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AEROBIC FORMATION OF FUMARIC ACID IN THE MOID *RHIZOPUS NIGRICANS*: SYNTHESIS BY DIRECT C_2 CONDENSATION*

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Recent studies[1] have demonstrated that fumaric acid formation from glucose by *Rhizopus nigricans* No. 45 involves at least two mechanisms, one of which is aerobic, the other anaerobic. The latter involves a bulk fixation of CO_2 via oxalacetate, in confirmation of the reaction qualitatively demonstrated in this mold eight years ago with radioactive carbon dioxide ($C^{11}O_2$).[2]

The aerobic mechanism is the subject of the present work. Methods of cultivation and handling of the mold, submerged mycelium, and analytical procedures, are those given in detail by Foster and Davis[1,3] and additional details will be given where necessary.

Experiments and Results. -- *Relation of C_2 compounds to fumarate formation from glucose*: Using washed submerged mycelium the essential surface culture results of Butkewitsch and Federoff[4,5] and Foster and Waksman[6] were confirmed, namely: aerobically ethanol accumulates in the early stages of the carbohydrate utilization, and gradually disappears, with a concomitant increase in fumarate, implying that alcohol is an intermediate between glucose and fumarate. Also confirmed was the formation of fumarate from alcohol as the sole carbon source, as well as from acetate, first noted by Takahashi and Asai in 1927.[7] A systematic study of fumarate formation from C_2 compounds (an aerobic process) was, therefore, undertaken.

Conversion of alcohol to fumarate: Extensive experimentation indicates the following conditions are essential for high efficiency of this conversion: (a) good aeration conditions, such as agitating thin layers on a

reciprocal-type shaking machine; (b) initial alcohol concentration of 2 to 3%; (c) the growth medium in which the mycelium for the alcohol experiment is obtained must be neutralized to prevent a sharp drop in pH due to fumarate formation from the sugar; if the pH falls below 5.0 the mycelium is inferior for this purpose; (d) phosphate cannot be used for the neutralization because it yields inferior mycelium; excess precipitated calcium carbonate has proved very satisfactory.

The washed mycelium, which is now furnished alcohol, must be allowed to become acid. In the neutral range alcohol consumption is less, and conversion to fumarate is lacking altogether or is small. Enough $CaCO_3$ can be carried over from the growth medium, trapped in the mycelial clumps, or adhering to the mucilaginous coatings on the hyphae, so as to maintain the pH high enough to suppress fumarate formation from alcohol. Washing the mycelium briefly two or three times in 0.2 N HCl dissolves this $CaCO_3$ without injuring the mycelium. Excessive contact with the HCl is injurious. After a water wash a portion of the mycelium placed in water and agitated violently should show no $CaCO_3$ shaken loose and settling out. The optimum acidity for conversion of alcohol to fumaric acid is about pH 3 to 4, yet maximum conversions are obtained only when the pH initially is 6 to 7 and is allowed to fall due to fumaric acid accumulation in the now unbuffered medium; under good conditions the value falls to about pH 2.5 before the conversion of alcohol is affected harmfully.

In properly conducted experiments aseptic techniques are unnecessary, due to the high acidity which developes. In these experiments, 1.2 g. wet weight (= 0.2 g. dry wt.) mycelium was used per 25 ml. of 2% ethanol in 250 ml. Erlenmeyer flasks. After 4 to 5 days shaking at 28° C. and correction for small evaporation losses, filtrates from these cultures contain from 4 to 6 mg. free fumaric acid per ml. The highest we have obtained is 7.9 mg. per ml. Doubtless higher amounts could be obtained by partial neutralization. Generally

ethanol consumption amounts to 4 to 10 mg. per ml. Analytical data show this is equivalent to about 50 to 80% weight conversion of ethanol to fumaric acid. This represents approximately 40 to 60% molar conversion efficiency. Actually, the three highest values we have observed have been, weight conversion = 84, 86 and 91% (= molar conversions of 67, 68, and 72%). Fumarate is never obtained from mycelium in the absence of substrate (alcohol).

Two things are remarkable about this process -- the high yields of fumaric acid and the purity of the product. Paper chromatography (solvent = butanol-propionic acid)[8] of a culture filtrate containing about 500 µg. fumaric acid showed no other detectable acid spots under our conditions. This means that other metabolic acidic impurities were present in amounts less than about 1% of the amount of fumaric acid. However, after substantial concentration, namely, ether extraction and crystallization of fumaric acid from a water solution of the residue, malic acid could be identified in the mother liquor from the crystallization. It showed an acid spot of RF = .43 on a paper chromatogram. The purified fumaric acid was identified as such by melting point and the $KMnO_4$ unsaturation test.

<u>Acetate conversion</u>: The conversion of acetate to fumarate has been studied under a great variety of conditions, the best weight conversion never exceeding 30% (= 29% on molar basis). Neutral conditions are essential here, as utilization of acetate is greatly reduced on the acid side and fumarate formation is not observed at all. In confirmation of Butkewitsch and Federoff's[9,10] studies with surface pads, some succinic acid is also formed with submerged mycelium. In one of our experiments 7.2 mg. acetate per ml. was consumed, yielding 1.92 mg. fumarate and 0.26 mg. succinate. The succinate was measured manometrically using a succinic dehydrogenase preparation from pig heart.

The discrepancy in yields from alcohol and acetate make it likely that

acetic acid itself is not the substance undergoing conversion to fumarate, at least in the pathway from ethanol. An active C_2 compound probably is generated more efficiently from ethanol than from acetate, and though we have established that phosphate is essential for the conversion,[11] assiduous tests for acetyl phosphate[12] have been consistently negative, both on filtrates and ground mycelium extracts.

Conversion of succinate: Fumarate formation from C_2 compounds and the fact that succinate accumulates in acetate cultures implies the intermediary formation of succinate followed by dehydrogenation to fumarate.

Demonstration of the dehydrogenation reaction is, therefore, essential for the premise that fumarate formation from C_2 compounds proceeds via succinate. Living *Rhizopus nigricans* mycelium is inert toward succinic acid, when tested manometrically for oxygen uptake. This is true at acid or neutral pH values. However, desiccated mycelium actively oxidizes succinate, indicating that permeability limitations account for the negative results with living mycelium. Judging from the amount of oxygen taken up, the oxidation of succinate does not proceed beyond fumarate in desiccated preparations, which by themselves display some endogenous O_2 consumption. In one experiment with 0.01 mM succinate in phosphate buffer at pH 7.4, the oxygen uptake leveled off sharply to the endogenous rate, at which point the following O_2 uptake values were obtained: endogenous flask = 96 µl.; succinate flask = 200 µl.; difference due to succinate = 104 µl. O_2; theoretical for oxidation of succinate to fumarate = 112 µl. O_2. Each vessel had 30 mg. dry mold material.

Experiments on CO_2 fixation: Because this organism can synthesize appreciable amounts of fumarate via CO_2 fixation with glucose as the substrate,[1] examination of CO_2 fixation mechanisms appeared desirable in the alcohol experiments. A double fixation, namely, $C_2 + CO_2 \longrightarrow C_3$; $C_3 + CO_2 \longrightarrow C_4$

would be involved, and of exceptional efficiency. The following experiments appear to preclude these mechanisms: (a) fumarate formation is undiminished in a CO_2-free atmosphere secured in a closed system by the presence of alkali and partial vacuum; (b) fumarate formation is not accelerated or enhanced by elevated CO_2 tensions; (c) experiments with radioactive carbon dioxide ($C^{14}O_2$) and unlabeled ethanol show that CO_2 could not possibly account for the major portion of the total fumarate formed. Acid-permanganate degradation (see later) of the fumaric acid formed in this experiment showed that the radioactivity was predominantly in the carboxyl groups as compared to methine carbon. In view of the fact that this mold has been demonstrated to possess oxalacetic acid decarboxylase, it is almost certain that the carboxyl radioactivity results from reversible decarboxylation of oxalacetate arising from fumarate via malate. Carbon dioxide is, therefore, a negligible factor in synthesis of fumarate from alcohol.

Theoretical consideration of the above results with respect to a tricarboxylic acid cycle mechanism: For two reasons the yield data seem to eliminate the tricarboxylic acid cycle in the formation of the C_4 dicarboxylic acids (fumaric).

First, theoretical molar yields of C_4 from C_2 via a C_6-cycle are 67%. In the mold system, yields equaling and possibly exceeding these have been obtained. That the Krebs cycle could operate in this case at its theoretical efficiency in producing C_4 is improbable, due to other degradation outlets for the intermediates. The fumarate yields from alcohol are in reality substantially higher than those reported above because they are based on total alcohol which had disappeared, after corrections from suitable evaporation controls. Actually some of the alcohol was assimilated by the mold mycelium. In one alcohol experiment the dry weight increase in mold mycelium was equivalent

to 15% of the alcohol which had been consumed. Since this occurred in the absence of a nitrogen source and minerals, it is considered to represent oxidative assimilation.

Second, for each molecule of C_4 formed, the tricarboxylic acid cycle would require a C_4 molecule to begin with. This would have to come from ethanol, i.e., a C_2, which is the only substrate available. Thus, a functioning Krebs cycle in this system would still require a C_4 synthesis from C_2 by some extremely efficient mechanism.

Since CO_2 fixation has been eliminated (see above) as a major mechanism, attention was, therefore, directed to a consideration of a $2C_2$-condensation origin of the fumarate. None of the above experimental data are inconsistent with this possibility, and indeed, the yield data are strongly suggestive of this mechanism. Labeled ethanol makes it possible to put this theory to the final test.

<u>Experiments Using C^{14}-Labeled Ethanol</u>. -- <u>Methods</u>: Methyl-labeled ethanol and carbinol-labeled ethanol were obtained from Tracerlab, Inc. To avoid volatilization hazards, as well as to obtain CO_2 measurements, these experiments were conducted in 10-liter desiccators with ordinary air atmosphere. Other details were exactly as in the previous experiments with nonlabeled ethanol.[1,3] The cocks were opened daily to compensate for oxygen consumption. Only one flask was placed in a desiccator. The whole apparatus, with the flask fastened in position was placed on a shaker. Both kinds of labeled ethanol were run with portions of the same batch of mycelium and at the same time for 4 days. The radioactive alcohols were diluted with ordinary ethanol to a final concentration of 2%. At the end of the experiments a 300-ml. aliquot of the gas phase was removed with an evacuated bulb, the CO_2 absorbed in alkali, precipitated and weighed as $BaCO_3$ on a porcelain filter disc. Radioactivity

measurements were made directly from the disc with a mica window Geiger-Müller counter. All radioactivity measurements on $BaCO_3$ were measured as close as possible to infinite thickness and corrected for self-absorption when necessary. All counts actually measured were at least 10 times the background, and most were 20 to 200 times. Radioactivity of other organic compounds was measured as $BaCO_3$ obtained from wet oxidation using persulfate-$AgNO_3$.[13] The liberated CO_2 was absorbed in NaOH and collected as $BaCO_3$ by addition of $BaCl_2$.

At time of analysis the culture with methyl-labeled ethanol had 5.4 mg. fumaric acid per ml., and that with carbinol-labeled ethanol 5.7 mg. per ml., determined by a mercurous fumarate method modified from Stotz.[14] Aliquots of the clear culture filtrates were adjusted to pH ~10 and about two-thirds the volume distilled off. The residue was acidified to pH ~2.0 with H_2SO_4 and extracted with ether overnight in a Kutscher-Steudel extractor. The ether in the extract was evaporated, and all remaining traces of volatile matter removed by maintaining the residue under high vacuum at 80° C. for 30 minutes. The light tan deposit of fumaric acid was dissolved in hot water, filtered and concentrated in an air stream on a hot plate to incipient crystallization. After standing in a refrigerator overnight, the crystals of radioactive fumaric acid were collected and washed on a sintered glass funnel. Yield from the carbinol-labeled alcohol was 52 mg. and from the methyl-labeled ethanol 32 mg. Both preparations melted at 274-275° C. in a sealed tube as compared to 279.5° C. for pure fumaric acid under our conditions.

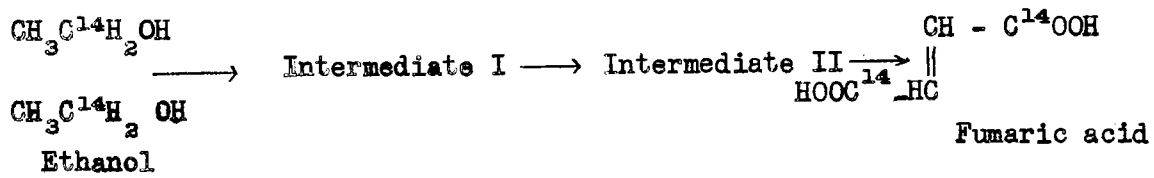
About 5 mg. of each kind of labeled fumaric acid was combusted for total radioactivity and another 10 to 12 mg. degraded with acid-permanganate for location of the labeled carbons. Allen and Ruben[15] have shown that this oxidation liberates 3 moles CO_2 and 1 mole of formic acid per mole of fumarate, and that the formic acid is derived exclusively from one of the methine-carbons of

the fumarate. The other methine-carbon and the two carboxyls make up the three CO_2 liberated. In confirmation, we have, with unlabeled fumarate, identified formic acid as a product of this oxidation under our conditions by ether extraction and its Duclaux distillation constants. Formate separation also has been done by steam distillation direct from the oxidation mixture after destruction of the residual $KMnO_4$ with H_2O_2. The oxidation mixture contained 11 mg. fumaric acid in 6 ml., 20 ml. 0.3 N $KMnO_4$ and 14 ml. 3 N H_2SO_4. The oxidation is complete in less than 10 minutes at room temperature, but the formate slowly is further oxidized. The oxidations were carried out in 160-ml. capacity Warburg vessels connected to manometers containing mercury, and when theoretical amounts of CO_2 had been liberated (= three-fourths of the fumarate carbon) the flask was placed in ice water to retard formate oxidation. Alkali was introduced through the vent in one side arm, and the CO_2 absorbed. The oxidation mixture was removed for formate separation, and the CO_2 precipitated as $BaCO_3$ and taken for radioactivity measurements.

In the case of radioactive fumarate, inactive carrier formic and acetic acids (0.3 mE of each) were added prior to ether extraction and Duclaux distillation. Two successive extractions with 10 volumes of ether netted about 90% recovery of the acids. The ratios of acetic/formic acid obtained in the Duclaux distillations from both kinds of labeled fumaric acid were so shifted by the ether extracts that formic acid definitely must have been contributed by the degradation of fumaric acid. The Duclaux constants are indispensable in ascertaining the radioactivity of the formic acid produced from fumarate. Radioactivity of the various Duclaux fractions should, of course, be proportional to the characteristic formic acid values in a Duclaux distillation, otherwise the radioactivity in the preparation cannot be due to formic acid. After the titration values were obtained, the various Duclaux fractions were oxidized to CO_2

for radioactivity determinations.

Results with fumaric acid from carbinol-labeled ethanol: The formic acid in this case was devoid of radioactivity even when measured as gaseous CO_2 with the vibrating reed electrometer which is ~10 times more sensitive than our solid counting procedure. There was a small amount of steam distillable radioactivity (~2% of the total) but it doubtless came from impurities in the fumarate, for it bore no relation to the formate distillation curve. The fumaric acid itself had a specific activity of 1.2 (counts/sec./mg. $BaCO_3$), located entirely in the carboxyls, in view of the inactivity of methine-carbon. This experiment demonstrates conclusively that a direct C_2 condensation has taken place, and furthermore, that no other reactions took place whereby carbinol-carbon of ethanol is converted to methine-carbon of fumaric acid. That is, no mixing takes place. It means the following conversion must have occurred:



Based on the fact that phosphate and succinate appear to be involved, according to the earlier experiments, the supposition is made that Intermediate I is an "active" C_2 compound, possibly phosphorylated, and Intermediate II is the primary product of the condensation reaction, presumably an "active" succinate.

The starting radioactive alcohol used in this experiment had a specific activity of 1.67. The reduced specific activity (1.2) of the fumarate formed from this alcohol could result only from a dilution of the radioactive carboxyls by inactive carboxyls. The probable mechanism: some of the alcohol is oxidized to CO_2. The CO_2 at the end of the experiment had a specific activity of 1.2, representing inactive CO_2 from methyl carbons as well as endogenous respiration,

and radioactive carbon from carbinol. Thus, due to dilution, the specific activity of the CO_2 is much less than that of the carbinol group of the alcohol ($2 \times 1.67 = 3.3$). After fumaric is formed via direct C_2 condensation, the initially highly radioactive carboxyl groups are diluted with lower specific activity CO_2, probably via reversible exchange by oxalacetate decarboxylase, an enzyme known to be active in this organism.[1]

Results with fumaric acid from methyl-labeled ethanol: The formic acid derived from this fumarate was decidedly radioactive, as was the CO_2 representing three-fourths of the fumarate. This latter contains, of course, one methine carbon, equivalent to the formic acid carbon. The radioactivity was unquestionably due to formic acid, since radioactivity measurements precisely paralleled titration values of the Duclaux fractions representing pure formic acid. The last fraction, the most accurate because of its size, contained by titration 65% of the total formic acid distilled. This fraction also was found to contain 65% of the total radioactivity of the formic acid.

The specific activity of the starting ethanol in this experiment was 1.83, and that of the isolated fumaric acid 2.18. This increase can be accounted for only by entrance of labeled carbon into the initially nonradioactive carboxyls of the fumarate. Here, also, an exchange between labeled CO_2 and the fumarate carboxyls must have occurred, again probably involving the participation of the oxalacetate decarboxylase in this organism. The labeled CO_2 had a specific activity of 0.8, and was derived from oxidation of some ethanol. The incorporation of $C^{14}O_2$ into unlabeled carboxyl naturally results in increased specific activity of the fumarate. It is interesting that the exchange reaction in the carbinol-labeled alcohol experiment reduced the specific activity of the fumarate formed, and increased it in this experiment. These experimentally observed results are, therefore, what one would expect.

The specific activities of the methine-carbon and carboxyl-carbon of this fumarate were 3.6 and 0.7 respectively as determined by measurements on formate and CO_2 from permanganate oxidation. Since the specific activity of the methyl group of the starting alcohol was determined to be 3.66, it is evident that a direct conversion of the ethanol methyl group to fumarate methine group must have occurred.

Discussion. -- In recent years experiments on bacteria, yeast, fungi, and animal tissue have suggested strongly the existence of the $2C_2$ condensation reaction (Thumberg-Wieland condensation). Although the evidence has, up to this point, been held in some dispute (see comprehensive literature reviews and critical analyses by Wood,[16] and Block,[17]) the experiments in this paper decisively establish the $2C_2$ condensation reaction. Thus, a third mechanism of formation of the C_4 dicarboxylic acids is added to the other two generally accepted as functioning in aerobic cellular respiration: CO_2 fixation yielding oxalacetic acid, and decarboxylation of α-ketoglutaric yielding succinate. Just what part, if any, the $2C_2$ condensation plays in various living systems in furnishing (eventually) oxalacetic acid for the tricarboxylic acid respiratory cycle is yet to be ascertained.

Apart from the reaction itself, of particular significance appear to be the results indicating entrance of CO_2 into carboxyl groups of the fumaric acid which itself is synthesized via a mechanism not concerned with CO_2 fixation. This CO_2 entrance is assumed to occur through reversible decarboxylation of pre-existing oxalacetate (or malate) in equilibrium with the fumarate. It would be of interest to determine if this mode of entrance of CO_2 could account for some of the carboxyl-labeled organic acids formed in the numerous Krebs type experiments which have heretofore been done with labeled CO_2 or $NaHCO_3$.

In view of the prominent role C_2 compounds play in the metabolism of fungi,[18] it is not surprising that the "$2C_2$ condensation reaction" should have a special

significance in these organisms. With adequate oxygen, carbohydrate in mold cultures is oxidized via oxidative decarboxylation of pyruvate, the resulting active C_2 (acetyl phosphate?) forming organic acids and/or CO_2 eventually. However, the physical nature of mold mycelium in masses almost automatically limits oxygen availability during carbohydrate utilization, and anaerobic alcoholic fermentation ensues. Upon exhaustion of carbohydrate, the mold now slowly oxidizes the alcohol, generating organic acids and/or CO_2 just as it does from pyruvate directly. This mechanism whereby the mold eventually obtains its full quota of energy and carbon from the original carbohydrate and accumulates acids is possible because ethanol oxidation yields apparently the same or similar active C_2 fraction as pyruvate oxidation. The oxidation merely proceeds via a roundabout route, which has been named the "alcohol excursion". These results also indicate why alcohol has so often been considered disputably the intermediate in organic acid formation from sugars by fungi.

The formation of pure fumaric acid in high yields labeled predominantly in methine-carbons should make possible the availability of similarly labeled analogues, such as oxalacetic acid, malic acid, succinic acid, aspartic acid, α-ketoglutaric acid, etc., having value as tracers in metabolism studies. It also provides now the only feasible method of synthesizing methine-labeled maleic anhydride, a vital intermediate in chemical synthesis of many suitably labeled ring-type organic compounds, including carcinogens.

Summary. -- Conditions for obtaining high yields of fumaric acid from ethanol by *Rhizopus nigricans* are specified. Yields of fumaric acid were so high that the tricarboxylic acid cycle as a mechanism of formation of fumarate is excluded. Yields of fumarate from acetate are one-third those from alcohol. Experiments with unlabeled ethanol and C^{14}-labeled carbon dioxide eliminates a CO_2 fixation mechanism of fumarate synthesis. The mold possesses an enzyme which oxidizes

succinate to fumarate.

Fumarate formed from C^{14} methyl-labeled ethanol contains the same specific activity in the methine-carbon as that of the methyl group from the starting ethanol. The carboxyls contain a small amount of radioactivity due to a reversible decarboxylation reaction probably involving oxalacetate decarboxylase.

Fumarate from C^{14} carbinol-labeled ethanol contained no radioactivity in the methine-carbons, but abundantly in the carboxyls. All the evidence proves unequivocally that the C_4 dicarboxylic acid synthesis occurs by direct $2C_2$ condensation as hypothesized originally by Thunberg. The implications of this reaction in the tricarboxylic acid cycle of respiration and in synthesis of tracer intermediates for biochemistry and synthetic organic chemistry are discussed.

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† On leave of absence from the University of Texas.

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