[CONTRIBUTION FROM THE CARNEGIE INSTITUTE OF TECHNOLOGY]

Fermentation of Cellulose and Cellulose Humic Acid and Lignin and Lignin Humic Acid

BY E. BERL AND W. KOERBER

Those scientists who believe that lignin is the parent material of bituminous coal, as well as of lignites, base their belief on the assumption that cellulose, and other carbohydrates which form the bulk of plants, are decomposed by bacteria much more quickly and more completely than lignin. This assumption has found friends and opponents. Olson and Peterson[1] found that very small amounts of lignin (1%) inhibit the fermentation of pulp by cellulose fermenting bacteria. One of us has expressed the opinion that lignin and its derivatives are not the exclusive parent material for bituminous coals. It has been shown that under conditions which may have existed geochemically (high temperature and pressure) and with *p*H values above 7, bituminous coals could be obtained by artificial incoalification of carbohydrates. Those artificial carbohydrate coals show exactly the same properties as natural bituminous coals. This result never could be obtained with lignin and its derivatives, like lignin humic acid.

During the first stage of their conversion, with the *p*H above 7, cellulose and other carbohydrates form partly saccharinic acids which, upon further incoalification (with the formation of carbon dioxide, water, and lower hydrocarbons), give carbohydrate (cellulose) humic acids.

Until now all those alkali-soluble materials present in peat and lignites resulting from the decomposition of plants have been considered as lignin humic acids, and not as mixtures of cellulose humic acids and lignin humic acids. As yet no method of determining the amount of cellulose humic acids in these mixed acids has been found. We know that cellulose humic acids, in their chemical composition and in their behavior toward further incoalification, are fundamentally different from lignin humic acids. Furthermore, as may be shown in another publication, their heat stability is completely different from the heat stability of carbohydrates and of lignin, and lignin humic acids.

It was of great interest to find out how certain anaerobic and aerobic bacteria which decompose cellulose[2,3] would behave toward cellulose (carbohydrate) humic acid, lignin humic acid, and lignin. These experiments, which are described below, have been carried out and show a remarkable result. Anaerobic and aerobic bacteria which decompose cellulose do not attack cellulose humic acids, lignin, and lignin humic acids at all. They do not attack natural humic acids isolated from peat.

It has been shown that rather little cellulose, as such, may be present in peat and lignites and that under certain conditions the carbohydrate content of peat and lignites decreases with age. This does not lead to the conclusion that cellulose and lower carbohydrates are completely converted into gases and water-soluble compounds. They are very likely transformed into carbohydrate humic acids which resist the action of bacteria because of their phenolic structure. That cellulose humic acids may be present in those alkali-soluble materials obtained from peat can be seen by the fact that the peat humic acid used in our experiments contains only a few per cent. of methoxyl compared with 10.6% of methoxyl found in the lignin humic acid produced under very drastic conditions at 250° in a rotating closed vessel. A certain amount of methoxyl may be split off by the action of bacteria (see Waksman[4]).

Experimental Part

Cellulose, cellulose humic acid, lignin humic acid, and humic acid from peat were used for the fermentation experiments. The preparation of these materials was carried out in the following way.

(a) **Cellulose** was used in the form of wadding.

(b) **Cellulose Humic Acids.**—Linters were heated at 250° for ten hours with *N*/sodium hydroxide in a revolving bomb. The product was filtered and the dark brown filtrate obtained was acidified with hydrochloric acid, the precipitated humic acids filtered and dried. The finely pulverized material was washed with water until the chloride reaction disappeared and then dried over calcium chloride. The dark brown humic acids were dissolved in acetone, the solvents evaporated, and the residue dried *in*

(1) Olson and Peterson, *Ind. Eng. Chem.*, **29**, 9, 1026 (1937).

(2) W. Omeliansky, *Zentr. Bakt.*, II, **36**, 472 (1913); II, **12**, 33 (1904); II, **11**, 369 (1904); II, **8**, 193 (1902).

(3) Van Tieghem, *Bull. soc. botan. France*, **1**, 24, 128 (1877).

(4) S. A. Waksman and H. W. Smith, THIS JOURNAL, **56**, 1225 (1934).

TABLE I

AEROBIC FERMENTATION OF CELLULOSE, *p*H 7.4, FIVE DAYS, 55°

Flask	Cell. G./200 cc.	Fermented cellulose Cell. dec. G.	Fermented cellulose Cell. dec. %	Total acid G.	Total acid % of cell. dec.	Volatile acid Calcd. as acetic G.	Volatile acid Calcd. as acetic % of cell. dec.	Non-vol. acid G.	Non-vol. acid % of cell. dec.	Alcohol G.	Alcohol % of cell. dec.	Gas % of cell. dec.	Culture
30	2.92	2.00	67.4	1.11	55.8	0.97	48.3	0.14	7.5	0.050	2.5	9.1	A
31	2.80	2.24	80.1	1.46	65.3	1.26	56.2	.22	9.1	.067	3.0	11.8	A
32	3.10	2.19	70.6	1.30	59.1	1.11	50.6	.18	8.5	.044	2.0	9.5	C
33	3.00	2.34	78.2	1.45	61.8	1.32	56.5	.12	5.3	.051	2.2	14.2	C
34	2.98	1.81	60.7	0.82	45.2	0.73	40.2	.09	5.0	.029	1.6	13.9	F
Av.	2.96	2.12	71.4	1.23	57.4	1.08	50.3	.15	7.1	.048	2.2	11.8	

TABLE II

AEROBIC FERMENTATION OF CELLULOSE *p*H 6.0, FIVE DAYS, 55°

Flask	Cell. g./200 cc.	Fermented cellulose Cell. dec. G.	Fermented cellulose Cell. dec. %	Total acid G.	Total acid % of cell. dec.	Volatile acid Calcd. as acetic G.	Volatile acid Calcd. as acetic % of cell. dec.	Non-vol. acid G.	Non-vol. acid % of cell. dec.	Alcohol G.	Alcohol % of cell. dec.	Gas % of cell. dec.	Culture
35	2.83	0.62	22.0	0.12	19.8	0.12	19.8	None		None		2.2	A
36	3.10	.56	18.1	.08	15.2	.08	15.2	None		None		2.9	A
37	3.00	.71	23.8	.14	20.3	.14	20.3	None		None		3.5	C
38	2.95	.74	25.2	.17	23.4	.17	23.4	None		None		1.8	C
39	2.98	.50	16.9	.06	12.7	.06	12.7	None		None		4.2	F
Av.	2.97	.63	20.9	.11	18.1	.11	18.1	None		None		2.8	

vacuo over phosphorus pentoxide: C, 68.2; H, 6.1; O, 25.7; CH_3O, 0.

(c) **Lignin.**—Lignin was prepared from sawdust according to the hydrochloric acid method of Willstätter. The product obtained was refluxed with 5% sulfuric acid for five hours, filtered, and washed. A treatment with copper ammonium hydroxide at room temperature for twenty-four hours was added. The residue was thoroughly washed with hot acidified water and dried over phosphorus pentoxide: C, 62.4; H, 5.8; O, 27.8; CH_3O, 14.6.

(d) **Lignin Humic Acids.**—The lignin as described under **c** was treated with *N* sodium hydroxide at 250° for three hours in a revolving bomb. The further preparation was carried out as described under **b**: C, 63.8; H, 6.7; O, 29.5; CH_3O, 10.5.

(e) **Humic Acids from Peat.**—Peat from Wisconsin was finely pulverized and extracted with *N* sodium hydroxide on a shaking machine for twenty-four hours. The alkali-soluble part was separated by filtration, acidified, and the humic acids isolated as described under **b**: C, 63.4; H, 4.8; O, 31.8; CH_3O, 1.9.

Isolation of Aerobic Cellulose Fermenting Bacteria.—A cellulose fermenting bacillus was isolated from horse-dung according to the method described by Snieszko.[5] Control experiments were carried out with cellulose in salt media of *p*H 7.4 and 6.

Conical flasks of 300 cc. were each charged with 200 cc. of a salt medium containing 0.1% KNO_3, 0.1% $MgSO_4$, 0.1% K_2HPO_4, 0.5% peptone, exactly 2 g. of calcium carbonate and 3 g. of cellulose (latter dried over phosphorus pentoxide), *p*H 7.4. After sterilization the flasks were inoculated with the above-mentioned cellulose fermenting culture and incubated for five days at 55°. After this time the flasks were removed from the incubator and each of them titrated with *N* hydrochloric acid. From the titrated calcium carbonate the total acid formed was calculated. More acid was added and the contents of the flasks filtered and washed. The residue was unattacked cellulose; 25 cc. of 80% H_3PO_4 was added to the filtrate, which was then steam distilled. The distillate was titrated with *N* sodium hydroxide (volatile acids expressed as acetic acid). In order to determine the alcohol, the neutral distillate was ether-extracted three times, the ether evaporated, and the extract refluxed with acetyl chloride for two hours. After neutralization the solution was ether-extracted again, hydrolyzed with 10 ml. of 0.5 *N* sodium hydroxide, and back-titrated with 0.5 *N* hydrochloric acid (Tables I, II).

Aerobic Fermentation Experiments on Cellulose Humic Acids, Lignin, Lignin Humic Acids, and Humic Acids from Peat.—The above materials were exposed to the action of the cellulose-fermenting bacteria under the same conditions as were used in the control experiments on cellulose. None of these products could be fermented by those cultures which were active on cellulose. Even after four weeks no fermentation could be obtained with *p*H 7.4 and 6.

Isolation of Anaerobic Cellulose Fermenting Bacteria.—*Amylobacter navicula* was isolated from human feces under anaerobic conditions as described by Clausen.[6] Control experiments were performed with cellulose in a salt medium with *p*H 7.4 and 6.

Tubes were each charged with 150 ml. of a salt medium containing 0.1% $(NH_4)_2SO_4$, 0.1% $MgHPO_4$, 0.05% NaCl, and 1 g. of calcium carbonate; 1.5 g. of cellulose (dried over phosphorus pentoxide) accurately weighed, was added, *p*H 7.4. After sterilization the tubes were inoculated with an active cellulose fermenting culture of *amylobacter navicula* and incubated at 37° for five days under anaerobic conditions. After this time the tubes were removed from the incubator, their contents filtered, the residue washed and dried over calcium chloride *in vacuo*. The only fermentation products being gases, no other determinations were carried out than the weighing of the undecomposed cellulose.

(5) S. Snieszko, *Zentr. Bakt.*, 11, **88**, S., 403 (1933).

(6) P. Clausen, *ibid.*, 11, **84**, A., 20–60 (1931).

TABLE III

ANAEROBIC FERMENTATION OF CELLULOSE, *p*H 7.4, FIVE DAYS, 37°

Expt.	Cellulose in medium g./150 cc.	Fermented g./150 cc.	% cellulose decomposed
1	1.52	0.95	62.5
2	1.40	.96	68.5
3	1.55	1.01	65.3
4	1.45	1.01	70.0
5	1.43	0.92	64.5
Av.	1.47	.97	66.1

TABLE IV

ANAEROBIC FERMENTATION OF CELLULOSE, *p*H 6, FIVE DAYS, 37°

Expt.	Cellulose in medium g./150 cc.	Fermented g./150 cc.	% cellulose decomposed
6	1.50	0.21	14.1
7	1.48	.28	19.0
8	1.47	.24	16.3
9	1.49	.27	18.1
10	1.46	.22	15.2
Av.	1.48	.24	16.5

Anaerobic Fermentation Experiments on Cellulose Humic Acids, Lignin, Lignin Humic Acids, and Humic Acids from Peat.—The above materials were exposed to the action of the anaerobic cellulose-fermenting bacteria under the same conditions as were used in the control experiments on cellulose. None of these products could be fermented by those cultures which were active on cellulose. Even after four weeks no fermentation could be obtained with *p*H 7.4 and 6.

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Summary

1. (a) A cellulose fermenting bacillus isolated from horse-dung showed in agreement with Sinesko a decomposition of approximately 70% of the cellulose used. Fermentation products are volatile acids, non-volatile acids, and alcohol in medium of *p*H 7.4. (b) The fermentation in medium of *p*H 6 by the same bacteria showed a decomposition of about 20% of the original cellulose.

2. Fermentation experiments carried out with the same culture on cellulose humic acids, lignin, lignin humic acids, and humic acids from peat showed that none of those products can be fermented, neither in medium of *p*H 7.4 or *p*H 6, even after four weeks of incubation.

3. (a) The anaerobic cellulose fermenting *amylobacter navicula* isolated from human feces as described by Clausen showed a fermentation of 63% of cellulose in salt medium of *p*H 7.4 under anaerobic conditions. (b) The same culture in medium of *p*H 6 fermented cellulose in an amount of approximately 15%.

4. Fermentation experiments carried out with the same culture on cellulose humic acids, lignin, lignin humic acids, and humic acids from peat showed that none of those products can be fermented, neither in medium of *p*H 7.4, nor *p*H 6, even after four weeks of incubation.

These experiments prove that derivatives of carbohydrates, like cellulose humic acids, which are formed from carbohydrates of plants are resistant toward the action of bacteria used in these experiments. These cellulose humic acids probably form bituminous coals and crude oil without further activity of bacteria.

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The Crystal Structure of Diketopiperazine

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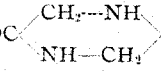
Introduction

For many years 2,5-diketopiperazine, "glycine anhydride,"  , has been of considerable interest to those concerned with the constitution of proteins. Substituted diketopiperazines have been shown to be present among the products of protein hydrolysis[1] and much experimental evidence has suggested that these compounds might play a major role in the elucidation of the structure of protein molecules.[2] Although this importance of diketopiperazine as a basic unit in protein chemistry is not now generally conceded, its relation to the amino acids and dipeptides renders a determination of its structure of fundamental value to further knowledge of these compounds. The present investigation was therefore

(1) E. Abderhalden and W. Stix, *Z. physiol. Chem.*, **132**, 238 (1924).

(2) B. Klarmann, *Chem. Rev.*, **4**, 51 (1927).