The analyses (micro-Dumas) were carried out by Mr. W. S. Ide. The melting points are corrected.

Summary

A series of 1-aryl-5,5-diethyl barbituric acids, and a series of l-aryl-5,5-ethyl-*n*-butyl barbituric acids, have been prepared, the substituent N-aryl groups in both series being phenyl, *o*-, *m*- and *p*-tolyl, *o*-, *m*- and *p*-anisyl, *o*-, *m*- and *p*-phenetyl, and α- and β-naphthyl. The aryl radicals were selected in order that comparisons might be made, pharmacologically, with a series of alkylaryl ureas having these radicals.

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Biochemical Studies in the Genus Rhizopus. I. The Production of Dextro-Lactic Acid[1]

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Introduction

The production of lactic acid by microörganisms has, in general, been regarded heretofore as an attribute of bacteria, and not characteristic of the fungi. The prevailing conceptions regarding the relation of lactic acid to microbiological metabolism were summarized by Raistrick[2] in 1932: "It is a striking fact that lactic acid has never been reported as a mould metabolic product, although it is produced in larger or smaller quantities by many bacteria." Raistrick and other authors who have made similar statements have probably had in mind the biochemical activities of organisms of genera such as Aspergillus, Penicillium and Fusarium, which, indeed, have never been known to yield lactic acid. However, several investigators have reported lactic acid production in varying quantities by certain organisms of the genera Rhizopus and Mucor.

Since an accurate summary of the literature has never before appeared, and since some of the work is not conveniently reached or translated, it is briefly summarized here. In 1894, Eijkmann[3] suggested that the small quantity of acid produced by *Mucor rouxii* (*rouxianus*) was probably lactic acid, and Chrzaszcz[4] later confirmed this view. Shortly thereafter there were issued patents[5] which described the production of lactic acid by a mold termed "Lactomyces." The culture was of doubtful authenticity, but was probably a Rhizopus. In 1911, Saito[6] reported that *Rhizopus chinensis* produced *l*-lactic acid when cultivated on glucose solutions, and in 1919, Ehrlich[7] stated that in cultures of certain Rhizopus species which produced principally fumaric acid, there occurred small quantities of *d*-lactic acid, succinic acid, and *l*-malic acid.

Takahashi and co-workers[8] showed that up to 38% of the fermented glucose was converted to *l*-lactic acid by certain species of Rhizopus. Varying quantities of fumaric acid, succinic acid, *l*-malic acid, formic acid, acetic acid and ethyl alcohol were also found in these cultures. In 1933, Takahashi and Asai[9] found that four species of Mucor produced traces of lactic acid, in addition to acetaldehyde, ethyl alcohol, pyruvic acid and succinic acid. Ethyl alcohol was the principal product of these fermentations.

In 1930, Kenji Miyaji[10] reported that a new Monilia species isolated from commercial cultures of soy sauce produced *d*-lactic acid and succinic acid when cultured on artificial media containing glucose.

The highest yields of *d*-lactic acid from carbohydrates by fungi heretofore obtained were recently reported by Kanel,[11] who found that a fungus resembling *Rhizopus japonicus* converted 38 to 40% of the consumed invert sugar or starch to this acid. Fumaric acid accumulated in older

(1) Presented in part before the Section of Biological Chemistry of the American Chemical Society, April, 1935, New York City, N. Y.

(2) Raistrick, *Ergebnisse Enzymforsch.*, **1**, 362 (1932).

(3) Eijkmann, *Zentr. Bakt. Parasitenk.*, **16**, 97 (1894).

(4) Chrzaszcz, *ibid. II*, **7**, 326 (1901).

(5) Boullanger, British Patent 13,439 (1899); German Patent 118,063 (1901).

(6) Saito, *Zentr. Bakt. Parasitenk. II*, **29**, 289 (1911).

(7) Ehrlich, *Ber.*, **52**, 63 (1919).

(8) Takahashi and Sakaguchi, *J. Agr. Chem. Soc .(Japan)* ,**1**, 46 (1925); Takahashi, Sakaguchi and Asai, *Bull. Agr. Chem. Soc. (Japan)*, **2**, No. 5, 61 (1926).

(9) Takahashi and Asai, *Zentr. Bakt. Parasitenk. II*, **89**, 81 (1933).

(10) Kenji Miyaji, *Gifu Imp. Coll. of Agr. (Japan) Research Bull.*, 10 (1930).

(11) Kanel, *Microbiology (U. S. S. R.)*, **3**, 259 (1934).

TABLE I

SUMMARY OF METABOLIC ACTIVITIES OF ACID-PRODUCING RHIZOPI

Organism	*Rhizopus oryzae* 394			*R. oryzae* 395			*R. arrhizus* 519		
Nitrogen source	NH_4NO_3	$(NH_4)_2SO_4$	Urea	NH_4NO_3	$(NH_4)_2SO_4$	Urea	NH_4NO_3	$(NH_4)_2SO_4$	Urea
Age at harvest, days	16	16	16	16	16	16	21	21	21
Weight of mycelium, g.	0.35	0.64	0.87	0.32	0.59	0.80	0.09	0.08	0.14
Glucose consumed, g.[12]	8.3	10.2	10.8	8.3	9.7	10.6	8.8	9.9	9.9
Lactic acid found, g.[13]	4.90	5.51	4.58	5.11	5.25	4.98	3.34	4.08	3.19
Fumaric acid found, g.[14]	None	None	None	None	None	None	None	None	0.017
Calcium in solution, g.	1.25	1.46	1.29	1.29	1.40	1.31	0.86	0.96	0.71
Calcium as lactate, %	87	84	79	88	84	85	86	95	*practically* 100
Calcium as fumarate, %	None	None	None	None	None	None	None	None	Trace
Yield of lactic acid based on glucose consumed, %	59	54	42	62	54	47	38	41	32
Water of crystn. of desiccated zinc lactate, %	...	13.05	...	...	13.00	...	12.91	...	...
Sp. rot. of anhyd. zinc lactate, 4% soln.	...	−8.4°	...	...	−8.3°	...	−8.2°	...	...

Organism	*R. oryzae* 610			*R. tritici* 488			*R. oryzae* 585		
Nitrogen source	NH_4NO_3	$(NH_4)_2SO_4$	Urea	NH_4NO_3	$(NH_4)_2SO_4$	Urea	NH_4NO_3	$(NH_4)_2SO_4$	Urea
Age at harvest, days	21	21	21	20	20	20	21	21	21
Weight of mycelium, g.	0.73	0.91	1.20	Lost	Lost	Lost	0.46	0.78	1.12
Glucose consumed, g.[12]	7.3	10.4	10.6	7.0	9.0	9.0	6.2	9.0	10.5
Lactic acid found, g.[13]	2.97	4.32	4.22	1.74	3.90	2.96	[a]	2.05	2.00
Fumaric acid found, g.[14]	0.28	0.34	0.46	0.74	0.89	0.41	[a]	1.03	1.28
Calcium in solution, g.	0.98	1.25	1.26	0.77	1.17	0.87	[a]	1.08	1.12
Calcium as lactate, %	67	77	75	51	74	76	[a]	43	39
Calcium as fumarate, %	10	9	13	33	26	16	[a]	33	34
Yield of lactic acid based on glucose consumed, %	41	41	40	25	43	33	[a]	23	19
Water of crystn. of desiccated zinc lactate, %	...	13.28	...	...	12.81	...	Zinc lactate not isolated		
Sp. rot. of anhyd. zinc lactate, 4% soln.	...	−7.1°	...	...	−7.3°	...			

[a] Relatively poor development. Complete analysis not made.

cultures, in addition to small quantities of other unidentified acids.

Discussion

In the course of a survey of the biochemical activities of fungi of the genus Rhizopus conducted in this Division, it was found that certain organisms, when cultivated on a glucose-nutrient medium in the presence of calcium carbonate, induced a vigorous fermentation, with the formation of soluble calcium salts, which were found to consist principally of calcium *d*-lactate, with varying quantities of calcium fumarate. Two of the organisms (*Rhizopus oryzae* 394 and *R. oryzae* 395) were outstanding in their ability to convert glucose to *d*-lactic acid, the yields of up to 62% far exceeding those previously reported for fungi.

Ammonium nitrate, ammonium sulfate, and urea were satisfactory nitrogen sources, but none of the organisms developed well or utilized the glucose to an appreciable extent when sodium nitrate was supplied. When cultivated in the absence of calcium carbonate, the organisms generally utilized only a small quantity of the glucose, and developed in the medium only a slight acidity, usually equivalent to not more than 30 cc. of 0.1 *N* potassium hydroxide per flask (75 cc. of culture solution).

Certain strains (two strains of *Rhizopus sp.*, four strains of *R. nigricans*, one of *R. microsporus* and one of *R. chinensis*) produced very little acid under any of the conditions studied. Some strains, particularly one *R. nigricans* and the *R. chinensis*, formed appreciable quantities of ethyl alcohol when urea was used as the nitrogen source and calcium carbonate was absent.

A summary of the activities of organisms which were noteworthy in acid production is presented in Table I. Calcium carbonate was present in all these cases.

From the data presented, it is evident that several Rhizopi are able to convert the major portion of the glucose substrate to *d*-lactic acid, and that two organisms, *R. oryzae* 394 and *R. oryzae* 395, are outstanding in this respect. Fumaric acid is often formed in small quantities by the good lactic

(12) Shaffer and Hartmann, *J. Biol. Chem.*, **45**, 365 (1921).
(13) Friedmann and Graeser, *ibid.*, **100**, 291 (1933).
(14) Hahn and Haarmann, *Z. Biol.*, **87**, 107 (1927).

acid producers, and is formed in considerable amounts by certain other species, especially by *R. oryzae* 585. It should be mentioned that subsequent work has shown that fumaric acid production by *R. oryzae* 394 and *R. oryzae* 395 is a variable function, and small amounts may or may not be present, dependent upon such factors as temperature, age of the cultures, variations in the components of the medium, etc. Data involving this phenomenon and further physiological studies will be presented in a later communication.

All the products of the fermentation have not been identified. The species listed in Table I develop some acidity due to compounds other than lactic and fumaric acids. Many of the solutions give a positive Denigès test for malic acid, and occasionally there have been obtained traces of an acid melting at 177–180°, which suggests the presence of succinic acid. The acidity due to volatile acids is exceedingly small. It is conceivable that there might exist in these biological systems an equilibrium between succinic acid, fumaric acid, malic acid, and perhaps oxalacetic acid, such as has been the subject of recent intensive investigations of tissue metabolism. The authors are studying this question more fully from the standpoint of fungus metabolism.

The 62% yields of *d*-lactic acid given by *R. oryzae* 395 are not only reproducible, but the authors have occasionally attained a yield of 65 to 67% based on the glucose consumed.

Experimental Part

All the cultures were grown at 30° on approximately 15% solutions of commercial hydrated glucose (91.5% *d*-glucose, 8% moisture, 0.4% dextrins), with other nutrient constituents as $MgSO_4 \cdot 7H_2O$, 0.25 g. per liter; KH_2PO_4, 0.30 g. per liter; NH_4NO_3, $(NH_4)_2SO_4$, $NaNO_3$ or urea as nitrogen sources, to yield 0.5 g. of nitrogen per liter.

Each variation was studied in triplicate using 75 cc. of nutrient solution in 200-cc. Pyrex Erlenmeyer flasks. Each variation of nitrogen source was studied with and without the addition of calcium carbonate at the rate of 4 g. per flask. The calcium carbonate was sterilized separately, and added to the sterile nutrient solution at the time of inoculation.

At the conclusion of the incubation period, the culture liquors were analyzed for residual glucose, calcium ion, lactic acid and fumaric acid, as indicated in Table I.

In addition to the examination of its zinc salt, the lactic acid was also identified by the preparation of ethyl lactate (b. p. 154°) and the vacuum distillation of the acid itself (b. p. 119° at 12 mm.).

By virtue of its favorable partition coefficient, the fumaric acid could be separated qualitatively from lactic acid in the first ether extracts of the acidified culture solution, and could then be purified by recrystallization from water. The melting point (285° dec.), neutralization equivalent (58), and melting point of the *p*-nitrobenzyl ester (150°) proved its identity.

In conclusion, the authors wish to mention that the study of the 14 species of Rhizopus reported here represents only the beginning of an investigation of the biochemical activities of the entire genus. About eighty species of the authors' collection have not yet been investigated, it having been considered desirable to study in more detail the production of *d*-lactic acid by the vigorous organisms already encountered.

Summary

Several species of Rhizopus are able to convert glucose to *d*-lactic acid when cultivated in the presence of calcium carbonate. The yields of 62%, based on glucose consumed, are noteworthy in view of the 40% yields which are the highest previously reported. The lactic acid is accompanied by variable quantities of unidentified acids, and may or may not be associated with variable quantities of fumaric acid.

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