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An attempt has been made to elucidate the biosynthetic precursor of ethylene in the mold P. digitatum.   By application of basic radiotracer methodology and the utilization of several specifically labeled radiochemical substrates, it has been possible to demonstrate that the internal carbon atoms of C-4 dicarboxylic acids such as succinate, fumarate and malate contribute to the ethylene carbon skeleton.   In attempting to further elucidate the pathway of ethylene formation, β-alanine, acrylic acid and propionic acid were employed as metabolic substrates to test the hypothesis that acrylic acid could be the precursor of ethylene. β-alanine and propionic acid were found to be metabolized by other pathways unrelated to acrylic acid formation.   However, when acrylate was used as a test substrate, a significant incorporation of the C-2 and C-3 carbon atoms into the ethylene molecule was demonstrated.   Thus, this work strongly suggests that acrylic acid is the immediate precursor of ethylene in P. digitatum.

THE BIOSYNTHETIC PRECURSOR OF ETHYLENE
IN <u>PENICILLIUM</u> <u>DIGITATUM</u> SACCARDO

by

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THIS THESIS IS DEDICATED TO MY PARENTS
AND MY WIFE WHOSE CONTINUING ENCOURAGEMENT
RESULTED IN ITS COMPLETION.

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THE BIOSYNTHETIC PRECURSOR OF ETHYLENE
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INTRODUCTION

The biological occurrence and physiological effect of ethylene

gas have been recently documented in a comprehensive review by

Burg (7). The mode of action underlying biochemical and physiologi-

cal responses invoked by ethylene remain obscure. In a more recent

report (21), Lyons and Pratt suggested that the respiratory response

induced in plants by ethylene may be the result of increased permea-

bility of mitochondrial membrane. Meanwhile, considerable effort

has been devoted by several laboratories to elucidate the biosynthetic

mechanism of ethylene formation in fruit and in the mold Penicillium

digitatum.

Fergus (12), using the triple response of etiolated pea seedlings

as a means of estimating ethylene, reported that, with the mold P.

digitatum, enhanced yield of ethylene was realized when the mold was

grown on D-mannose, D-mannitol, D-xylose, D-galactose, or citrate

as compared to other carbon sources. Phan-Chon-Ton (24) relying

on a manometric method to detect ethylene released from a mercuric

perchlorate trap (23), demonstrated that glucose, sodium malate,

glycerol, ethanol, and L-alanine were good carbon sources for pro-

duction of ethylene by the mold.

Burg and Thimann (10, 11) and Burg (11) made use of radio-tracer techniques to demonstrate that, with apple tissue sections, ethylene carbon skeleton could be derived preferentially from C-6 of glucose than from any other carbon atom. They also demonstrated that tritium labeled ethylene was produced when tritium labeled water was introduced into the apple tissue sections. This observation led these authors to suggest that a reversible hydration-dehydration step was involved in the biosynthetic mechanism of the ethylene molecule (9).

In 1964 Burg and Burg (8) examined the formation of ethylene in apple tissue from a number of C^{14} substrates. It was found that C-2 of acetate was a better carbon source than C-1 of acetate. Similarly, fumarate-2, 3-C^{14} contributed to a greater extent to ethylene than fumarate-1,4-C^{14}. It was further observed that $C^{14}O_2$ could be incorporated into the ethylene molecule.

In an attempt to elucidate the metabolic intermediates of ethylene, Jansen (16, 17) fed C^{14} ethylene and H^3 ethylene to avocado fruit by gas exposure. While less than one percent of the radioactivity was incorporated, it was possible, nevertheless, to demonstrate that a significant amount of labeled ethylene resided in benzene and toluene isolated from the fruit. In contrast, Buhler, et al. (6), using C^{14} ethylene, found significant incorporation into the organic acids of avocado, particularly succinate and fumarate.

Abeles and Rubinstein (1) have isolated a crude enzyme from etiolated pea seedlings that catalyzes ethylene production making use of constituents in the supernatant of the enzyme preparation. The involvement of linolenic acid in the biosynthetic mechanism of ethylene in apples has been recently reported by Lieberman and Mapson (20).

In this laboratory, the biosynthesis of ethylene in P. digitatum has been extensively examined by means of radiotracer techniques using C^{14} specifically labeled substrates such as D-glucose, DL-alanine, DL-glutamic acid and DL-aspartic acid (35). It was demonstrated in 1962 that the precursor of ethylene carbon skeleton is closely related to Krebs cycle intermediates. The operation of glycolysis and the Krebs cycle pathway has been previously demonstrated in this mold (27, 28). The findings were supported not only by the results of Burg and Burg (8) in 1964 but also by the report of Gibson (14) in 1964, revealing that C-3 of pyruvate was preferentially incorporated into the ethylene molecule produced by P. digitatum. These results are also in line with the observations of Freebairn and Buddenhagen (13) that fumarate enhances the production of ethylene by Pseudomonas solanacearum and with the reports of Spencer, et al. (22, 25, 26, 30) demonstrating that, with tomatoes, the site of ethylene formation is associated with the mitochondria fraction and that ethylene production is related to the respiratory

mechanism of this fruit.

In the present work, radiotracer techniques have been used to elucidate more exactly the biosynthetic pathway of ethylene in P. digitatum.

MATERIALS AND METHODS

Culture Conditions

Stock cultures of P. digitatum (ATCC-10030) were maintained at 4° C on 25 ml of Difco potato dextrose agar buffered to pH 3. 5 with D-tartaric acid (37). Several of the serum bottle (200 ml size) slants were inoculated at one time and allowed to develop at 22° C for four days before refrigeration. Stock cultures were routinely reactivated by transfer to new agar slants every three to four months.

Experimental inocula were obtained from 1 cc suspensions of mycelia, grown on the potato dextrose agar. Growth medium consisted of: $(NH_4)_2HPO_4$, 1. 0 g; $NaNO_3$, 1. 0 g; $MgSO_4$, 0. 25 g; KCl, 0. 1 g; $CaCl_2$, 0. 1 g; $FeCl_3$, 5 mg; $MnCl_2$, 0. 1 mg; $ZnCl_2$, 0. 05 mg; H_3BO_3, 0. 05 mg; $CuCl_2$, 0. 01 mg; KI, 0. 01 mg; Difco yeast extract, 2. 0 g; glucose, 20 g; all dissolved in one liter of water. The pH of the medium was adjusted to 3. 2 with hydrochloric acid. Standing aerobic cultures were grown at 22° C in 100 ml of the medium contained in 500 ml Erlenmeyer flasks. Under these conditions, ethylene production was noticeable on the third day after inoculation and reached peak production rate on the ninth or tenth day. There followed a decline to lower rates accompanying the exhaustion of glucose substrate. At the end of 14 days the mean dry weight of mold was 425 mg.

A stream of sterile air at 10 cc per minute continually swept out gaseous emanations from the culture flask. Respiratory CO_2 was trapped in sodium hydroxide and ethylene was adsorbed on silica gel at -79° C according to the method previously reported by Wang, Persyn and Krackov (35).

Radiochemical Substrate Administration

Results obtained in preliminary studies indicated that organic acids could be utilized by P. digitatum in the presence of a primary carbon source such as glucose. Therefore, all radiochemical substrates, being tested as potential ethylene precursors, were added aseptically to medium containing essential salts, yeast extract and glucose seven to eight days after inoculation. Samples of respiratory CO_2 and ethylene were individually collected at intervals of 24 hours after the addition of a given labeled substrate.

Time Course Studies

The evaluation of relative contributions of various carbon sources to the biosynthesis of ethylene skeleton is a difficult task. When the radiotracer technique is used, the criteria for evaluation relies on either the mean specific activity of ethylene derived from a C^{14} labeled substrate or the overall radiochemical yield of the substrate radioactivity in the ethylene molecules. The former

criterion is semi-quantitative in nature. This is true since one is

uncertain of the rate of permeability, the size of the endogenous

pool, the rates of metabolism and other parameters relative to a

specific substrate. The latter criterion is more reliable, but cross-

comparison of data observed in experiments with different substrates

cannot be made unless complete utilization of a given amount of each

of the substrates by the mold can be realized. This fact, consequent-

ly, necessitates the information on the time course of the utilization

of substrates by mold, making use of such indices as the production

of respiratory $C^{14}O_2$.

In the present work, the level of C^{14} labeled substrates in each

of the experiments has been determined in preliminary experiments

so that the administered substrate can be completely utilized within

a prescribed duration of five days. Consequently, the yields of

$C^{14}_2H_4$, from various C^{14} labeled substrates, observed at

the end of these experiments can be cross-compared since any dif-

ferences in metabolic behaviors among different substrates such as

rate of permeability, rate of metabolism, etc. would have been elim-

inated with the complete utilization of each of these substrates.

Moreover, by following the time course of the production of

respiratory $C^{14}O_2$ and $C^{14}_2H_4$ from mold utilizing a given sub-

strate, it is possible to gain further insight with respect to the re-

lationship between ethylene biosynthesis and the respiratory

mechanism of the organism.

Measurement of Radioactivity

Respiratory $C^{14}O_2$, trapped as $Na_2C^{14}O_3$, was converted to $BaC^{14}O_3$ for radioactive assay. The method for the collection and measurement of C^{14} ethylene has been previously reported by Wang, Persyn and Krackov (35). In essence ethylene gas was swept from the silica gel adsorption column at $50^{\circ}C$ into a Beckman GC-2 gas chromatograph. The separation of ethylene from other gaseous components, namely air and traces of carbon dioxide, was facilitated with the use of a continuous chromatographic column consisting of molecular sieve 4A (Wilkens Instrument and Research, Inc.) and silica gel (Beckman #70010). Pure ethylene fraction was solidified in a fraction collector submerged in a liquid nitrogen bath. Pre-cooled scintillator solution (35) was added to the ethylene collection tube while still immersed in liquid nitrogen. The contents of the collection tube, upon melting, were transferred quantitatively to a liquid scintillation counting vial for determination of C^{14} activity. The residual radioactivity in the media was determined by the liquid scintillation counting of thixotropic gel preparation (36).

All counting processes were carried out to a standard deviation no greater than one percent except for the samples of low counting rate. In those cases no significant conclusions are needed to be drawn from the counting data. Authenticity of the ethylene peak in the gas

chromatographic separation was determined by co-chromatography

with pure ethylene (obtained from the Olin Matheson Company).

Radiochemical Substrates

Many of the specifically labeled substrates used in the experi-

ments were obtained from the respective commercial sources given

in the following: succinate-1,4-C^{14}, succinate-2,3-C^{14}, sodium

propionate-1-C^{14}, sodium propionate-2-C^{14}, sodium propionate-3-

C^{14}, sodium acetate-1-C^{14}, and sodium acetate-2-C^{14}, from New

England Nuclear Corporation; DL-malate-3-C^{14}, fumarate-1,4-

C^{14} and β-alanine-2-C^{14}, from Volk Radiochemical Company;

β-alanine-1-C^{14}, β-alanine-3-C^{14}, from Atomic Accessories In-

corporated; and fumarate-2,3-C^{14}, from Nuclear Chicago Cor-

poration.

Potassium acrylate-1-C^{14}, potassium acrylate-2-C^{14} and po-

tassium acrylate-3-C^{14} were prepared in this laboratory from the

corresponding labeled β-alanine. The starting material was first

converted to β-hydroxypropionic acid with nitrous acid and then de-

hydrated by means of 50 percent concentrated sulfuric acid at 100°C

to acrylic acid (19). The three specifically labeled acrylates were

purified by means of column chromatography, using Celite columns,

according to a procedure described by Swick (32) and by means of

preparative thin-layer chromatography using silica gel (approximate

thickness: 350 microns). The solvent system used was that of

Braun and Geenen (4) which consisted of 95 percent ethanol, ammonium hydroxide (28 to 30 percent ammonia) and water (100:16:12). The resolved acrylate fraction $(R_f = 0.69$ to $0.73)$ was detected with ultraviolet light illumination and the pure compound was extracted from the silica gel with 100 percent ethanol.

RESULTS AND DISCUSSION

The results presented in this work represent average values

from replicate experiments, the discrepancy of which is generally no

greater than ten percent.

Krebs Cycle Intermediates as Ethylene Precursors

On the basis of the findings of Wang, Persyn and Krackov (35),

it was speculated that the conversion of glucose carbon atoms to

ethylene followed a route involving glycolysis and the Krebs cycle

pathway. These authors, using C^{14} specifically labeled DL-alanine,

DL-aspartate, and DL-glutamate as substrates, demonstrated that

the internal carbon atoms of dicarboxylic acids, such as succinate,

fumarate, etc., might be the origin of ethylene carbon skeleton.

In order to verify this speculative mechanism, in the present

work, time course studies were first carried out with specifically

labeled succinate, fumarate, DL-malate and acetate as substrates.

As mentioned previously, these acids can be utilized by P. digitatum

if a sufficient amount of glucose is present in the incubation medium.

Data of a typical time-course study with fumarate-2, 3-C^{14}

(21 μmole) as the substrate is given in Figure 1. These results re-

veal the close correlation between the rates of production of respir-

atory $C^{14}O_2$ and that of $C_2^{14}H_4$ and hence support the contention
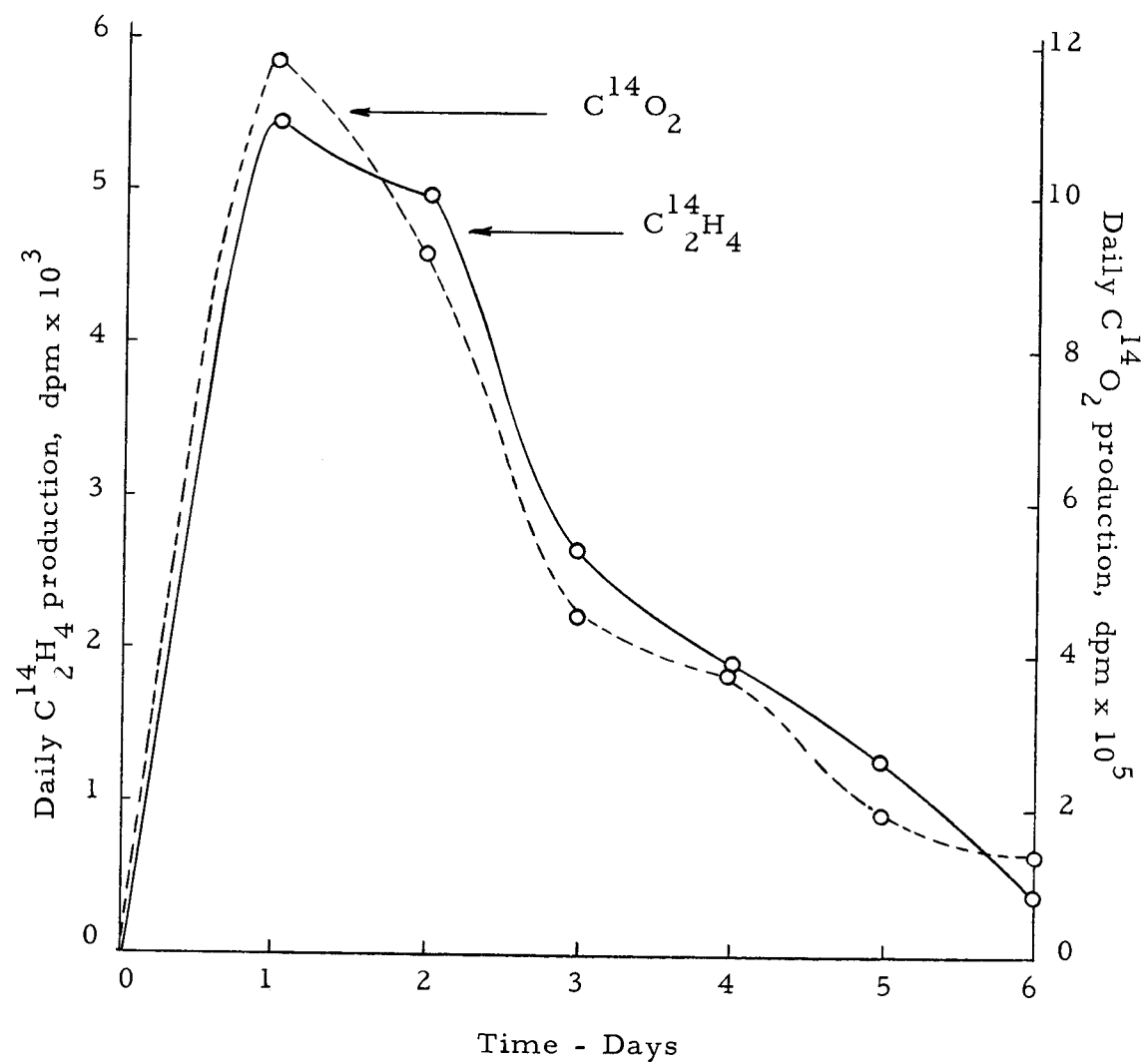
Figure 1. Time course of $C_2^{14}H_4$ and $C^{14}O_2$ production from fumaric-2, 3-C^{14} acid.

that ethylene biosynthesis is directly linked with the respiratory

pathways in the mold. Similar situations were also observed with

other substrates.

The results of other time-course studies relative to the pro-

duction of $C^{14}O_2$ and $C_2^{14}H_4$ are given in Table I. It is noted that

the yields of respiratory $C^{14}O_2$ reach a peak within 48 hours and

then decline to low levels reflecting the exhaustion of substrate in

the medium. The overall yields and the mean specific activity of

$C_2^{14}H_4$ are given in Table II along with the five-day cumulative

yields of $C^{14}O_2$ and data on the radioactivity detected in the medium.

The data given in Table II show that the internal carbon atoms

of fumarate, succinate, and malate contribute heavily to the form-

ation of ethylene carbon skeleton. The findings are also in line

with those observed in the labeled acetate experiments, in which C-2

of acetate is found to be preferentially incorporated into ethylene.

These results thus provide direct support to the contention reported

by Wang, Persyn and Krackov (35). Recent studies by Gibson (14),

using C^{14} specifically labeled pyruvates as substrate to P. digitatum,

are also consistent with the results obtained in the present work.

Table I. Time course for the production of $C^{14}O_2$ and $C_2^{14}H_4$ by <u>P.</u> <u>digitatum</u> utilizing several C^{14} labeled substrates.

	Substrate							
	Succinate-1,4-C^{14}		Succinate-2,3-C^{14}		Fumarate-1,4-C^{14}		Fumarate-2,3-C^{14}	
	Yield in %		Yield in %		Yield in %		Yield in %	
	$C^{14}O_2$	$C_2^{14}H_4$	$C^{14}O_2$	$C_2^{14}H_4$	$C^{14}O_2$	$C_2^{14}H_4$	$C^{14}O_2$	$C_2^{14}H_4$
Time (Day)	%	% x 10^{-2}	%	% x 10^{-2}	%	% x 10^{-2}	%	% x 10^{-2}
1	29	0.2	6	1.8	40	...	21	9.9
2	31	0.2	15	2.9	17	...	17	8.9
3	18	0.1	7	4.2	13	...	8	4.8
4	10	...*	7	3.3	8	...	7	3.4
5	6	0.1	6	1.6	6	...	3	2.3

	DL-Malate-3-C^{14}		Acetate-1-C^{14}		Acetate-2-C^{14}	
	Yield in %		Yield in %		Yield in %	
	$C^{14}O_2$	$C_2^{14}H_4$	$C^{14}O_2$	$C_2^{14}H_4$	$C^{14}O_2$	$C_2^{14}H_4$
Time (Day)	%	% x 10^{-2}	%	% x 10^{-2}	%	% x 10^{-2}
1	23	14.0	36	0.5	27	23.0
2	17	5.6	13	0.1	8	4.3
3	13	9.6	7	0.1	6	2.3
4	12	6.6	3	0.1	4	2.0
5	6	2.2	2	0.1	3	2.4

* < 0.001%

Table II. Utilization of C^{14} labeled substrates by $\underline{P}$. <u>digitatum</u> relative to production of $C^{14}O_2$ and $C_2^{14}H_4$.

Substrate	μc	μmole	$C_2^{14}H_4$ ($\times 10^{-2}$)	$C^{14}O_2$	Media	Mean $C_2^{14}H_4$ Specific Activity (dpm/μmole)*
			Percent Distribution of Substrate Radioactivity			
Succinate-1,4-C^{14}	1.78	10	0.7	72	17	199
Succinate-2,3-C^{14}	2.50	10	20.0	40	28	1074
Fumarate-1,4-C^{14}	1.42	21	$<$0.1	85	8	1
Fumarate-2,3-C^{14}	2.50	21	32.0	57	4	3723
DL-Malate-3-C^{14}	2.50	30	41.0	79	8	4488
Acetate-1-C^{14}	2.20	50	1.0	61	5	200
Acetate-2-C^{14}	2.02	50	33.0	49	5	4827

* Normalized to 1μc/μmole substrate specific activity.

The Biosynthetic Pathway of Ethylene
from the Krebs Cycle

From these findings one is led to speculate that acrylate may

be involved in ethylene biosynthesis as an intermediate since a

single decarboxylation reaction of fumaric acid would give rise to

acrylic acid directly. Alternatively, acrylate could originate from

malic acid through a concerted non-hydrolytic elimination -- decar-

boxylation reaction, similarly thought to occur for the formation of

Δ^3-isopentenyl pyrophosphate from (R)-mevalonate-5-pyrophosphate

in cholesterol biosynthesis (3). Such a reaction would involve the

requirement of ATP. The requirement of ATP in ethylene bio-

synthesis has been suggested in at least two independent studies (10,

22). It should also be noted that Varner (34) had previously proposed

the possible sequence in ethylene production: β-alanine $\rightarrow$ β-alanyl-

CoA $\rightarrow$ acrylyl-CoA $\rightarrow$ acrylic acid $\rightarrow$ ethylene. Moreover, acrylic

acid was shown to be a product of β-alanine metabolism in several

organisms (5, 33).

In order to further elucidate the mechanism for ethylene bio-

synthesis, C^{14} specifically labeled β-alanine samples were first

used as test substrates to the mold. The results of this series of

experiments for the utilization of β-alanine is given in Table III

and Table IV. The production of respiratory $C^{14}O_2$ from individual

carbon atoms of β-alanine was found to follow the order of

Table III. Time course for the production of $C^{14}O_2$ and $C_2^{14}H_4$ by P. <u>digitatum</u> utilizing several C^{14} labeled substrates.

	Substrate					
	β-Alanine-1-C^{14}		β-Alanine-2-C^{14}		β-Alanine-3-C^{14}	
	Yield in %		Yield in %		Yield in %	
Time	$C^{14}O_2$	$C_2^{14}H_4$	$C^{14}O_2$	$C_2^{14}H_4$	$C^{14}O_2$	$C_2^{14}H_4$
(Day)	%	% x 10^{-2}	%	% x 10^{-2}	%	% x 10^{-2}
1	41	...*	33	4.1	36	0.1
2	22	...	16	2.4	17	...
3	7	...	7	1.3	8	...
4	4	...	4	1.5	3	...
5	14	...	2	0.9	4	...

	Acrylate-1-C^{14}		Acrylate-2-C^{14}		Acrylate-3-C^{14}	
	Yield in %		Yield in %		Yield in %	
Time	$C^{14}O_2$	$C_2^{14}H_4$	$C^{14}O_2$	$C_2^{14}H_4$	$C^{14}O_2$	$C_2^{14}H_4$
(Day)	%	% x 10^{-2}	%	% x 10^{-2}	%	% x 10^{-2}
1	30	...	9	3.3	46	11.1
2	28	...	9	2.7	28	4.2
3	12	...	7	2.3	10	2.1
4	4	...	5	3.8	4	1.9
5	7	...	5	4.0	< 1	1.3

	Propionate-1-C^{14}		Propionate-2-C^{14}		Propionate-3-C^{14}	
	Yield in %		Yield in %		Yield in %	
Time	$C^{14}O_2$	$C_2^{14}H_4$	$C^{14}O_2$	$C_2^{14}H_4$	$C^{14}O_2$	$C_2^{14}H_4$
(Day)	%	% x 10^{-2}	%	% x 10^{-2}	%	% x 10^{-2}
1	41	0.2	22	1.3	17	6.9
2	17	...	15	0.9	11	3.0
3	5	...	8	0.5	7	2.3
4	2	0.1	3	0.2	4	1.8
5	1	...	4	0.3	2	1.0

* < 0.001%

Table IV. Utilization of C^{14} labeled substrates by P. digitatum relative to production of $C^{14}O_2$ and $C_2^{14}H_4$.

Substrate	µc	µmole	Percent Distribution of Substrate Radioactivity			Mean $C_2^{14}H_4$ Specific Activity (dpm/µmole)*
			$C_2^{14}H_4$ (x10^{-2})	$C^{14}O_2$	Media	
β-Alanine-1-C^{14}	2.00	50	0.1	71	9	25
β-Alanine-2-C^{14}	2.50	50	31.0	48	15	3120
β-Alanine-3-C^{14}	2.50	50	0.2	64	8	28
Acrylate-1-C^{14}	3.68	12	0.1	82	12	8
Acrylate-2-C^{14}	2.04	16	18.0	34	18	1330
Acrylate-3-C^{14}	0.30	2**	21.0	89	8	177
Propionate-1-C^{14}	2.50	25	0.4	67	3	60
Propionate-2-C^{14}	2.50	25	3.0	52	11	370
Propionate-3-C^{14}	2.50	25	15.0	40	14	2140

* Normalized to 1µc/µmole substrate specific activity.

** The low specific activity of acrylate-3-C^{14} made it impossible to raise the substrate level used in other acrylate experiments.

C-1 > C-3 > C-2 and for $C^{14}_2H_4$ production the order appeared as

C-2 >> C-3 > C-1. These facts are in contrast to the results obtained with DL-α-alanine as substrate which revealed that $C^{14}_2H_4$ yields follow the order of C-3 >> C-2 > C-1 (35).

To facilitate the analysis of these data, the major metabolic pathways of β-alanine are outlined in Figure 2. First, if the reversed enzymatic reaction catalyzed by aspartate 1-decarboxylase is operative (Figure 2, A), then synthesis of aspartic acid from β-alanine and CO_2 could take place (5). Second, if a β-alanine acylase is present (Figure 2, B) in the organism, β-alanine-CoA can be formed and can be in turn converted by means of the reversed reaction catalyzed by acrylyl-CoA aminase to yield acrylyl CoA (33). Via the third catabolic pathway (Figure 2, C) the action of a transaminase could convert β-alanine to malonic semialdehyde and eventually to acetyl-CoA (2, 15, 29). By way of the foregoing described sequential reactions, the key intermediates, shown in Figure 2, can be further metabolized to yield CO_2 and C_2H_4.

The aspartate 1-decarboxylase pathway and the acrylyl-CoA aminase pathway appear to be nonoperative in P. digitatum judging from the relative yields of $C^{14}O_2$ from individual carbon atoms of substrate β-alanine.

The preferential conversion of C-3 of β-alanine to respiratory $C^{14}O_2$ over that of C-2, in fact, suggests strongly that conversion

$$H_2N-CH_2-CH_2-COOH$$

β-alanine

$\overset{NH_2}{\underset{|}{}}$

$HOOC-CH-CH_2-COOH$

Aspartic acid

$H_2N-CH_2-CH_2-CO{\sim}SCoA$

β-alanyl-CoA

$CHO-CH_2-COOH$

Malonic semi-
aldehyde

Krebs cycle

$CH_2{=}CH-CO{\sim}SCoA$

Acrylyl - CoA

$CH_3-CO{\sim}SCoA$

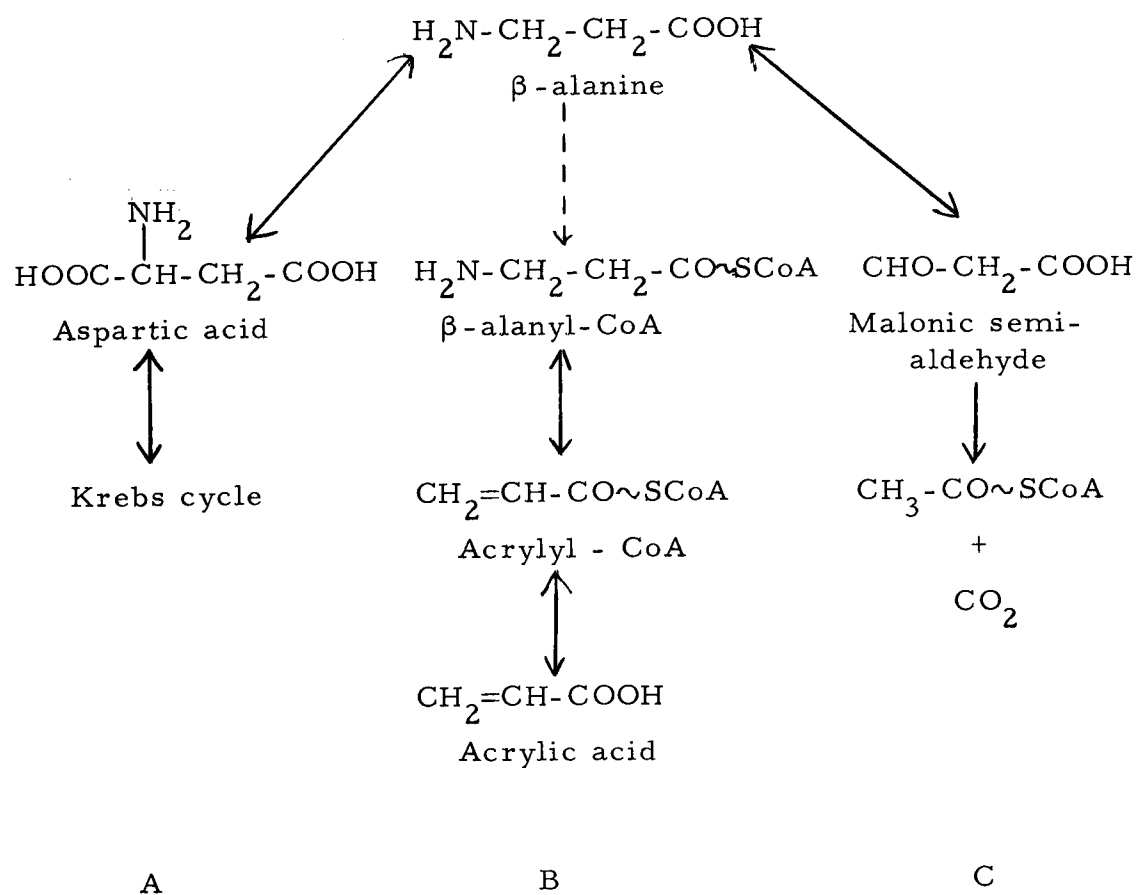$+$

CO_2

$CH_2{=}CH-COOH$

Acrylic acid

A

B

C

Figure 2. The metabolism of β-alanine.

of β-alanine to malonic semialdehyde appears to be the most likely

catabolic pathway for this substrate. Thus, malonic semialdehyde

can be further metabolized to acetyl-CoA (equivalent to C-2 and C-3

of β-alanine) and CO_2 (equivalent to C-1 of β-alanine). The oper-

ation of this pathway can also account for the observation that C-2 of

β-alanine is preferentially incorporated into the ethylene produced

by the mold. The preferential incorporation of C-2 of acetate into

ethylene has been previously observed (Table II).

These findings, however, do not rule out the possible involve-

ment of acrylate in ethylene biosynthesis. Consequently, C^{14} speci-

fically labeled acrylate was prepared and used as the test substrate

to the mold. As indicated by results given in Table III and IV, acry-

late can be actively metabolized by the mold giving rise to the for-

mation of respiratory $C^{14}O_2$. It is interesting to note that, whereas

C-1 of acrylate was not incorporated into the ethylene skeleton, C-2

and C-3 of acrylate contributed significantly and to approximately

the same extent to the formation of ethylene. These findings suggest

that ethylene can be produced by the mold via a decarboxylase re-

action of acrylic acid. It is evident that acrylic acid can also be

metabolized by the mold via other pathways since the yield of $C^{14}O_2$

from C-3 is much greater than that of C-2.

The exact fate of acrylate in the metabolism of P. digitatum

mold cannot be ascertained without further experimentation.

However, possible pathways are summarized in Figure 3.

As shown in Figure 3, an alternate pathway for acrylate metabolism involves the formation of propionyl-CoA which can, in turn, be converted to succinyl-CoA (18) and eventually lead to the formation of ethylene from the middle carbon atoms of succinic acid. Such a sequence (as shown in Figure 4) would also give rise to equal labeling in ethylene from either C-3 or C-2 of acrylic acid. To investigate whether such a reaction sequence is operative in P. digitatum, C^{14} specifically labeled propionates were used as substrates to the mold.

The result obtained in the propionate experiments, as given in Table III and Table IV, indicate that this compound can be actively metabolized by the mold giving rise to the formation of respiratory $C^{14}O_2$ in good yields. However, insofar as the formation of $C^{14}_2H_4$ is concerned, only the label of C-3 of propionate was found, to a significant extent, in the ethylene produced by the mold. These findings more than likely rule out the possible involvement of propionic acid as an intermediate in acrylate metabolism in the mold. The mechanism responsible for the preferential incorporation of C-3 of propionate to ethylene carbon skeleton is not certain. However, it can be speculated that the mechanism may have involved the formation of acetate via an α-oxidation as observed by Stumpf (31). The metabolism of propionate in which pyruvate is involved as an

$$CH_2=CH\text{-}COOH \longrightarrow CH_2=CH_2 + CO_2$$

Acrylic acid

$$CH_3\text{-}CH_2\text{-}CO\sim SCoA \longleftrightarrow CH_2=CH\text{-}CO\sim SCoA$$

Propionyl - CoA   Acrylyl - CoA
(See Figure 4)

$$H_2N\text{-}CH_2\text{-}CH_2\text{-}CO\sim SCoA \qquad HO\text{-}CH_2\text{-}CH_2\text{-}CO\sim SCoA \qquad CH_3\text{-}\overset{\overset{\displaystyle OH}{|}}{CH}\text{-}CO\sim SCoA$$

                                     β - OH propionyl - CoA   Lactyl - CoA

$$H_2N\text{-}CH_2\text{-}CH_2\text{-}COOH \qquad CHO\text{-}CH_2\text{-}CO\sim SCoA \qquad CH_3\text{-}\overset{\overset{\displaystyle OH}{|}}{CH}\text{-}COOH$$

β - alanine   Malonic semialdehyde-CoA   Lactic acid

$$CHO\text{-}CH_2\text{-}COOH \qquad HOOC\text{-}CH_2\text{-}CO\sim SCoA \qquad CH_3\text{-}\overset{\overset{\displaystyle O}{\|}}{C}\text{-}COOH$$

Malonic semialdehyde   Malonyl - CoA   Pyruvic acid
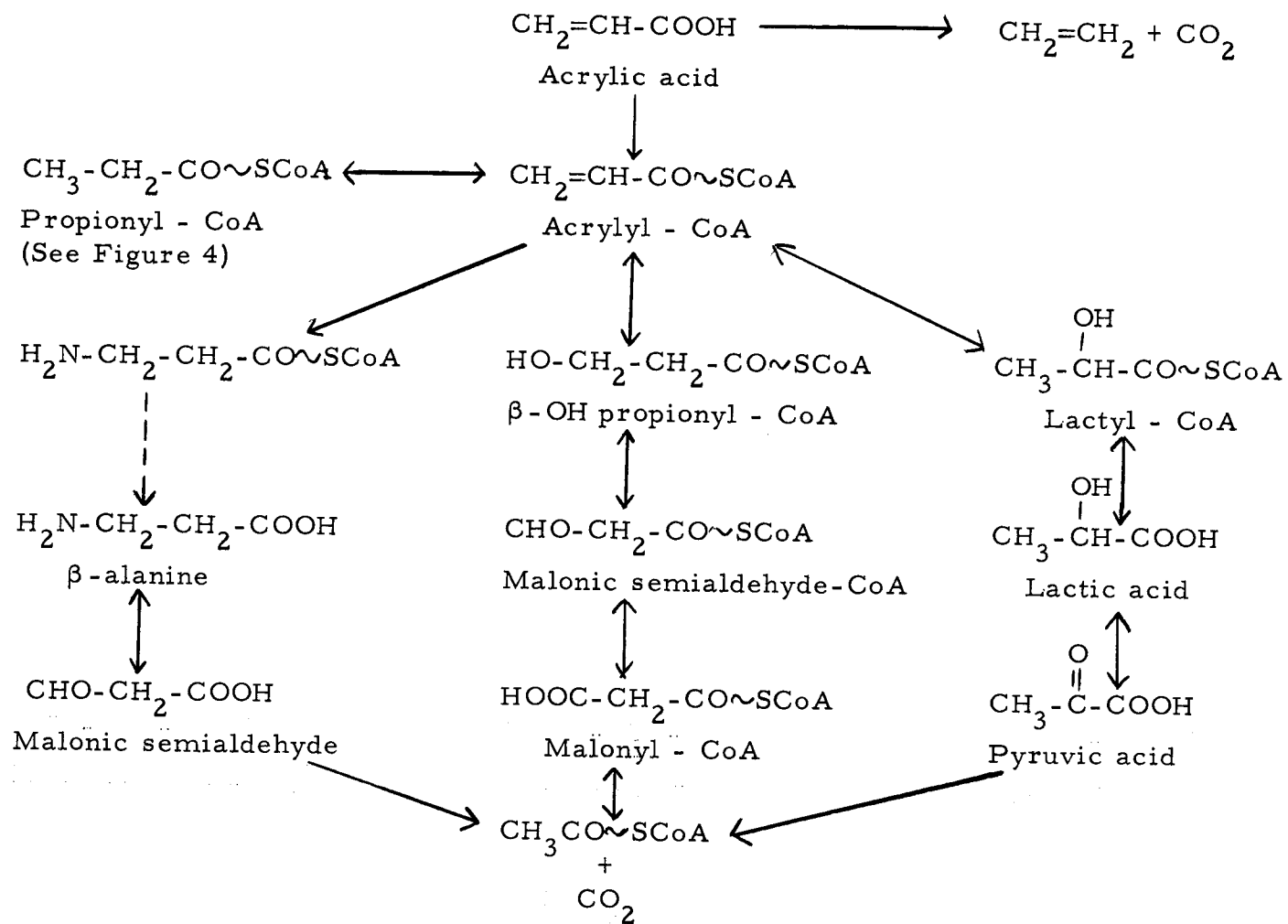
$$CH_3CO\sim SCoA$$
$$+$$
$$CO_2$$

Figure 3. The metabolism of acrylic acid.

intermediate is also a possibility. The preferential incorporation of C-2 of acetate into ethylene has been demonstrated (see Table II). Similarly, C-3 of pyruvate is preferentially incorporated into ethylene in $\underline{P}$. digitatum (14). The possible metabolic fate of propionate is depicted in Figure 4.

On the basis of the experimental results obtained in the present work, it is proposed that in $\underline{P}$. digitatum, ethylene is biosynthesized by way of the reaction sequence in Figure 5.
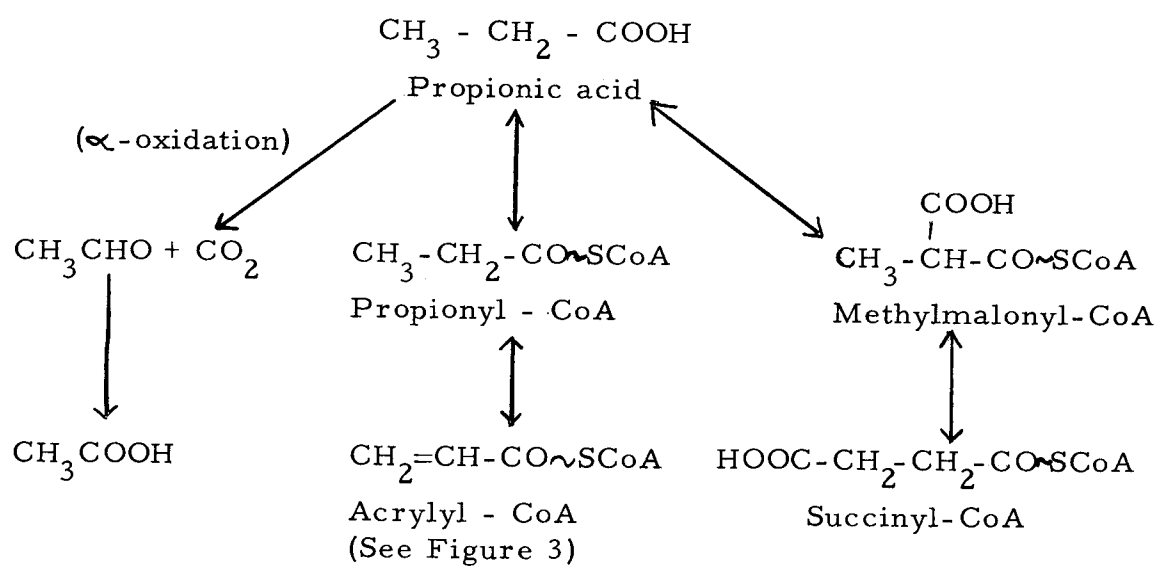
Figure 4.   The metabolism of propionic acid.

$$\begin{array}{c}
\quad\quad\quad\quad OH \\
\quad\quad\quad\quad | \\
HOOC - CH - CH_2 - COOH \\
\quad o \quad * \quad\uparrow\quad * \quad\quad o \\
\quad\quad\quad\quad \updownarrow \\
HOOC - CH = CH - COOH \longrightarrow CH_2 = CH\text{-}COOH \longrightarrow CH_2 = CH_2 \\
\quad o \quad * \quad\uparrow\quad * \quad\quad o \quad\quad\quad\quad * \quad\quad * \quad o \quad\quad\quad\quad\quad * \quad\quad * \\
\quad\quad\quad\quad \updownarrow \quad\quad\quad\quad\quad\quad\quad\quad + \quad\quad\quad\quad\quad\quad + \\
HOOC - CH_2 - CH_2 - COOH \quad\quad CO_2 \quad\quad\quad\quad\quad\quad CO_2 \\
\quad o \quad * \quad\quad * \quad\quad o \quad\quad\quad\quad\quad\quad o \quad\quad\quad\quad\quad\quad\quad o
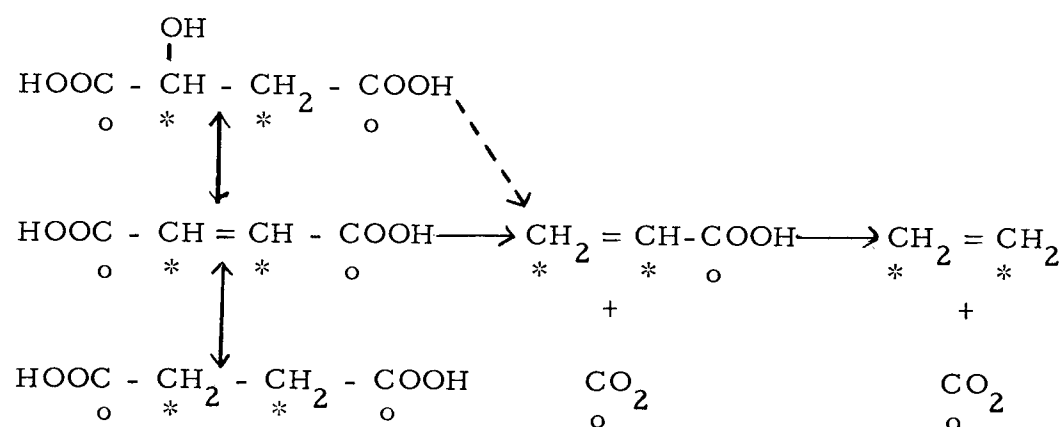\end{array}$$

Figure 5.   The biosynthesis of ethylene in P. digitatum.

SUMMARY

The biosynthesis of ethylene in <u>P</u>. <u>digitatum</u> has been examined by means of radiotracer method. Findings obtained in a series of experiments employing C^{14} specifically labeled substrates such as succinate, fumarate, DL-malate, acetate, β-alanine, acrylate and propionate lead one to believe that acrylate may be the immediate precursor of ethylene. Acrylic acid is thought to be derived from either fumarate or malate, two key intermediates of the Krebs cycle pathway.

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