogen av. vol., cc. | Maximum hydrogen in evolved gas for any 2 hour period, % |
|---|---|---|---|
| Dextrose | 48 | 932 | 11.0 |
| Levulose | 72 | 925 | 15.6 |
| Sucrose | 60 | 3893 | 30.7 |
| Starch | 120 | 2080 | 22.0 |

acids in the series formic to valeric, with the exception of a little from formic. Further evidence that the nature of the compound fermented rather than the culture at hand is the dominating factor in hydrogen production is shown in the following experiment: 50 g. of sucrose was fed to an active culture and allowed to ferment until gasification ceased. Acetic acid–sodium acetate mixture was then fed under the same conditions, allowed to ferment, and finally sucrose was fed a second time. The amounts of hydrogen produced from sucrose, acetate and sucrose were 4000, 120, and 3587 cc., respectively.

## Discussion

The decomposition of carbohydrates appears to take place in two stages: (1) a period of high carbon dioxide and hydrogen, low methane production, together with production of volatile acids and possibly some other non-volatile substances. This is followed by (2) decomposition of fatty acids with production of low carbon dioxide, high methane and no hydrogen.

The manner of decomposition of fatty acids has not been proved definitely because in the trial method outlined above of feeding phenyl-substituted fatty acids, there was complete deposition to gas of the acids fed. The reactions involved in an alpha oxidation of a fatty acid to the next lower one would be as follows

$$RCH_2COOH + H_2O = RCHOHCOOH + H_2$$
$$RCHOHCOOH + H_2O = RCOCOOH + H_2 + H_2O$$
$$RCOCOOH + H_2O = RCOOH + HCOOH$$
$$\overline{RCH_2COOH + 2H_2O = RCOOH + HCOOH + 2H_2}$$

The hydrogen produced in the above could either react with carbon dioxide to give methane and water or it could bring about the observed reduction of other substances.

Evidence obtained in favor of an alpha-oxidation of fatty acids, which are the principal intermediate in the methane fermentation, is as follows: (1) butyric, propionic, and acetic acids were found at the same time in dextrose fermentation. (2) Acetic and formic acids were indicated at one time during the fermentation of propionic acid. (3) Formic acid was detectable in a number of the fermentations studied.

The observed reduction of lactic to propionic acid is not at variance with an alpha-oxidation theory because in the oxidation by water of lactic acid to pyruvic, the hydrogen produced would be available for reduction of the remaining unchanged lactic acid to propionic. Since no hydrogen is produced in the hydrolysis of pyruvic acid, that acid is not reduced.

## Summary and Conclusions

An investigation has been made of the methane fermentation of thirty-six pure substances including fatty, alpha-hydroxy, keto, and amino acids, phenyl-substituted fatty acids, aromatic compounds and carbohydrates.

With the exception of a few resistant aromatics there was complete quantitative decomposition of all the above compounds, including the ring and attendant side chains of benzoic, phenylacetic, hydrocinnamic, and cinnamic acids, and tyrosine. There was also some decomposition of phthalic and salicylic acids and phenol. Benzaldehyde, benzene, bromobenzene, toluene, and aniline were not attacked.

The following general types of reactions were observed: (1) decarboxylation, (2) deamination, (3) hydrolysis, (4) reduction, and (5) hydrolytic oxidation–reduction.

The progressive changes in composition of gases evolved during fermentation have been used as a means for determining the course of the reaction. This method is advantageous since relatively small weights of material can be determined with considerable accuracy and it is not necessary to withdraw a sample of the fermentation mixture for analysis, or at most a very small sample will suffice. Application of the method to the fermentation of dextrose, levulose, sucrose, and starch indicates that aliphatic acids resembling butyric are first formed in the fermentation and later undergo decomposition to carbon dioxide and methane. The amounts of hydrogen produced from different carbohydrates indicate that there is some difference between the manner of fermentation of dextrose and levulose, as compared with sucrose and starch.

Lower fatty acids appear to be the principal type of intermediate formed in the methane fermentation and an alpha oxidation of these is indicated in their decomposition.

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