ANAEROBIC FORMATION OF FUMARIC ACID BY THE MOLD RHIZOPUS NIGRICANS[1]

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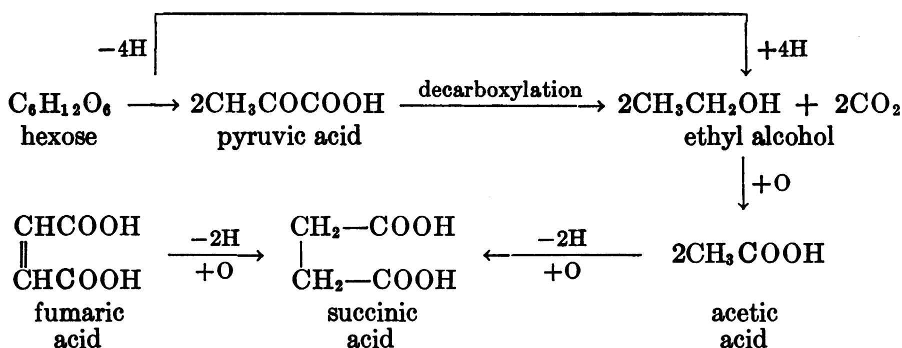
Fumaric acid formation in high yields from sugar by fungi belonging to the genus *Rhizopus* has been known for a long time and studied extensively (Gottschalk, 1926; Butkewitsch and Federoff, 1929*a,b*, 1930*a,b*; Foster and Waksman, 1939*a,b*). The following scheme first proposed by Gottschalk epitomizes present concepts of the mechanism of fumarate formation from hexose.



A typical alcoholic fermentation is the first stage. The alcohol (or the acetaldehyde) undergoes aerobic oxidation to acetate, this in turn being oxidized via the Thunberg-Wieland condensation yielding succinate, thence to fumarate. With an exceptionally high fumarate-yielding strain (no. 45) of *Rhizopus nigricans* we observed that the rate of conversion of alcohol into fumarate by preformed mycelium was too slow to account for its formation from glucose, indicating a possible alternative mechanism of fumarate synthesis. This has been demonstrated, and it differs from the mechanism above in that it is independent of oxygen.

EXPERIMENTAL DATA

Methodology was in general similar to that employed previously (Foster and Waksman, 1939*a,b*), except that fumarate was determined according to Stotz (1937), glucose by Shaffer and Somogyi's method (1933), and alcohol by dichromate oxidation. *R. nigricans* no. 45 proved distinct from other fumaric-acid-forming strains in two respects: (1) it produces the highest yields aerobically, amounting on a weight basis to 45 to 55 per cent of the sugar consumed (Foster and Waksman, 1939*b*); (2) fumarate formation from glucose by washed surface pads is not eliminated anaerobically, the yields under this condition ranging from

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one-third to one-half of those obtainable aerobically (table 1). Calcium carbonate was present to neutralize the accumulated acidity.

The fumaric acid was isolated by ether extraction and identified by melting point, unsaturation, and neutralization equivalent. If one calculates all the products in terms of C_2 equivalents, the total is in excess of the amount of C_2 theoretically obtainable from hexose by the alcoholic fermentation mechanism. As seen from column 4 in table 1, 16.66 mM C_2 are available but a total of 19.17 mM C_2 was recovered as alcohol, lactate, and fumarate. The C_2 equivalency of the alcohol and lactate is unmistakable; the fumarate apparently causes the discrepancy and one can only conclude that it does not arise via the assumed condensation of $2C_2$.

TABLE 1

Action of Rhizopus nigricans no. 45 on glucose solution in hydrogen atmosphere*

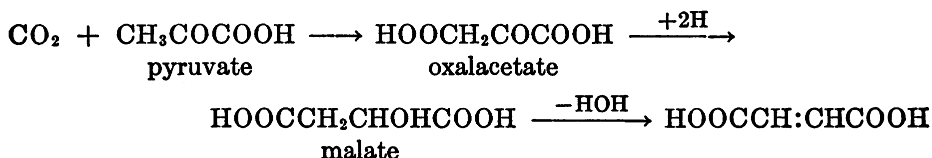
	GLUCOSE CULTURE			WATER CONTROL
	g	mM	mM C_2	
Glucose consumed	1.494	8.33	16.66	—
Fumaric acid	.320	2.76	5.52	None
Calcium in solution†	.130	—	—	None
Calcium accountable as fumaric acid‡	.110	—	—	—
Ethyl alcohol	.582	12.65	12.65	—
Lactic acid‡	.090	1.00	1.00	—
Volatile acids	Trace	—	total: 19.17	None
Weight conversion of glucose to fumaric acid, per cent	21.4	—	—	—

* Fifty ml of 3 per cent glucose.

† No free acidity was present as judged by titration of a 5-ml aliquot. Complete neutralization of the acids in an aliquot was achieved by warming the solution before analysis.

‡ Later work showed lactic acid to be the other acidity. In this case the remaining Ca in solution was considered as Ca-lactate, i.e., 0.090 g lactic acid (=1.00 mM).

An alternative mechanism and one that could proceed independently of oxygen is the $C_3 + C_1$ condensation (Wood-Werkman reaction) between pyruvate and carbon dioxide to yield oxalacetate, which, by means of well-known secondary reactions, is converted to fumarate.



$$CO_2 + \underset{\text{pyruvate}}{CH_3COCOOH} \longrightarrow \underset{\text{oxalacetate}}{HOOCH_2COCOOH} \xrightarrow{+2H}$$

$$\underset{\text{malate}}{HOOCCH_2CHOHCOOH} \xrightarrow{-HOH} HOOCCH{:}CHCOOH$$

None of the evidence available is inconsistent with this premise. A C_3 origin of fumarate is in agreement with the amount available from the sugar. The

balance now becomes:

C_3 available from sugar, 16.66 mM

C_3 equivalency of	
alcohol	12.65 mM
lactate	1.00
fumarate	2.76
Total found	16.41

It has not been possible to demonstrate an actual uptake of CO_2 because a great excess of CO_2 is formed concomitantly during the alcohol fermentation stage. Gas exchange experiments were done in Warburg respirometers. Homogenous cell material was obtained on a shaker in 5 per cent glucose salts medium containing precipitated $CaCO_3$ as a neutralizing agent. The mycelial suspensions obtained thereby were not ideal, for they consisted of clumps and small balls of varying sizes, but this technique did allow apportionment of equal amounts of mycelium. As will be seen later, these clump structures proved to be a source of difficulty. Three-day-old mycelium was collected on a filter, dispersed in water to allow excess $CaCO_3$ to settle out, and washed in dilute (1 per cent) HCl to dissolve adhering carbonate. Evidently $CaCO_3$ is bound very tenaciously by the mycelium, for sometimes repeated or prolonged washings failed to remove all of it. It was a mistake to assume that all the carbonate was eliminated even after it appeared that no more could be shaken out in water. Following a final water wash, the mycelium was sucked free of excess moisture on a Buchner. The thin pad of mycelium disintegrates easily, and aliquots are weighed on the analytical balance. Dry weight equals about 16 per cent.

Several anaerobic flask experiments were conducted with mycelium, prepared as above, acting on glucose in desiccators with a natural gas (CH_4) atmosphere. To minimize diffusion limitations, the desiccators were set on a shaking machine. Anaerobiosis was checked periodically by placing freshly inoculated slants of *Bacillus subtilis* in the desiccator; not even the slightest visible growth was ever observed on these slants indicating absence of oxygen. Decolorized methylene blue glucose solution also was used as an indicator of anaerobiosis. An alcohol solution was supplied to control mycelium in each desiccator as a check against fumarate formation via C_2 condensation. This was never observed.

Age of mycelium. For both shaker or surface culture, the age of the mycelium influences its capacity to form fumarate from glucose anaerobically. Thus, 1-, 2-, and 3-day-old shaker mycelium produced, respectively, 10.3, 13.1, and 17.4 per cent weight conversion of glucose to fumarate. The effect was much more marked with surface mycelium: 5-, 7-, and 9-day-old mycelium gave conversions of 0.6, 3.3, and 18.1 per cent, respectively.

Effect of pH. Mycelium harvested from a neutral medium is superior in anaerobic fumarate formation to mycelium from acid media. But, in the anaerobic replacement phase, both neutral- and acid-grown mycelium require acid conditions for maximum fumarate formation (table 2). Neutrality was maintained

by $CaCO_3$. Treatments with no $CaCO_3$ rapidly become acid. The yield in acid environment was 3 to 4 times that under neutral conditions (excess $CaCO_3$).

Excessively low pH values (below pH 2.5) are inhibitory to further accumulation of fumarate. The pH drop to 2.5 in the acid treatments is due solely to the organic acidity resulting from fermentation of the glucose. Neutral-grown mycelium always traps a small amount of carbonate, which prevents the pH from falling to an inhibitory level in the replacement phase when no added neutralizing agent is present. The optimum pH range is about 3.0 to 4.0. When sufficient carbonate is added to maintain the pH significantly above 4.0, there is a sharp reduction in fumarate yield. Thus $CaCO_3$ added at the rate of 0.1 to 0.4 mg per ml maintained the pH between 3.4 and 3.8 with a maximum conversion yield of fumarate of 14.5 per cent.

One mg $CaCO_3$ per ml maintained the pH at 4.75, resulting in only one-half the maximum fumarate yield. This pH effect is emphasized here because it

TABLE 2

*Acidity and anaerobic fumarate formation**

TYPE OF MYCELIUM	REPLACEMENT PHASE	FINAL pH	FUMARIC ACID FORMED	WT. CONVERSION OF GLUCOSE TO FUMARIC
			mg/ml	*per cent*
Acid	Acid	2.5	2.98	8.7
Acid	Neutral	6.4	1.11	3.2
Neutral	Acid	3.7	4.20	12.3
Neutral	Neutral	6.5	1.07	3.1
Neutral Control †	Acid		0	—
	Neutral		0	—

* Glucose consumed in each case was 34.2 mg per ml. Incubation, 2 days.

† Water only in these treatments.

underscores the difference in the optimum pH for the formation of fumarate by *R. nigricans* aerobically and anaerobically, additional evidence for two different mechanisms in this organism. Aerobically excess carbonate (i.e., nearly neutral pH) is optimum where highest yields are crucial (Kane *et al.*, 1944; Waksman, 1944).

The fact that an acid range was optimum for anaerobic fumarate formation facilitated greatly subsequent experimental work, for it was possible to dispense with aseptic precautions in handling the mycelium and solutions because of the rapid development of acidity, which effectively prevents bacterial contamination.

Mycelium cultivated in phosphate-buffered medium was definitely inferior to that from $CaCO_3$-buffered medium with respect to anaerobic fumarate formation.

Storage of mycelium. The storage of washed mycelium in water suspension in an icebox results in rapid loss of anaerobic fumarate-producing ability within a week. When the mycelium is held in its original growth culture fluid, however, its activity is maintained over a week or more.

Attempts to produce biotin-deficient mycelium. Direct proof of the origin of anaerobic fumarate via CO_2 fixation would be obtained if this reaction could be specifically inhibited. At present there are no poisons specific in this respect,

and the only tool available is to secure a deficiency in biotin, the coenzyme of the fixation reaction (Potter and Elvehjem, 1948; Shive and Rogers, 1947). This problem is not so simple in *R. nigricans* as it is in certain other systems because the fungus is prototrophic with respect to biotin, whereas the other systems require exogenous biotin. Three different approaches all were uniformly negative: (a) Storage of mycelium at pH 4.5 according to the method of Lichstein and Umbreit (1947), whereby *Escherichia coli* can be rendered biotin-deficient by enzymatic destruction of the cellular biotin. (b) Growth in the presence of analogues of desthiobiotin (Rogers and Shive, 1947). The following desthiobiotin analogues[2] known to be effective in certain bacteria were ineffective when tested at 1 mg per ml with very small inocula of spores or vegetative mycelium of *R. nigricans* no. 45: 4 methyl-5-(w-carboxyoctyl)-2-imidazolidone, 4 methyl-5-(w-carboxyamyl)-2-imidazolidone, and 5-(w-carboxyamyl)-2-imidazolidone. (c) Biotinless mutants. Notwithstanding the fact that *R. nigricans* sporangiospores are multinucleated (3 to 5 average) and that no segregation of nuclei is possible via sexual reproduction, an attempt was made to obtain mutants using a nitrogen mustard according to techniques especially designed to predispose the best chances for asexual segregation. Some 2,000 sporangiospores were tested[3] in complete and in minimal synthetic media without revealing a single deficiency mutant. About a dozen distinctive morphological mutants were obtained and several slow growers, which on the second or third transfer grew at the normal rate.

CO_2 deprivation. Another possible way of repressing specifically the carboxylation of pyruvate is to reduce the CO_2 concentration to a point where the fixation enzyme is unsaturated with respect to this particular reactant. Though the solubility of CO_2 is negligible in the pH range 3 to 4, which is optimum for anaerobic fumarate formation, it has not been possible to achieve a CO_2 deficiency because the mycelium vigorously generates CO_2 by means of alcoholic fermentation. This can only mean that the interior of the fungus cells contains significant amounts of CO_2 at all times, enough to keep the fixation reaction going at an undiminished rate. This was true even when the fermentation was conducted in a high vacuum, to remove intracellular CO_2 as quickly as possible. The vigor of the CO_2 formation was evidenced by a continuous stream of bubbles emanating from the hyphae kept in a vacuum. Though evacuation did remove some of the CO_2, there could be three reasons why it did not remove enough to suppress CO_2 fixation: (1) the cell sap probably is well buffered near neutrality, which means that considerable CO_2 would be retained in solution as bicarbonate; (2) loss of CO_2 from the cells is impeded by the cell membrane and wall; (3) the cells of this coenocytic organism are thick. It is questionable that CO_2 could be removed instantly and completely from the interior of hyphae of such large diameter. We assume reassimilation occurs before the CO_2 has a chance to diffuse out of these large fungus cells.

[2] We wish to thank Dr. R. Duchinsky and Dr. J. A. Aeschlimann of Hoffman-LaRoche, Inc., for generous gifts of these derivatives.

[3] We wish to thank Messrs. James Norman and Kenneth Roemer and Miss Ruby Rae Allen for valuable assistance in the mutation work.

Manometric studies of gas balance. For every mole of alcohol formed there should be formed 1 mole of CO_2. Any reutilization of the latter in the synthesis of C_4 dicarboxylic acids should result in a CO_2:alcohol ratio of less than 1. Several Warburg manometric experiments showed peculiarly wide deviations in the CO_2:alcohol ratio. Only in a few cases were the ratios less than unity and most of these only slightly less. Frequently the ratio exceeded unity—and to varying degrees. In order to get sufficient fumarate formed for its determination in the manometer vessel contents, it was essential to use 3 per cent glucose solution; mercury was used as the manometer fluid. About 70 mg mycelium, moist weight, were used per vessel, the experiments generally requiring about 12 hours. The manometric studies were completed using HCl-washed mycelium before the significance of the difficulties of removing residual-trapped $CaCO_3$ was fully appreciated. We interpret the varying high CO_2:alcohol ratios obtained as being

TABLE 3

Fermentation balance of Rhizopus nigricans no. 45

	mg/ml	mMols
Glucose consumed	23.14	0.129
Fumaric acid	3.23	0.028
Ethyl alcohol	8.55	0.186
Lactic acid*	4.0	0.044
CO_2	7.90	0.180
Carbon recovery	103.3 per cent	
O/R index	1.12	
C_3 available from glucose	0.258 mMols	
C_3 found in products	0.258 mMols	

* By the method of Barker and Summerson (1941).

due to dissolution of this carbonate by the acids formed, giving that much excess CO_2. The variation in results is in accord with the differing amounts of trapped carbonate that would be expected. In some experiments, however, this source of difficulty did not obscure the nature of the events taking place. For example, in one case in which the fumarate yield from glucose was 8.2 per cent there were formed 0.0845 mM CO_2 and 0.0913 mM ethanol. The ratio of 0.92 in this case indicates reutilization of CO_2. In one case "acid" mycelium was used, that is, a limited amount of carbonate was furnished in the growth medium, and the mycelium was used 1 day after visible carbonate was dissolved. Ostensibly this mycelium was carbonate-free, and a manometric experiment in duplicate clearly showed a substantial CO_2 deficiency. The ratio was 0.81; the fumarate yield was 4.9 per cent. As noted earlier, "acid" mycelium is inferior in anaerobic fumarate formation, as is mycelium from medium buffered with phosphate. Hence the use of mycelium from carbonate-buffered media.

Table 3 gives the balance of products in another manometric experiment. The CO_2:alcohol ratio here is 0.97, but the data show this to be artificially high, because of extraneous CO_2 from residual carbonate. The C_3 balance happens to be theoretical (0.258:0.258 = 100 per cent). However, both the carbon recovery and the O/R index of the fermentation products are in excess of theory;

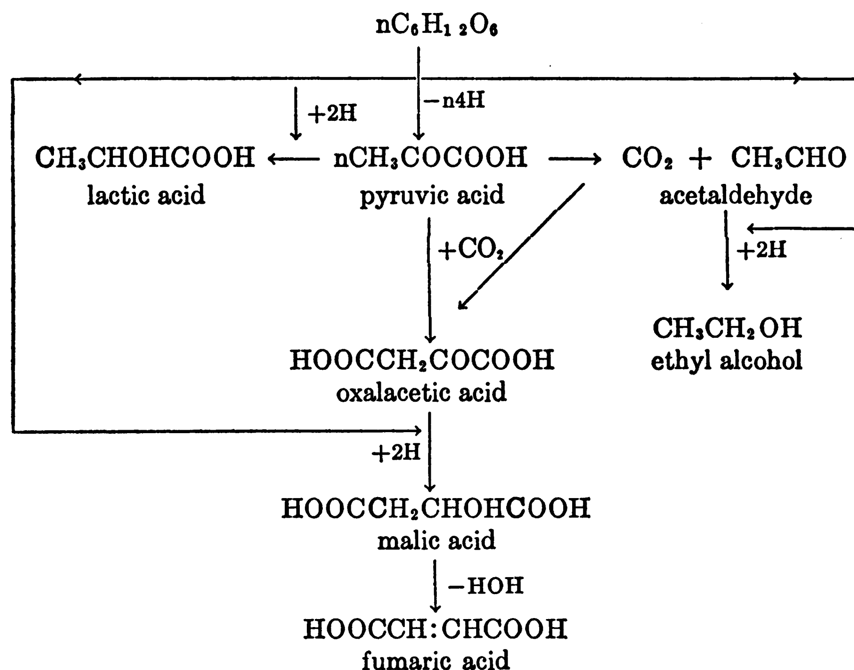
and in view of the theoretical C_3 balance, extra oxidized carbon must be present, i.e., CO_2 from trapped carbonate. Thus, the actual CO_2:alcohol ratio must be appreciably less than unity.

Oxalacetate decarboxylase. The presence of the enzyme responsible for CO_2 fixation has been demonstrated. The reverse action was studied, namely, the decarboxylation of oxalacetic acid (OAA). The decomposition was linear for the 1-hour duration of the experiment (table 4). Mycelium desiccated *in vacuo* over P_2O_5 was considerably more active in decarboxylation of OAA than was fresh mycelium of corresponding dry weight, presumably a permeability matter (Krampitz and Werkman, 1941). Cell material dehydrated with acetone-ether or with dioxane was inactive.

The enzymatic decarboxylation was calculated by deducting the endogenous and the boiled cell controls from the total obtained with unboiled material, and it probably represents the minimum value, because cell extractives in the boiled control contain substances that catalyze spontaneous decomposition of OAA.

In addition to all the foregoing evidence for origin of the anaerobic fumarate via CO_2 fixation in *R. nigricans* there is to be considered the direct evidence resulting from the use of radioactive carbon as a tracer (Foster *et al.*, 1941), when the carbon resided exclusively in the carboxyl groups of the fumarate. However, the tracer experiments merely indicated the qualitative nature of the reaction. There was no indication of a bulk fixation taking place, as the foregoing has demonstrated. Fumarate is formed anaerobically also by animal tissue and by *Escherichia coli* (Krebs and Eggleston, 1940), but not in bulk as above.

Based on the findings above the fermentation of glucose by *Rhizopus nigricans* no. 45 may be represented as follows:



In essence this scheme represents a reduction of pyruvic acid in three ways: before decarboxylation (lactate), after decarboxylation (alcohol), and after carboxylation (fumarate via malate).

DISCUSSION

The discovery that bulk formation of a C_4 dicarboxylic acid from sugar by fungi can take place independent of oxygen and that it arises via fixation of carbon dioxide has important implications for our understanding of the carbohydrate metabolism of fungi in general, and in particular for those typified by the formation and accumulation of organic acids. It offers clarification of a number of seemingly anomalous results in the literature dealing with fumaric acid forming fungi of the *Rhizopus nigricans* type and also citric acid formation by *Aspergillus niger* strains (Wang, 1941; Barinova, 1941; Butkewitsch and Gaevskaya, 1935; Gudlet 1935*a*,*b*; Zhuravskiĭ, 1939; Wells, Moyer, and May, 1936).

TABLE 4

Oxalacetate decarboxylase in Rhizopus nigricans mycelium

TREATMENT	CO_2 IN 1 HR
	µl
30 mg dry wt. mold, no OAA	126
30 mg dry wt. mold + 0.01 mM OAA	377
30 mg dry wt. mold boiled, + 0.01 mM OAA	58
CO_2 due to enzymatic decarboxylation	193

The anomalies arise from the fact that physiological balances between carbohydrate consumed and CO_2 and organic acids produced were not consistent with values expected according to the mechanisms conventionally used to interpret their formation. These mechanisms all predicate an initial alcoholic fermentation and subsequent aerobic condensation of the C_2 moieties to C_4 and, in the case of citrate, of $C_4 + C_2$. Because of acid yields exceeding those possible by these schemes, mechanisms such as a direct $C_4 + C_2$ split of the hexose chain and even a closing of the C_6 chain have been postulated.

These novel theories do not have to be resorted to if one considers that all the available data can be reconciled to the fact that in various fungi two mechanisms for the synthesis of C_4 decarboxylic acid can take place concomitantly, namely, $C_2 + C_2$ and $C_3 + C_1$. The C_1 is reutilized as a by-product of the C_2 formation. This is a logical explanation for the high yields of fumarate and citrate and the low CO_2 yields reported for certain strains. This idea was suggested on the basis of qualitative CO_2 fixation studies with C^{11} (Foster *et al.*, 1941) in which the fixed CO_2 was located in the carboxyl groups of fumarate and citrate. The bulk formation of C_4 reported now lends added weight to this idea.

Special note must be made of the fact that four other strains of *Mucorales* that produce fumaric acid aerobically do not produce fumarate anaerobically. This

affords one explanation of strain specificity with regard to organic acid production by fungi, at least for fumarate and citrate, and possibly for others. That is, strain specificity, in part at least, depends on the possession of a bulk CO_2 fixation mechanism that supplements the regular C_2 condensation mechanism for synthesis of C_4 and C_6 acids. Different strains may possess this mechanism to varying degrees. Doubtless all exceptionally high yielding strains will be found to possess a bulk CO_2 fixation mechanism. In the case of citric acid, it is well to keep in mind the possibility of bulk CO_2 fixation via oxalosuccinic acid.

There is reason to believe a similar situation holds for the formation of oxalic, itaconic, and succinic acids by fungi.

SUMMARY

A strain of *Rhizopus nigricans* (no. 45) forms fumaric acid anaerobically from glucose in yields approximating 20 per cent of the carbohydrate consumed. The other fermentation products are ethyl alcohol, lactic acid, and CO_2. Evidence is presented that indicates that the fumarate is formed by CO_2 fixation and involves the Wood-Werkman reaction (pyruvate + CO_2). Various factors influencing this fermentation are reported. The fungus mycelium is shown to contain the enzyme that is responsible for CO_2 fixation, oxalacetate decarboxylase. This organism possesses two mechanisms for fumarate formation: (1) the above-described $C_3 + C_1$ fixation, which can proceed anaerobically; (2) a $C_2 + C_2$ condensation that occurs only aerobically. Probably both reactions proceed concomitantly in the presence of air. Existence of the bulk $C_3 + C_1$ mechanism offers an explanation of the yields of organic acids (fumaric, citric, and oxalic) formed by fungi in excess of those compatible with an origin exclusively from C_2 intermediates.

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