[CONTRIBUTION FROM ILLINOIS STATE WATER SURVEY DIVISION]

THE ANAEROBIC OXIDATION OF FATTY ACIDS

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Studies on anaerobic metabolism are of interest not only to explain anaerobiosis in general, but also as guides to the early steps in the series of organic transformations constituting aerobic respiration. Among the facultative anaerobes, there is no proof that the initial changes undergone by a food material on entering the cell are governed by free oxygen. In animal biochemistry, also, recent progress has emphasized the role of organic molecules as hydrogen donors and acceptors, and relegated free oxygen to the function of sweeping up the debris, thus permitting the biologically reversible processes to run to completion. Whether the organic food molecule has its energy content enhanced by the protoplasm[1] or is activated by enzymes,[2] the result is a degradation resulting aerobically in relatively simple end-products of low available energy, and anaerobically in more or less complex organic fragments of higher energy content than the aerobic products. The two considerations, the simpler fragmentation of organic molecules and the probable analogy between initial aerobic and anaerobic transformations, add interest to the study of anaerobic metabolism.

Literature on the biochemistry of anaerobic microörganisms deals primarily with protein and carbohydrate cultures; that is, with systems of great chemical complexity which involve enzymic selectivity of optical isomers. The anaerobic degradation of fatty acids is largely free from these complications, and was accordingly chosen for the present studies. Anaerobic bacteria, capable of decomposing fatty acids, are widely distributed in nature; they give almost stoichiometric yields of methane and carbon dioxide, in a medium composed otherwise only of inorganic salts.

Hoppe-Seyler[3] found that calcium acetate could be fermented by inoculation with river mud, the products being methane and carbon dioxide in a 1:1 ratio. Mazé[4] identified a sarcina in similar acetate cultures and found it to produce gas only when supplemented by either of two accompanying rod-forms, though the latter alone could not produce gas. Omelianski[5] recognized this sarcina in cultures fed with other lower fatty acids; for example, butyric salts gave a 5:3 ratio of methane to carbon dioxide. Söhngen[6] studied fatty acid decomposition by cultures containing a sarcina

[1] Mathews, in Cowdry's "General Cytology," **1924**.

[2] Quastel, *Biochem. J.*, **20**, 166 (1926).

[3] Hoppe-Seyler, *Z. physiol. Chem.*, **9**, 561 (1887).

[4] Mazé, *Compt. rend.*, **137**, 887 (1903); *Compt. rend. soc. biol.*, 398 (1915).

[5] Omelianski, *Centr. Bakt. Parasitenk.*, Abt. II, **15**, 673 (1905).

[6] Söhngen, "Proefschrift," Delft, **1906**; *Rec. trav. chim.*, **29**, 238 (1910).

and a rod-form, these cultures being built up from canal or sewer slime by repeated treatment and decantation with a medium composed of 2% of calcium acetate in tap water to which 0.05% each of sodium chloride and dipotassium hydrogen phosphate had been added. Cultures active for most lower fatty acids were soon obtained and easily maintained, the organisms being lime-encrusted and predominantly in the sediment. Söhngen reports the following methane to carbon dioxide ratios: formate, 1:3; acetate, 2:2; butyrate, 5:3; caproate, 8:4; caprylate, 11:5; caprate, 14:6. Propionic, valeric, heptylic and nonylic acids failed to ferment. Coolhaas[7] has similarly studied formate and acetate fermentation by a thermophilic rod, obtaining ratios of 1:2 for formate and 2:2 for acetate.

In all of these investigations, one mole of carbon dioxide is assumed to remain in the sediment as calcium carbonate, the equations being written, for example, $Ca(C_2H_3O_2)_2 = 2CH_4 + CO_2 + CaCO_3$; a quantitative balance was possible, therefore, only in terms of methane, and the important transformations in combined oxygen had to be assumed. We have extended these experiments to include a complete balance of the participating substances, and in addition have collected some data on fatty acids with an uneven number of carbon atoms.

Experimental

Active Söhngen cultures were obtained from an actively fermenting sewage-sludge digestion tank. In preliminary experiments, 5 to 10 g. of the acid under investigation, as the sodium or calcium salt, was used in 1-liter quantities of medium, the gas being collected over saturated brine and analyzed in an Illinois gas apparatus for carbon dioxide, hydrogen and methane. Since the inoculating organic matter equaled 50 to 75% of the fatty acid decomposed, controls on the inoculum alone were deducted from the final gas data. However, even controls, supplemented by analytical determinations of initial and final organic solids, do not completely guard against misleading gas ratios, and subsequent experiments have been, and are being, conducted in 8-liter reaction vessels, equipped for gas collection, to which several hundred grams of fatty acid can be fed. To avoid lethal osmotic effects and hydroxyl-ion concentrations, either the calcium salt, or the sodium salt plus some free acid, is fed in daily rations in the form of Söhngen medium, the displaced supernatant liquor being saved for analysis. Twenty-five to 50 g. of acid per week can be metabolized in such an apparatus, and the experiment continued until the organic matter in the initial inoculum is a negligible percentage of the total metabolism. Much of the carbon dioxide remains in the reaction vessel as carbonate or bicarbonate, the evolved gas showing about 30% of carbon dioxide, 65% of methane and small amounts of hydrogen and nitrogen. To the gaseous carbon

[7] Coolhaas. *Centr. Bakt. Parasitenk*, Abt. II, **75**, 161 (1928).

dioxide must be added the final dissolved and combined carbon dioxide in the medium after deducting that determined initially in the reaction mix-

TABLE I

ANAEROBIC FERMENTATION OF SODIUM PROPIONATE

		G. 5.71 of sodium propionate	Control on inoculum alone, g.
Total gas evolved	CH_4	2.177	0.533
	CO_2	1.333	0.553
	H_2	0.009	0.002
Carbon dioxide in medium	Initial	1.26	1.28
	Final	4.12	1.45
Increase added to gas		2.86	0.17
Propionic acid	In inoculum	0.46	0.47
	Added	4.40	0.00
	Sum	4.86	0.47
	Recovered at end	0.21	0.11
	Propionic metabolized	4.65	0.36
Organic solids in inoculum	Initial	5.18	5.28
	Final	4.89	3.77

Theoretical CH_4 from 4.65 g. of acid = 1.76 g.
CH_4 found (2.177 − 0.533) = 1.644 g.; yield = 93.4%
Theoretical CO_2 = 3.45 g.
CO_2 found (1.333 + 2.86) − (0.553 + 0.17) = 3.470 g.; yield = 101.0%
Volume ratio, CH_4:CO_2 = 7:5.3.

TABLE II

ANAEROBIC FERMENTATION OF CALCIUM ACETATE (RESULTS IN GRAMS)

Added	Acetic acid	CH_4	CO_2	Total organic matter	Calcium as Ca	Phosphate as P_2O_5	Nitrogen as N
Initial inoculum	Trace	...	3.07	37.8	3.52	1.284	4.04
As Ca acetate	159.69	...	...	159.7	42.49	...	..
As tap water	...	...	0.68	...	0.29	...	0.01
As K_2HPO_4	...	...	...	...	...	1.753	..
Total	159.69	...	3.75	197.5	46.30	3.037	4.05
Recovered							
In reaction vessel	38.27	...	37.51	80.5	41.36	2.819	2.55
In displaced liquor	7.24	...	12.64	14.9	5.81	0.216	1.92
In gas	...	28.08	32.81	107.4	...	...	..
Total	45.51	28.08	82.96	202.8	47.17	3.035	4.47

Summary:
Acetic metabolized 114.18 g.
Theoretical CH_4 30.489 g.
CH_4 produced 28.08 g.; yield, 92.9%
Theoretical CO_2 83.691 g.
CO_2 produced 79.22 g.; yield, 94.7%
Hydrogen produced 0.106 g.
Volume ratio, CH_4:CO_2 = 1:1.03

TABLE III

ANAEROBIC FERMENTATION OF SODIUM PROPIONATE (RESULTS IN GRAMS)

Added	Propionic acid	Total solids	Organic matter
Initial inoculum	0.00	53.06	45.07
As sodium propionate	115.20	151.04	....
As propionic acid	53.28	53.28	168.48
As tap water	....	1.51	....
As inorganic salts	....	6.96	....
Total	168.48	265.85	213.55
Recovered			
In reaction vessel	38.42	110.64	80.96
In displaced liquor		32.90	11.84
In gas	....	145.15	145.15
Total	38.42	288.69	237.95
Correction for H_2O reacting		21.90	15.82
Corrected solids		266.79	222.13

Summary			
	Propionic acid metabolized	130.06	
	CH_4 produced	48.587;	yield, 98.7%
	CO_2 produced	96.320;	yield, 99.6%
	H_2 produced	0.243	
	Volume ratio, CH_4:CO_2 =	7:5.05	

TABLE IV

SUMMARY OF CH_4:CO_2 RATIOS FOR FATTY ACID FERMENTATIONS

Fatty acid	Number of detns.	Average H_4 : CO_2 ratio found	Theoretical ratio (see below)
Acetic	2	1:0.97	1:1
Propionic	8	7:5.04	7:5
n-Butyric	3[a]	5:2.7	5:3
n-Valeric	1[a]	13:6.7	13:7
Oleic	1[a]	25:12.0	...
Lactic	1[a]	1:1.06	...

[a] Preliminary data; these data are being extended to more precise determinations particularly of the anabolic phase of the process.

ture and that added as tap water in the daily rations of Söhngen medium. Olefins, carbon monoxide or higher homologs of the methane series were not present in determinable amounts, because the methane analytically determined in the combustion pipet checked the carbon dioxide produced in the combustion. Table I illustrates the more essential points in the preliminary tests with 1-liter cultures and Table II those with 8-liter daily feeding experiments. In the latter case, calcium, phosphate and nitrogen balances are included to indicate the magnitude of sampling errors. Table III gives the results of a propionate feeding experiment. A summary of results in terms of CH_4:CO_2 ratios is shown in Table IV.

Oleic and lactic acids are included merely for comparison; these types are reserved for future consideration when the saturated normal and branched-chain acids have been fully studied.

Discussion of Results

The lower fatty acids are characterized by (1) a high yield of methane from the acid metabolized; (2) a much larger yield of carbon dioxide than can be contributed by simple decarboxylation (except acetic); and (3) traces of hydrogen in the gas evolved. Of the fatty acid metabolized, 90 to 95% appears as gas; the remainder presumably is consumed in anabolism through intermediary production of amino acids, because the simultaneous feeding of amino acid (glycocoll) temporarily stops gas production.

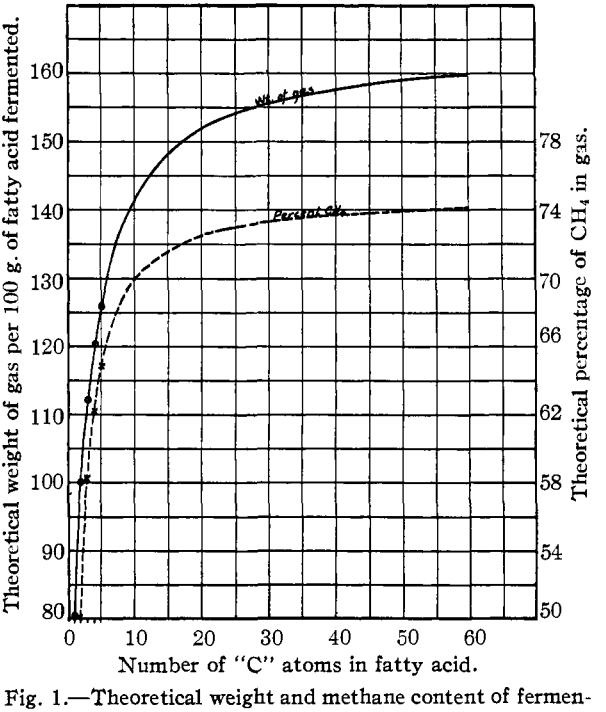

Fig. 1.—Theoretical weight and methane content of fermentation gases.

With the exception of acetic acid, the respiratory degradation yields carbon dioxide in excess of the carboxyl group; changes in the weight of organic inoculum are negligible, phosphates are not reduced, sulfates are absent from this tap water, and the total nitrate and dissolved oxygen introduced in the daily ration represent quantitatively only a fraction of a gram of carbon dioxide. The additional oxygen, therefore, must come from water, and the number of molecules of water needed to balance equations expressing the observed gas ratios is found to increase regularly with the length of the carbon chain, and to be expressed by the simple relation: moles of water required per mole of acid = $(n - 2)/2$, where n is the number of carbon atoms in the acid. Thus the general equation is

$$C_nH_{2n}O_2 + \frac{(n-2)}{2} H_2O = \frac{(n+2)}{4} CO_2 + \frac{(3n-2)}{4} CH_4$$

or for propionic acid it is

$$4C_3H_6O_2 + 2H_2O = 5CO_2 + 7CH_4$$

While ascending the homologous series, above acetic, therefore, the weight of gaseous products exceeds the weight of acid (as shown in Fig. 1) by an increasing percentage, attaining a purely hypothetical maximum of 164% of the acid decomposed for an infinite number of carbon atoms. The percentage of methane in the gaseous products also increases to a hypothetical limit of 75% by volume, although, due to the solubility and chemical retention of carbon dioxide in the medium, the evolved gases often contain 80 to 85% of methane.

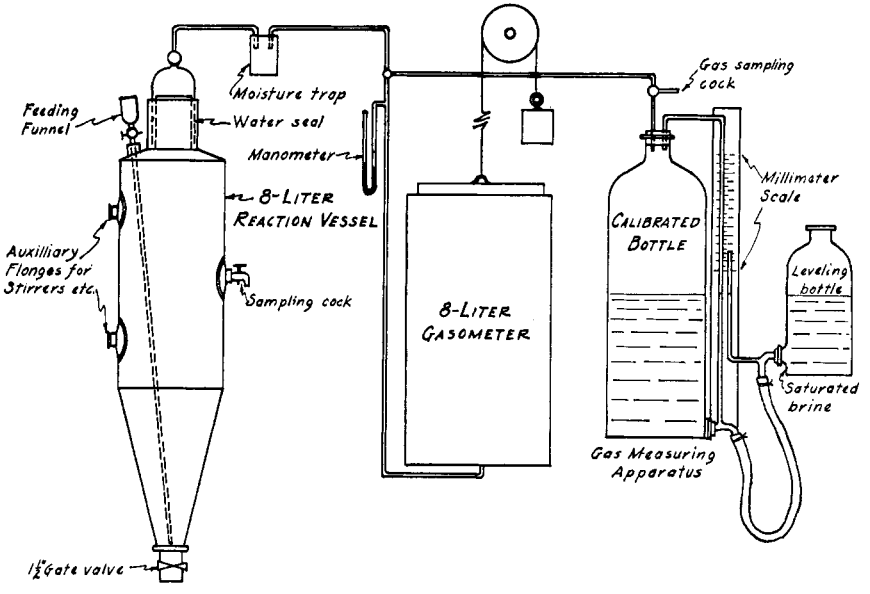

Fig. 2.—Apparatus for anaerobic metabolism studies.

The intermediate steps in this oxidation by water are yet to be revealed, but they are evidently not hydrolysis. The feeding of sodium propionate, for example, involves hydrolysis to liberate the free acid and due allowance must be made for this in analytically balancing the reactants, but the utilization of half a molecule of water by each molecule of propionic acid involves not simple hydroxylation but carboxylation of one of the carbon atoms; it is a process of intermolecular oxidation and reduction.

Most of our fermentation tests have shown the production of slightly more carbon dioxide than is predicted by the above respiratory mechanism. No extra-cellular side reactions have been detected to explain the excess of carbon dioxide and the evolution of hydrogen; accordingly, both of these

substances are regarded as clues to the anabolic processes, and the following three types of reactions are being investigated: (a) $CH_3COOH + 2H_2O = 2CO_2 + 4H_2$; (b) $RCH_2COOH + NH_3 = RCH(NH_2)COOH + H_2$; (c) the production of higher homologs, such as the formation of succinic from acetic, which Quastel[2] mentions, followed by decarboxylation to propionic acid. The experimental evidence favors the first two reactions, operating probably in the form of a cycle with hydroxy acid, then amino acid production representing the anabolic phase, as in the animal body;[8] the reverse process, including oxidation by water, would be the catabolic phase. The production of hydroxy and amino acids would yield hydrogen, and the catabolic oxidation would explain the excess carbon dioxide observed in these experiments. Such a cycle has the added interest of involving alpha, and not beta, oxidation of the carbon chain.

Summary

1. A study has been made of the anaerobic breakdown of lower fatty acids by microörganisms converting them into methane and carbon dioxide.

2. Water has been shown to act as an oxidizing agent in this degradation, and a simple relation to exist between the number of carbon atoms in the acid and the number of participating water molecules.

3. Concurrent side reactions are demonstrated and their probable mechanisms discussed.

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THE PREPARATION OF MERCURY DIALKYLS FROM ORGANOMAGNESIUM HALIDES

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Introduction

Some improvements were described recently[1] for the preparation of mercury dialkyls from mercuric chloride and the Grignard reagent. This is one of the best methods for the synthesis of this class of compounds. The improvements suggested then were: first, the use of a Soxhlet extractor to facilitate manipulation of the mercuric halide; second, the use of a larger volume of ether to reduce caking; and, third, a marked extension in the time of heating.

In those experiments a liberal excess of Grignard reagent was used. We have now shown that it is possible to synthesize some typical mercury dialkyls with practically an equivalent quantity of organomagnesium

[8] Knopp, *Science*, **71**, 23 (1930).

[1] Gilman and Brown, THIS JOURNAL, **51**, 928 (1929). This article contains references to earlier studies, particularly those of Marvel and co-workers.