[CONTRIBUTION FROM THE DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF TEXAS, AUSTIN, TEXAS, AND THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

Formate as a Precursor of Carboxylic Acids in Fungi[1]

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C^{14} formate was used as a tracer of glucose metabolism by washed mycelial suspensions of lactic acid and fumaric acid producing strains of *Rhizopus* shaken in air. Fumaric and lactic acids and ethanol were isolated and purified. Each had significant radioactivity, indicating that the formate–carbon entered these molecules. Degradation of each compound showed radioactivity was present in each carbon of all the compounds studied. Specific activity of non-carboxyl carbons of the C_4 dicarboxylic acids was equal to or greater than that of carboxyls. Specific activity of the β-carbon of lactic acid was many times greater than that of the α-carbon and the carboxyl. Specific activity of the methyl carbon of ethanol was higher than that of carbinol carbon. These results indicate that formate can function as a precursor of methyl and methene groups in organic acid metabolism of fungi. The data for lactate stress the possibility of synthesis of the 3-carbon chain by the following type of reaction: $HCOOH + C\text{-}C \longrightarrow CH_3CHOHCOOH$. (a b c a b c)

Introduction

Within the past few years it has been established that formic acid or formaldehyde can be converted to the β-carbon of serine by rat liver homogenates[3,4] and into the α- and β-carbons of propionic acid by propionic acid bacteria.[5] Since formate is known to be metabolized by certain fungi,[6] it seemed of value to test its possible participation in the formation of carboxylic acids produced aerobically from glucose by species of fungi belonging to the genus *Rhizopus* and under study in these laboratories.

Experimental

The organisms used were: *Rhizopus nigricans* No. 45, which produces from glucose predominantly fumaric acid and ethanol, and *Rhizopus* MX which produces predominantly lactic acid and ethanol, together with small amounts of fumaric acid. They were cultivated on a shaking machine for 24 hours on a medium consisting of 0.5% glucose, 1.0% phosphate buffer at *p*H 6.8, 0.2% $(NH_4)_2SO_4$, 0.5% $MgSO_4 \cdot 7H_2O$, and trace amounts of Fe, Zn, Mo, Mn and Cu. The mycelia were filtered and washed twice with 1% KH_2PO_4. Aliquots of mycelium (0.25 g.) freed of excess moisture on a buchner funnel were placed in 250-ml. flasks containing 35 ml. of 1.5% glucose solution. C^{14}-Formic acid was added to each flask (0.35 mg. containing 20 μ curies = 118.2×10^6 c./min./mM. C for *Rhizopus* MX and twice that amount for *R. nigricans* No. 45). The flasks were fixed in 10-l. desiccators which were placed on a reciprocating shaker for 18 hours at 30°.

The flasks were then removed and the contents filtered. Carrier ethanol was added to the filtrates which were then distilled at *p*H 9. Ethanol in the distillates was oxidized with a $K_2Cr_2O_7$–H_2SO_4 mixture and the resulting acetic acid, recovered by steam distillation, was degraded by the method of Phares.[7,8]

Carrier formic acid was added to the residues from the alkaline distillations. These were adjusted to approximately *p*H 1.0 to obtain the carboxylic acids. The acids in the ether extracts were separated on celite–sulfuric acid partition columns developed with ether.[9,10]

Fumaric acid was degraded to formic acid by a modification of the $KMnO_4$–H_2SO_4 oxidation described previously.[11,12] After isolation by steam distillation the formate was combusted for radioactivity measurements.[7]

Lactic acid was degraded by the method of Phares.[7,8] C^{14}-Measurements were made by methods previously de-

TABLE I

Rhizopus MX ON GLUCOSE + $HC^{14}OOH$

(Principal product—lactic acid)

	Respiration product	Counts/min./mM. $C \times 10^{-3}$ corrected for dilution
A.	CH_3—CHOH—COOH	130
	CH_3—CHOH—	175
	CH_3—	326
	—CHOH—	18.2
	—COOH	22.7
B.	HOOC—CH=CH—COOH	245
	—CH=CH—	379
	—COOH[13]	111
C.	CH_3—CHOH—COOH	1.58
	CH_3—CHOH—	1.39
	CH_3—	3.11
	—CHOH—	.15
	—COOH[13]	1.72
D.	CH_3—CH_2OH	29.6
	CH_3—	38.0
	—CH_2OH	7.3

TABLE II

Rhizopus nigricans No. 45 ON GLUCOSE + $HC^{14}OOH$

(Principal product—fumaric acid)

Respiration product	Counts/min./mM. $C \times 10^{-3}$ as determined
HOOC—CH=CH—COOH	75.7
—CH=CH—	57.9
—COOH	94.2
CH_3CH_2OH	13.8
CH_3COOH (from CH_3CH_2OH)	11.6
CH_3—	5.5
—COOH	16.6

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scribed.[7] The data obtained are presented in Tables I (A and B) and II; they represent experiments performed at the Oak Ridge National Laboratories.

Confirmatory experiments were conducted independently at the University of Texas, and the lactic acid produced by *Rhizopus* MX was isolated and degraded by methods different from those used at Oak Ridge. As shown in Table I the results obtained independently in the two laboratories were comparable in the important features. An important modification in the isolation procedure for lactic acid was adopted in the Texas experiments. It was found that lactic acid could be separated from succinic but not from α-ketoglutaric acid with a celite partition column developed with ether; a similar column developed with chloroform containing 10% butanol separated lactate from α-ketoglutarate but not from succinate. Although α-ketoglutarate could have been present only in traces at best (paper chromatography), the isolation procedure was altered to eliminate this acid. The lactic acid in the culture filtrate was recovered from the ether developed column as described above. At this point carrier α-ketoglutarate was added to the lactic acid fraction and the mixture was resolved on a celite column developed with chloroform containing 10% butanol. Lactic acid obtained in this experiment was degraded to acetaldehyde by a modification of the method described by Wood, Lifson and Lorber.[14]

The acetaldehyde, representing α- and β-carbons of lactic acid, was swept out of the oxidation mixture with an air stream and trapped in 200 ml. of ice-water. It was then degraded to iodoform and formic acid with sodium hypoiodite at room temperature. The iodoform was purified by centrifuging and washing 3 times, then taken up in a small volume of ether; the ether was later removed *in vacuo*. Formic acid in the supernatant was recovered as follows: the supernatant was boiled down to remove all remaining dissolved iodoform. After being cooled in an ice-bath, the solution was acidified by dropwise addition of dilute sulfuric acid. Free iodine was destroyed as it was liberated, by the addition of sodium thiosulfate. The formic acid was separated by steam distillation and purified on a celite partition column developed with chloroform containing 10% butanol. By this procedure the β-carbon of lactate is converted to iodoform and the α-carbon to formic acid.

Radioactivity measurements on the specimens from the Texas experiment were made with a vibrating reed electrometer.[7] The results of this experiment are given in Table I (C).

In the experiment described immediately above, the ethanol yield was so low that it was not suitable for degradation after addition of carrier. For that reason the experiment was repeated with a shorter (18-hour) incubation period to procure a higher ethanol yield. The radioactivity composition of this ethanol is given in Table I (D).

Discussion

The findings demonstrate that formate carbon can function as a precursor of the methyl groups of lactic acid and ethanol, and of the methylene groups of fumaric acid in the metabolism of species of *Rhizopus*. Of particular interest is the preponderance of radioactivity in the β-carbon of lactate. The lactate described in Table I (A) had radioactivity in the β-, α- and carboxyl carbons in the ratio 17.9:1:1.25.

Malic and succinic acids formed in these fermentations were labeled similarly to the fumaric acid. Thus it seems likely that formate participates in the synthesis of various members of the di- or tricarboxylic acid cycle. Presumably this is one mode of oxidation of formate, at least in these fungi.

The radioactivity of the carboxyl groups of the various organic acids studied very likely is derived to some extent from cycling *via* the di- and tricarboxylic acid respiratory cycles and to some extent from CO_2 representing completely oxidized formate. The CO_2 in the gas phase in the desiccators at the end of the incubation periods always contained considerable radioactivity. Several experiments with labeled CO_2 as a tracer in this fungus have shown that CO_2 enters the carboxyls of all the organic acids synthesized, but it does not enter significantly the non-carboxyl carbons of those acids. The special significance of these experiments is the labeling of non-carboxyl carbons.

A close relation appears to exist between the distribution of labeling in the ethanol and the fumarate in each organism, suggesting a mutual precursor of these compounds or an interchange between them. The ratio of radioactivity in the methine-C to that in the carboxyl-C of fumarate from *Rhizopus* MX = 3.8:1; of methyl-C to carbinol-C of ethanol = 5.2:1. The corresponding ratios from *Rhizopus nigricans* No. 45 are 0.6:1 and 0.33:1.

In the *Rhizopus* MX experiments the observed distribution of radioactivity in the lactate, in the fumarate, and in the ethanol suggests an origin of these compounds from a common precursor synthesized in part from formate, namely, pyruvate 3-C^{14}. When pyruvate 3-C^{14} is used as a tracer, the observed distribution of radioactivity in these three metabolic products is indeed similar to that obtained in the above formate experiments.[7] However, more evidence for the role of pyruvate in formate metabolism is needed.

The results obtained with labeled formate permit an alternative interpretation of the data from ethanol 2-C^{14} experiments which previously[11,12] were interpreted to mean that two 2-carbon molecules (derived from ethanol) condensed *via* the methyl groups (Thunberg–Wieland condensation) yielding succinate then fumarate. It is conceivable that C^{14}-formate could be produced by the oxidation of some ethanol 2-C^{14}, the formate then condensing with 2-carbon molecules derived from ethanol 2-C^{14}, producing eventually an α–β labeled 3-carbon compound. This 3-carbon compound might be serine[15] known to be synthesized from formate plus glycine in rat liver. Serine could then be converted to α–β labeled pyruvate, a reaction known to occur in bacteria and in rat liver.[16,17] Or the pyruvate itself conceivably may be formed without intermediate glycine and serine participation. In any case the α–β labeled pyruvate could then fix CO_2, yielding 2,3-labeled C_4-dicarboxylic acids. These possibilities are being studied.

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