keto group. Another instance of this was encountered by Pfeiffer and co-workers,[29] who made particular use of the absence of double compound formation between sarcosine anhydride and molecules containing hydrogen bonds. Since one hydroxyl group in 2,2'-dihydroxybenzophenone was involved in forming a double compound with sarcosine anhydride the other was considered to be present in a hydrogen bond. Attention should also be called to the failure of many phenolic compounds containing hydrogen bonds to form brown colored addition compounds with triphenylmethyl chloride. Comparison of other type reactions indicates differences in degree rather than in kind. Although rates of reactions of the potential hydroxyl or keto groups in the simpler hydrogen bonded molecules, as salicyl aldehyde or methyl salicylate, are not greatly different than those in normal aromatic keto hydroxyl compounds, there is a marked depression in the rate of reaction when the freedom of rotation of the keto group has been restricted. Well-known examples in which the hydroxyl group is difficult to methylate and also usually difficult to esterify are the 1-hydroxyanthraquinones, 1-hydroxyflavones and compounds in the rotenone series, where the OH is ortho to the carbonyl group.[30]

(29) Pfeiffer, Angein, Wang, Seydel and Quehl, *J. prakt. Chem.*, **126**, 97 (1930).

Summary

Infra-red absorption characteristic of hydroxyl groups has been measured quantitatively for a number of selected alcohols and phenols. Since the absence of characteristic OH absorption is found to be closely correlated with the presence of the hydrogen bond as indicated by evidence from other sources, it has been inductively concluded that the absence of such OH absorption constitutes a good criterion for the presence of a hydrogen bond. In the present work this method is used for testing various factors believed to influence the formation of a hydrogen bond between oxygen atoms.

(30) This has usually been attributed to "steric hindrance."

WASHINGTON, D. C. RECEIVED JANUARY 2, 1936

[257TH CONTRIBUTION FROM THE INDUSTRIAL FARM PRODUCTS RESEARCH DIVISION, BUREAU OF CHEMISTRY AND SOILS, UNITED STATES DEPARTMENT OF AGRICULTURE]

The Chemistry of the Citric Acid Fermentation. I. The Carbon Balance

BY P. A. WELLS, A. J. MOYER AND O. E. MAY

Various theories have been proposed to explain the formation of citric acid from sugars by fungi. Challenger[1] has reviewed the mechanisms suggested up to 1929. An appreciable portion of the more recent work recorded in the literature dealing with the citric acid fermentation has been concerned with mechanisms involving acetic acid as one of the important intermediate substances. Naturally the acceptance of such a role for acetic acid has led to the assumption that the appearance of this acid is preceded by a breakdown of glucose similar to that encountered in the alcoholic fermentation of yeast. However, there has been a growing tendency in the past few years to assume that the initial phases of the citric acid fermentation follow the general chemical equation which represents the alcoholic fermentation without any direct experimental evidence upon which to base such an assumption. The purpose of this communication is to indicate that there is no direct evidence for this assumption and that experimental data show that the usual alcoholic breakdown of glucose plays no part in the formation of citric acid by molds.

Chrzaszcz and Tiukow[2] first reported the biochemical transformation of acetic acid to citric acid by mycelia of *Aspergillus niger* and on the basis of this evidence proposed the theory that the first stage in the formation of citric acid proceeded in a manner similar to that of the alcoholic fermentation.

There is considerable evidence which apparently

(1) F. Challenger, *Ind. Chem.*, **5**, 181 (1929).

(2) T. Chrzaszcz and D. Tiukow, *Biochem. Z.*, **229**, 343 (1930).

supports this mechanism.[3-10] However, the role of acetic acid as an intermediate in this fermentation has been under dispute.[11,12]

The theory can be tested directly by preparing a carbon balance for the organism when grown on solutions containing glucose as the sole source of carbon. Certain quantitative relationships should exist, namely, (1) the citric acid : CO_2 weight ratio should not exceed 1.45 : 1 and (2) the weight yield of citric acid from glucose should not exceed 71.1%. Such a study has never been carried out with a vigorous citric acid producing organism. Currie[13] and Buchner and Wüstenfeld[14] reported experiments in which carbon dioxide and citric acid were determined but no conclusions can be drawn from their results because exact carbon balances were not made.

The organism used in our experiments was a strain of *Aspergillus niger* which, as a rule, has given consistently high yields of citric acid when grown on glucose solutions containing the proper nutrient salts.[15]

The results obtained are shown in Table I, and the distribution of the carbon among the products is shown in Table II. The non-aerated or control cultures are similar with respect to mycelial weight and acid production, and the results were included merely to emphasize the fact that the fermentation in the aerated flasks was entirely normal.

The calculation of the weight yields of citric acid (Col. 11, Table I) requires some explanation. In order for these weight yields to have any real significance it is necessary to determine the quantity of glucose actually used for the production of citric acid and carbon dioxide. These values, Col. 10, Table I, were obtained by subtracting from the original glucose content the sum of the residual glucose, the glucose used for mycelium, and the carbon in solution unaccounted for by glucose and citric acid (calculated as glucose). No correction is made for the glucose used for the production of carbon dioxide since, according to the theory under discussion, at least part of it is involved in the formation of citric acid.

It is apparent from these results that that portion of the proposed mechanism governing citric acid formation by molds which involves the breakdown of glucose to alcohol does not agree with the facts. The theoretical ratio of citric acid : CO_2 (1.45) is greatly exceeded in spite of the fact that no correction can be made for the carbon dioxide due to metabolic processes not involving decarboxylation of pyruvic acid. In fact, this ratio is so high that it seems very probable that the decarboxylation processes involved in the ordinary alcoholic breakdown of glucose do not occur in this fermentation.

The weight yields of citric acid obtained, Col. 11, Table I, are much greater than the maximum yield calculated according to the theory outlined above.[16]

On the basis of these results it can be safely stated that the initial reactions leading to the formation of citric acid by molds do not occur in the manner proposed by Bernhauer.[3] Likewise, the mechanism recently proposed by Emde,[17] in which quinic acid was suggested as an intermediate, cannot be accepted, since both the theoretical yield of citric acid and carbon dioxide production required by that theory do not agree with experimental data.

These results do not exclude the possibility of mechanisms in which decarboxylation is not involved. Carbon and oxygen balance experiments similar to those presented later might possibly yield results which would more clearly explain the mechanism of this reaction. Such a study is now under way.

The nature of the carbon compound in solution not accounted for by glucose and citric acid has not been established completely. Neither oxalic acid nor gluconic acid has ever been detected in

(3) K. Bernhauer, "Ergebnisse der Enzymforschung," Vol. III, 185–227 (1934).

(4) K. Bernhauer and H. Siebenauger, *Biochem. Z.*, **240**, 232 (1931).

(5) K. Bernhauer and N. Böckl, *ibid.*, **253**, 16–24 (1932).

(6) P. Mazé and A. Perrier, *Ann. Inst. Pasteur*, **18**, 553 (1904).

(7) T. Chrzaszcz, D. Tiukow and M. Zakomorny, *Biochem. Z.*, **250**, 254 (1932).

(8) A. J. Suthers and T. K. Walker, *Biochem. J.*, **26**, 317 (1932).

(9) K. Bernhauer and H. Thelen, *Biochem. Z.*, **253**, 30 (1932).

(10) K. Bernhauer and N. Böckl, *ibid.*, **253**, 25 (1932).

(11) V. S. Butkewitsch, E. V. Menzschrinskaya and E. I. Trofinova, *ibid.*, **272**, 290–307, 364–370 (1934).

(12) K. Bernhauer and F. Slanina, *ibid.*, **274**, 104 (1934).

(13) J. N. Currie, *J. Biol. Chem.*, **31**, 15–37 (1917).

(14) E. Buchner and H. Wüstenfeld, *Biochem. Z.*, **17**, 395–443 (1909).

(15) A study of the optimum culture requirements was presented in a paper entitled, "Citric Acid Fermentation of Glucose by *Aspergillus Niger*," by A. J. Moyer, O. E. May and H. T. Herrick, before the Division of Biological Chemistry of the American Chemical Society, Spring, 1933. A report of this work will appear elsewhere.

(16) After this paper was prepared for publication Chrzaszcz and Peyros [*Biochem. Z.*, **280**, 327–336 (1935)] described some optimum conditions for the production of citric acid by *Aspergillus niger*. The yields of citric acid from sucrose reported by them (Table XI) exceed the maximum yield which could be obtained from this sugar, according to the theory discussed above, thus substantiating our results.

(17) H. Emde, *Biochem. Z.*, **275**, 373 (1935).

TABLE I

CITRIC ACID FERMENTATION BY *Aspergillus niger*

Glucose 19.45%. Volume 75 ml. Total carbon/flask (calculated from glucose value) 5.85 g. Total carbon/flask (found by analysis) 5.80 g.

Expt.	Duration of expt. days 1	CO_2, g. 2	Mycelium, g. 3	Residual glucose, g. 4	Glucose used, g. 5	Citric acid: Ml. N/10 KOH 6	Citric acid: Acid from KOH, g. 7	Citric acid: Acid from Ca salt, g. 8	Citric acid/ carbon dioxide 9	Glucose used for citric acid and carbon dioxide, g. 10	Wt. yield of citric acid[a] % 11
1	4	0.67	0.375	11.94	2.64	260	1.66	1.61	2.40	2.11	76.3
2	4	.66	.356	11.94	2.64	260	1.66	1.60	2.42	2.08	76.9
3	4	.60	.331	12.23	2.35	232	1.48	1.44	2.40	1.89	76.2
4	7	1.55	.676	5.80	8.78	913	5.84	5.82	3.75	6.66	87.4
5	7	1.39	.603	7.10	7.48	774	4.96	4.96	3.57	5.83	85.0
6	7	1.44	.607	6.94	7.64	791	5.06	5.06	3.51	5.91	85.6
7	10	2.19	.825	2.46	12.12	1335	8.54	8.50	3.88	9.74	87.3
8	10	2.26	.879	1.84	12.74	1435	9.18	9.05	4.00	10.31	87.8
9	10	2.39	.910	1.59	12.99	1465	9.38	9.26	3.87	10.46	88.6
10	11	2.50	.919	0.92	13.66	1568	10.03	9.86	3.94	11.18	88.2
11	11	2.58	.911	1.36	13.22	1485	9.50	9.52	3.69	10.92	87.2
12	11	2.46	.903	0.98	13.60	1530	9.79	9.74	3.96	11.03	88.3
Control flasks											
1	4	..	0.346	11.97	2.61	232	1.48	1.42	..	1.95	72.8
2	4	..	.351	11.66	2.92	247	1.58	1.51	..	2.03	74.4
3	4	..	.321	12.00	2.58	237	1.52	1.46	..	1.83	79.8
4	7	..	.652	5.91	8.67	892	5.71	5.76	..	6.65	86.6
5	7	..	.657	6.03	8.55	865	5.54	5.51	..	6.43	85.6
6	7	..	.657	5.68	8.90	925	5.92	5.97	..	6.80	87.8
7	10	..	.820	1.92	12.66	1445	9.24	9.14	..	10.08	90.7
8	10	..	.845	2.67	11.91	1295	8.25	8.14	..	9.33	87.3
9	10	..	.871	1.63	12.95	1375	8.80	8.64	..	10.10	85.5
10	11	..	.878	0.70	13.88	1585	10.15	9.96	..	11.10	89.7
11	11	..	.889	1.01	13.57	1475	9.44	9.36	..	10.80	86.7
12	11	..	.908	0.76	13.82	1465	9.38	9.39	..	10.98	85.5

[a] Based on acid from Ca salt.

TABLE II

DISTRIBUTION OF CARBON IN THE CITRIC ACID FERMENTATION

Expt.	Duration of expt. days 1	Carbon in: CO_2, g. 2	Carbon in: Mycelium, g. 3	Carbon in: Residual glucose, g. 4	Carbon in: Citric acid, g. 5	Carbon in: Soln., g. 6	Carbon accounted for Cols. 2, 3, 6, g. 7	Carbon accounted for Cols. 2, 3, 6, % 8	Carbon in soln. unaccounted for by glucose and citric acid, g. 9
Aerated									
1	4	0.18	0.17	4.78	0.60	5.42	5.77	98.5	0.04
2	4	.18	.17	4.78	.60	5.43	5.78	98.6	.05
3	4	.16	.15	4.89	.54	5.46	5.77	98.5	.03
4	7	.42	.32	2.32	2.18	5.03	5.77	98.5	.53
5	7	.38	.28	2.84	1.86	5.08	5.74	97.9	.38
6	7	.39	.29	2.78	1.90	5.08	5.76	98.3	.40
7	10	.60	.38	0.98	3.19	4.74	5.72	97.6	.57
8	10	.62	.41	.74	3.40	4.70	5.73	97.8	.56
9	10	.65	.42	.64	3.47	4.70	5.77	98.5	.59
10	11	.68	.43	.37	3.70	4.63	5.74	97.9	.56
11	11	.70	.42	.54	3.57	4.61	5.73	97.8	.50
12	11	.67	.42	.39	3.65	4.64	5.73	97.8	.60

any of the cultures. Volatile neutral and acidic products are entirely absent, as shown by distillation tests of the culture solution. The material precipitated from the culture solution by the addition of alcohol has proved to be a non-reducing dextrorotatory carbohydrate which yields glucose on hydrolysis. The quantity of this carbohydrate amounts to approximately one-sixth of the "carbon unaccounted for," as shown by the copper reducing values of the culture solution before and after hydrolysis. The remainder of this "carbon unaccounted for" very probably

consists of substances synthesized by the organism, such as proteins, polyhydric alcohols, etc. Raistrick and his co-workers[18] reported similar results with certain species of Penicilium which produce citric acid from glucose. In view of the recent work of Bennet-Clark and La Touche,[19] who have shown that citric acid is reduced to carbohydrate substances by mycelia of *Aspergillus niger* and not oxidized in the generally accepted manner, it is possible that this material may have some real significance with respect to the formation of citric acid from glucose.

Experimental

The organism, our *Aspergillus niger* strain 67, used in these experiments was secured from the collection of Dr. Charles Thom.

The experiments were carried out in 200-ml. Erlenmeyer flasks as culture vessels. Seventy-five ml. of culture solution for each flask was used containing 14.58 g. of glucose and the following nutrient salts.:

	Grams/liter of solution
$MgSO_4 \cdot 7H_2O$	0.25
KH_2PO_4	.30
NH_4NO_3	2.25

Two ml. of $N/4$ HNO_3 was added to each flask at the time of inoculation.

The culture solution was pipetted to ensure an accurate and constant quantity of glucose in the individual flasks. The solutions were sterilized for twenty minutes at 15 pounds steam pressure and after cooling were inoculated with spores of the organism. The cultures were incubated at 30°. At the end of the culture period they were harvested, the mycelium was dried and weighed, and the solution was diluted to a definite volume. Aliquot samples were then used for the various determinations.

Each experiment in Table I represents the results obtained with a single culture. The non-aerated cultures, grown under conditions otherwise similar to those for the aerated cultures, represent control experiments.

Carbon dioxide was determined by passing a slow stream (15 cc./min.) of sterile, carbon dioxide-free, humidified air over the cultures. The carbon dioxide produced was absorbed in 20% potassium hydroxide and subsequently determined according to the modified method of Winkler.[20]

The carbon in solution and in the mycelium was determined according to the method of Friedemann and Kendall,[21] as modified by Wells, May and Senseman.[22] Glucose was determined by the method of Shaffer and Hartmann[23] using the carbonate-citrate reagent. Citric acid was detected qualitatively with Denigés' reagent and quantitatively by titration with 0.1 N potassium hydroxide and as the calcium salt according to the method of Sotnikov.[24]

The addition of four volumes of 95% alcohol to the culture solution caused the formation of a white precipitate, which was purified by several precipitations with alcohol; 0.101 gram of material was obtained from 580 ml. of culture solution which contained 1.31 g. of carbon not accounted for by citric acid and glucose. The substance was neutral to litmus, gave a positive naphthol Molisch test, and gave no reduction of copper with Benedict's reagent. After hydrolysis with hydrochloric acid it reduced Benedict's reagent and yielded glucosazone when treated with phenylhydrazine; 0.0805 g. in 5 ml. of solution had a rotation corresponding to $[\alpha]^{20}D$ +93°.

Summary

1. A carbon balance has been prepared for a strain of *Aspergillus niger* which produces high yields of citric acid when cultured on glucose solutions.

2. The quantity of carbon dioxide produced in relation to the citric acid formed shows that citric acid formation does not occur by a process involving the breakdown of glucose in a manner analogous to that occurring in the alcoholic fermentation.

3. The weight yields of citric acid obtained greatly exceed the maximum yield obtainable from glucose by any process which involves the decarboxylation of pyruvic acid as one of the intermediate stages in the reaction.

WASHINGTON, D. C. RECEIVED NOVEMBER 23, 1935

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