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Metabolism of Triacetic Acid and Triacetic Acid Lactone*

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ABSTRACT: Triacetic acid lactone (TAL) has been identified as a metabolite of two species of *Penicillium*. [^{14}C]Carboxyl-labeled triacetic acid (TAA) and TAL were prepared.

The *in vivo* conversion of [^{14}C]TAL and [^{14}C]TAA to other acetate metabolites was investigated. Low levels of incorporation of both compounds into 6-methylsalicylic acid (MSA), patulin, and fatty acids were observed. The radioactive MSA was degraded to CO_2 and 2,4,6-tribromo-*m*-cresol. The observed distribution of radioactivity indicated that TAL and TAA both had undergone degradation to C_2 units prior to incorporation rather than being directly utilized as C_6 units. Similar levels of incorporation into fatty acids supported this conclusion. TAL probably arises by cyclization of a thiol ester of TAA. Chromatographic and spectral evidence suggest that TAL is the "275 compound" of Bressler and Wakil [Bressler, R., and Wakil, S. J. (1962), *J. Biol. Chem. 237*, 1441] formed by the avian liver fatty acid synthetase in the absence of reduced nicotinamide–adenine dinucleotide phosphate (NADPH), thus supporting other published evidence that TAL is a by-product of fatty acid synthesis.

An increasing body of evidence indicates that many fungal aromatic metabolites are derived by condensation of acetate and malonate units (Richards and Hendrickson, 1964). Recently, Gatenbeck and Hermodsson (1965) obtained a cell-free system from *Alternaria tenuis* which converted both acetyl-CoA[1] and malonyl-CoA into alternariol. Earlier Bassett and Tanenbaum (1960) reported synthesis of patulin from acetyl-CoA by a cell-free extract from *Penicillium patulum;* in 1962, Tanenbaum and Bassett found that cell-free extracts from *Penicillium stipitatum* incorporated both acetyl-CoA and malonyl-CoA into the tropolone, stipitatic acid. Lynen (1961) reported that incorporation of [^{14}C]acetyl-CoA into MSA by extracts of *Penicillium patulum* required NADPH and malonyl-CoA. Lynen postulated by analogy with fatty acid synthesis that protein-bound polyketo chains were intermediates. At least a 6-carbon polyketo chain can be formed by the fatty acid complex. Brodie *et al.* (1964) identified the product produced by purified pigeon liver fatty acid synthetase in the absence of NADPH as 3,5-dioxohexanoic acid (TAA). Labeling evidence indicated that the product was produced from one acetyl-CoA and two malonyl-CoA units. However, they could not have distinguished TAA from its lactone (TAL) in their paper chromatographic system (Harris *et al.*, 1966). Brock and Bloch (1966) isolated TAL from incubations of the crude *Escherichia coli* fatty acid synthetase containing acyl carrier protein, acetyl-CoA, malonyl-CoA, NADPH, and thiol compounds. They were not certain whether the metabolic product was TAA or TAL, but our experiment described below with [^{14}C]TAA indicates that it is unlikely that TAA would have been converted to TAL under the conditions of their isolation.

Ehrensvärd (1955) observed that TAL stimulated the formation of aromatic compounds in *Penicillium urticae*. Harris *et al.* (1966) showed that small quantities of TAL are produced from [1-^{14}C]acetate by *P. patulum*. Bentley *et al.* (1966) found that TAL, its 6-acetyl derivative (4-hydroxy-6-(2-oxopropyl)-(2H)-pyran-2-one), and orsellinic acid accumulated in cultures of *P. stipitatum* in which the production of tropolones was inhibited by the addition of ethionine. Tanenbaum and coworkers (Brenneisen *et al.*, 1964; Acker *et al.*, 1966) isolated the methyl derivative of TAL, 3,6-dimethyl-4-hydroxy-(2H)-pyran-2-one, from another strain of the

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[1] Abbreviations: MSA, 6-methylsalicylic acid (2,6-cresotic acid; 2-hydroxy-6-methylbenzoic acid); tlc, thin layer chromatography; TAL, triacetic acid lactone (4-hydroxy-6-methyl-(2H)-pyran-2-one); TAA, triacetic acid (3,5-dioxohexanoic acid); CoA, coenzyme A; NADPH, reduced nicotinamide–adenine dinucleotide phosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

same organism. These observations implicated TAL in the biosynthesis of the acetate-derived fungal aromatic metabolites. This paper reports the chemical syntheses of carboxyl-labeled [^{14}C]TAL and [^{14}C]TAA and their incorporation into several fungal metabolites including MSA. The results indicate that it is unlikely that either compound is an obligatory intermediate between acetate and MSA.

Experimental Section

Materials. Sodium [1-^{14}C]acetate was obtained from New England Nuclear Corp. and from Volk Radiochemical Co. Barium [^{14}C]carbonate was obtained from Volk Radiochemical Co. Boron trifluoride–methanol reagent was obtained from Applied Science Laboratories. Diazomethane was generated from bis(*N*-methyl-*N*-nitroso)terephthalamide (EXR-101, E. I. duPont de Nemours and Co.) (Moore and Reed, 1961). PPO, POPOP, and Hyamine hydroxide (in methanol solution) were obtained from the Packard Instrument Co. Nonradioactive TAA and TAL were prepared by the method of Harris and Harris (1966). TAL was also purchased from Aldrich Chemical Co. and recrystallized from water before use. MSA was synthesized by the method of Eliel *et al.* (1953). Solvents for column chromatography and recrystallization were distilled before use.

For silicic acid column chromatography, Mallinckrodt 100 mesh silicic acid was washed with water, methanol, and acetone and air dried for 24 hr. Material of 100–270 mesh size was collected by screening, dried at 100°, and stored at room temperature in capped bottles. Tlc plates were prepared from silica gel with 10% $CaSO_4$ binder (Adsorbosil-1 from Applied Science Laboratories, State College, Pa.).

Microbiological Experiments

Culture Techniques. Penicillium patulum, NRRL strain 2159A, was maintained on malt agar (20 g of malt extract, 20 g of glucose, 1 g of peptone, 20 g of agar, and 1 l. of H_2O) or Czapek-Dox agar slants (50 g of glucose, 2 g of $NaNO_3$, 1 g of KH_2PO_4, 0.5 g of KCl, 0.37 g of 65% $MgSO_4$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 15 g of agar, and 1 l. of H_2O). For surface cultures, spores from slants were used to inoculate 50-ml erlenmeyer flasks containing 20 ml of Czapek-Dox medium (same as above without agar). For submerged cultures, spores were used to inoculate 100 ml of a starch–peptone medium (20 g of peptone, 10 g of malt extract, 40 g of glucose, 20 g of soluble starch, 3 g of $NaNO_3$, 1 g of KH_2PO_4, 0.5 g of 65% $MgSO_4$, 0.5 g of KCl, 0.02 g of $FeSO_4 \cdot 7H_2O$, and 1 l. of H_2O), and after growing for 24 hr on a rotatory shaker, the mycelium was collected by filtration, washed, and transferred to 100 ml of nitrate-free Czapek-Dox medium ($NaNO_3$ omitted from the previously described Czapek-Dox medium). *Penicillium griseofulvum*, ATCC 11885 white variant described by Bayan *et al.* (1962), was maintained on malt agar slants and grown as a submerged culture as described above.

Isolation of Secondary Metabolites. The isolation and purification of [^{14}C]TAL formed from [1-^{14}C]-acetate by submerged cultures of *P. patulum* was previously described (Harris *et al.*, 1966).[2] For the isolation of TAL from *P. griseofulvum*, 9 g of wet mycelium was suspended in 100 ml of nitrate-free Czapek-Dox medium and incubated for 17 hr at room temperature. Sodium [1-^{14}C]acetate (40 μc) was then added. After 11 hr the mycelium was removed by filtration and washed with water. The combined filtrate was extracted continuously overnight with ether. Nonradioactive TAL (154 mg) was added before extraction. The ether extract was evaporated on a steam bath and the residue was washed two times with 8 ml of benzene. The residue was dissolved in 12 ml of acetone and applied to a column containing 50 g of silicic acid. Before draining the sample onto the column 10 ml of hexane was added. The column was eluted with 200 ml of ether–hexane (1:1, v/v), 200 ml of ether–hexane (8:2, v/v), and finally with ether. Fractions of 20 ml were collected. Column elution was monitored with tlc (developing solvent, ether–hexane–acetic acid, 80:20:2, v/v/v). Tubes 1–23 were pooled as fraction A (68 mg of TAL) and tubes 24–57 as fraction B (74 mg of TAL). Each fraction was dissolved in 5 ml of acetone and 0.2-ml aliquots were removed for counting. The acetone was removed under a stream of nitrogen and each fraction was recrystallized three times from ethyl acetate and three times from water.

Two control experiments showed that TAL was not a trace contaminant in the [1-^{14}C]acetate and that the TAL was not produced by cyclization during the isolation procedure. An aqueous solution of nonradioactive TAL and sodium [1-^{14}C]acetate was continuously extracted with ether. The ether was evaporated and the radioactivity was removed from the residue by only two recrystallizations from ethyl acetate. In the second experiment 2 mg of [^{14}C]TAA was added to an aqueous solution of 100 mg of TAL. The solution was brought to pH 2.3 with sulfuric acid and continuously extracted with ether. After recrystallization of the lactone twice from ethyl acetate and twice from water, 1.2% of the initial TAA radioactivity remained associated with the TAL and after further purification by chromatography on silicic acid only 0.96% remained. This result is consistent with the conclusion of Witter and Stotz (1948a) that spontaneous lactonization of TAA does not occur.

MSA was isolated from acidified culture media by extraction with four portions of ether. When patulin and MSA were isolated together, the acidic culture medium was extracted continuously with ether for 12–24 hr. The ether was evaporated and warm benzene

[2] Confirmatory evidence for the formation of TAL by *P. patulum* has now been obtained by radioautography. Carrier TAL (2 mg) was added to the ^{14}C-labeled metabolites and the appropriate fraction isolated by column chromatography. Tlc in two solvent systems followed by radioautography of the tlc plates revealed a radioactive spot with an R_F corresponding to that of authentic TAL.

extracts of the residue were transferred to a column of 5 g of silicic acid. The column was eluted with 60 ml of benzene and 100 ml of hexane–ether (9:1, v/v). Fractions (20 ml) were collected. MSA was located by tlc of aliquots of each fraction (developing solvent: hexane–ether–acetic acid, 80:15:2, v/v/v) and usually appeared in the last benzene and first two hexane–ether fractions. MSA was recrystallized from chloroform. When patulin isolation was also required, the original benzene-extracted residue was dissolved in a few milliliters of ether, placed on the same column bed, and diluted with four volumes of hexane. The column was eluted with 40 ml of hexane–ether (4:1, v/v) followed by hexane–ether (7:3, v/v). Patulin elution was monitored by tlc (developing solvent: hexane–ether, 1:3, v/v); patulin appeared in a slightly tailing peak between 100 ml and 400 ml of eluent.

Isolation of Fatty Acids. Fatty acids were isolated from the filtered *P. patulum* mycelium by saponification of the mycelial pad with 50% aqueous methanol containing 5% KOH for 2 hr at 60° under nitrogen. The hydrolysate was acidified and extracted with ether. The fatty acids were converted to their methyl esters with diazomethane (Schlenk and Gellerman, 1960) or with boron trifluoride–methanol (Metcalfe and Schmitz, 1961). The esters were dissolved in hexane and transferred to a column of 10 g of silicic acid. After elution with 50 ml of hexane, the esters were eluted with 100 ml of hexane–ether (19:1, v/v). The presence of fatty acid esters in this fraction was verified by gas–liquid partition chromatography; the fatty acid content was very similar to that reported for *P. griseofulvum* (Light, 1965).

Chemical Experiments

Preparation of [2-^{14}C]-4-*Hydroxy-6-methyl-(2H)-pyran 2-one* ([^{14}C]*TAL*). The procedure is an adaptation of the method of Harris and Harris (1966) for preparation of diketo acids and the corresponding pyranones (lactones). The preparation was carried out in an efficient hood. The reaction flask (three neck) was fitted with a rubber septum and an air condenser topped with a stopcock and vacuum connection. The contents of the flask were stirred magnetically with an uncoated iron bar. The CO_2-generating flask (three neck) was fitted with an addition funnel and a stopcock for vacuum connection. This flask was stirred magnetically with a Teflon-coated bar. The flasks were connected by a glass tube containing a stopcock. The apparatus was carefully checked for leaks before each reaction. The reaction flask was closed off from the CO_2-generating flask but open through the air condenser to the atmosphere. Into this flask were introduced 70 ml of commercial, anhydrous, liquid ammonia, 246 mg (0.0106 g-atom) of sodium, and 2–3 mg of hydrated ferric nitrate. After conversion of the sodium to a grey suspension of sodium amide,[3] 500 mg (0.005 mole) of acetylacetone in 5 ml of anhydrous ether was introduced through a septum. After 50 min, the ammonia was carefully evaporated by means of a water bath. Residual ammonia was removed *in vacuo* (<1 mm) to leave disodioacetylacetone as a free-flowing powder. The reaction flask was then cooled in an ice bath and sealed under vacuum by closing the stopcock on the condenser.

In the CO_2-generating flask was placed 985 mg (0.005 mole, *ca.* 50 μc) of $Ba^{14}CO_3$. The flask was evacuated and sealed. The flask was cooled with an ice bath as small portions of sulfuric acid were added to the barium carbonate. After each addition the dust was allowed to settle before the stopcock between the flasks was opened to establish pressure equilibrium. After 25 ml of acid had been added the resulting barium sulfate was completely in solution. Finally the stopcock between the flasks was opened permanently. After the powdery reaction mixture had been stirred 2 hr, 15 ml of anhydrous ether was added to the reaction flask and the stirring was continued an additional 3 hr. The system was then opened. The reaction flask was cooled in an ice bath and 10 ml of 6 M HCl was added. The layers were separated and the aqueous layer was extracted seven times with ether. The combined ether extract (*ca.* 100 ml) was dried over magnesium sulfate, filtered, and evaporated. Residual acetylacetone was removed at 0.1 mm and 24° for 1 hr to leave 270 mg (38%) of relatively pure TAA. The product was a liquid which slowly crystallized in the freezer.

TAL was prepared from TAA by cyclization with HF as described by Harris and Harris (1966). A 63% yield (149 mg) of [2-^{14}C]-4-hydroxy-6-methyl-(2H)-pyran-2-one-([^{14}C]TAL), mp 185–187°, was obtained. To assure radiochemical purity, 122 mg of [^{14}C]TAL was transferred in 5 ml of acetone to the top of a column of 50 g of silicic acid (100–175 mesh), which was packed in hexane. Hexane (50 ml) was mixed with the acetone before draining the sample onto the column. The column was eluted with 150 ml of hexane, 200 ml of ether–hexane (1:4, v/v), 200 ml of ether–hexane (1:1, v/v), 500 ml of ether–hexane (4:1, v/v), and finally ether. The ether was collected in 12-ml fractions. Radioactivity appeared in a single, severely tailing peak extending from tube 27 beyond tube 110. The maximum activity was at approximately tube 42. Tubes 30–95 were combined and evaporated to yield 97.9 mg of TAL. Tlc (developing solvent: ether–hexane–acetic acid, 80:20:2, v/v/v), revealed one major spot corresponding to TAL and a minor spot of higher R_F. Scintillation counting of the silicic acid from the tlc plate indicated that the minor spot was nonradioactive and that the TAL spot was the only significantly radioactive spot present. This result was confirmed by radioautography of an identical tlc plate. The specific activity of the product was 74,000 cpm/mg (125,000 dpm/mg).

In a similar experiment conducted with 255 mg (0.011 g-atom) of sodium, 550 mg (0.0055 mole) of acetylacetone, and 1.082 g of $Ba^{14}CO_3$ (0.0055 mole,

[3] For similar procedures and for safety precautions to be observed in the preparation and handling of sodium amide see Hauser *et al.* (1954).

500 μc), there was obtained 175 mg (25% over-all yield) of the lactone. Chromatography afforded 93 mg of purified material. [^{14}C]TAA, which was isolated as above and crystallized in the freezer, was used without further purification.

Degradation of [^{14}*C*]*MSA*. A modification of the method of Light (1965) was employed for the degradation of MSA. MSA (50 mg) and 20 ml of water were placed in a 50-ml erlenmeyer flask. The flask, stirred magnetically, was closed with a syringe cap and flushed with nitrogen by means of two hypodermic needles. The nitrogen was introduced below the surface of the solution. Aqueous 1 M KOH (1.3 ml) was added through the cap with a syringe. The effluent nitrogen stream was bubbled through 3 ml of 1 M Hyamine in methanol as bromine water (9.7 ml containing 0.16 g of Br_2 and 1.5 g of KBr) was added to the MSA solution. After 15 min, 0.65 ml of 0.5 M sulfuric acid was added to the reaction flask by syringe and the nitrogen sweeping was continued for 1 hr in order to collect the carbon dioxide formed. Sweeping was continued for an additional period with a fresh Hyamine solution but negligible additional carbon dioxide was collected. The Hyamine solutions were diluted to 50 ml with toluene scintillator solution. Duplicate 15-ml aliquots were counted. The aqueous suspension of tribromo-*m*-cresol was extracted with ether. The ethereal solution was evaporated, and the residue was dissolved in benzene, transferred to a column of silicic acid (10 g), and eluted with benzene. Appropriate fractions, as determined by tlc, were combined to give a 68–73% yield of 2,4,6-tribromo-*m*-cresol. The material was sublimed at *ca.* 80° and 0.5 mm before its specific activity was determined. The purity of the material was confirmed by redetermination of its specific activity after recrystallization from hexane. Internal standardization and channel ratios of the MSA, tribromocresol, and Hyamine solutions indicated that only the Hyamine solutions were significantly quenched, and corrections were made for this quenching with internal standards.

Radioactivity Measurements

Assay of radioactivity was made with a Packard Tri-Carb Model 3214 liquid scintillation counter using one of the following scintillator solutions. The toluene solution contained 4 g of PPO and 50 mg of POPOP/l. of toluene. The dioxane solution contained 7 g of PPO, 300 mg of POPOP, and 100 g of naphthalene/l. of dioxane. [^{14}C]TAL and [^{14}C]TAA were assayed in the dioxane solution at an efficiency of 59%. All other materials were assayed in the toluene solution at an efficiency of 70%. Quenching was checked with an internal standard.

Results

Incorporation of Sodium [^{14}*C*]*Acetate into TAL by P. griseofulvum.* As can be seen in Table I the specific activity of the TAL fractions purified by chromatography decreased rapidly upon recrystallization with both fractions attaining essentially the same activity. This represents a conversion of [1-^{14}C]acetate to TAL of 0.003% which is somewhat lower than the 0.016% conversion found with *P. patulum* (Harris *et al.*, 1966).

TABLE I: Recrystallization of TAL Isolated from *P. griseofulvum.*

	Solvent	Specific Activity (cpm/mg)	
		Fraction A	Fraction B
0		121	39
1	ethyl acetate	26	17
2	ethyl acetate	18	13
3	ethyl acetate	19	13
4	water	16	10
5	water	14	12
6	water	13	11
7		—	12

Incorporation of TAA and TAL into Fungal Metabolites. The incorporation of [^{14}C]carboxyl-labeled TAA and TAL into MSA by surface cultures of *P. patulum* is shown in Table II. The incorporation was low and similar results were obtained with other cultures and other incorporation times. With identical cultures little variation in MSA activity could be detected over a range of 1–72 hr. Incorporation into patulin was also low and no trend with time was observed. It should be noted that incorporation of [^{14}C]TAL and [^{14}C]TAA into fatty acids is of the same order of magnitude.[4]

TABLE II: Incorporation of TAA and TAL into MSA, Patulin, and Fatty Acids by *P. patulum.*[a]

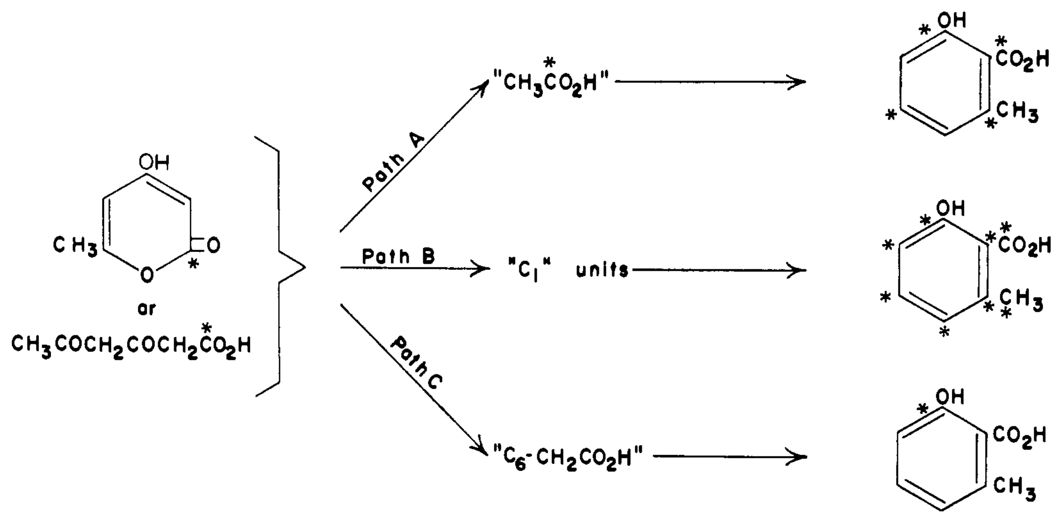
Precursor	Time (hr)	Per Cent Incorporation: MSA	Patulin	Fatty Acids
[^{14}C]TAA	40	0.08	0.66	1.1
[^{14}C]TAL	24	0.30	1.1	0.22

[a] Isotopic material (2 mg/culture of TAA, 472,000 cpm/mg, and of TAL, 1,000,000 cpm/mg) was added to 3-day surface cultures (20 ml) of *P. patulum* in which the absorbance at 276 mμ had reached *ca.* 80 (A = 0.8 in a 1:100 dilution). In each experiment eight cultures were pooled before isolation of metabolites.

[4] A preliminary study with *P. baarnense* indicated that [^{14}C]-TAL underwent conversion into orsellinic acid to the extent of 0.8% in surface cultures and 0.04% in submerged cultures.

TABLE III: Degradation of MSA Derived from [1-^{14}C]Acetate, [^{14}C]TAA, and [^{14}C]TAL.

Precursor	MSA, Sp Act. (cpm/μmole)	2,4,6-Tribromo-*m*-cresol Yield (%)	Specific Act. (cpm/μmole) Calcd Path A	Calcd Path B	Calcd Path C	Found	Recovery of ^{14}C in CO_2 % of Total Calcd (%) Path A	Calcd (%) Path B	Calcd (%) Path C	Found (%)
[1-^{14}C]Acetate	35.3	73	26.5	30.9	—	26.4	25.0	12.5	—	18.4
[^{14}C]TAA	8.1	76	6.1	7.1	8.1	6.2	25.0	12.5	0	19.0
[^{14}C]TAL	64.7	68	48.5	56.6	64.7	49.0	25.0	12.5	0	17.5



SCHEME I

Degradation of MSA Derived from [1-^{14}C]Acetate, [^{14}C]TAA, and [^{14}C]TAL. [^{14}C]MSA, which had been collected from still cultures (see above) of *P. patulum* to which [^{14}C]TAA or [^{14}C]TAL had been added, was degraded to determine the isotopic distribution pattern. For comparison a sample of MSA produced from [1-^{14}C]acetate by submerged cultures of *P. patulum* was obtained from a previously described experiment in which the incorporation of [1-^{14}C]acetate into TAL was being studied (Harris *et al.*, 1966). After chromatographic purification of the isolated [^{14}C]MSA, carrier MSA was added and the samples were recrystallized to constant specific activity from chloroform. The [^{14}C]MSA (from [^{14}C]TAA, [^{14}C]TAL, and [1-^{14}C]-acetate) was degraded to 2,4,6-tribromo-*m*-cresol and CO_2 to determine the isotopic distribution. The results are shown in Table III. It can be seen that the results of the experiment with [1-^{14}C]acetate are fully consistent with earlier studies of acetate labeling in this compound (Birch *et al.*, 1955). The low recovery (74% of theory) of carbon dioxide is believed to reflect either inefficient collection of carbon dioxide or incomplete reaction of the MSA. The second of these possibilities is supported by the yield (73% of theory) obtained of 2,4,6-tribromo-*m*-cresol. Three modes of labeling of MSA by [^{14}C]TAA and [^{14}C]TAL should be considered.[5] These are shown in Scheme I.

Degradation of TAA or TAL to acetate or other C_2 units before entering the pathway would afford a 25:75 distribution of label between the carboxyl group and the rest of the molecule (path A). Complete randomization would distribute the labeling 12.5:87.5 (path B).[6] On the other hand, direct introduction of TAA or TAL into the pathway by acylation of an additional C_2 unit would leave all radioactivity at position 2 in the aromatic ring (path C). The results are in good agreement only with path A. The specific activities of 2,4,6-tribromo-*m*-cresol were 75% those of the [^{14}C]MSA. Either of the other paths of incor-

[5] A fourth route in which TAA or TAL is converted to a C_8 compound by addition of an acetyl group to the terminal methyl position is unlikely from theoretical considerations. Moreover, this route is ruled out by the observed labeling pattern since it predicts that all ^{14}C would be incorporated at the carboxyl position.

[6] TAA undergoes decarboxylation quite readily. However, the possibility seems remote that $^{14}CO_2$ would eventually be converted to acetate metabolites. Rodig *et al.* (1966) found no incorporation of $^{14}CO_2$ into citrinin in cultures of *Penicillium citrinum*.

poration would have afforded cresol of higher relative specific activity than was obtained. In addition, more radioactivity was contained in the carbon dioxide than could be accounted for by either path B or C.

Discussion

The biosynthesis of fatty acids involves condensation between protein-bound malonyl and fatty acyl thiol esters to form 3-oxoacyl thiol esters, followed by reduction, dehydration, and further reduction to produce the next higher fatty acyl thiol ester (Vagelos, 1964). If a similar acetylpolymalonyl pathway exists for the other "polyketide" metabolites such as the fungal phenolic acids, there must be a similar condensation between a malonyl thiol ester and 3-oxoacyl or higher polyketoacyl thiol esters. Such a condensation does appear to occur slowly with the pigeon liver fatty acid synthetase when NADPH is deleted from the incubation mixture (Brodie *et al.*, 1964), and with the *E. coli* fatty acid synthetase in the presence of thiol compounds (Brock and Bloch, 1966). Brodie *et al.* (1964) identified the pigeon liver enzyme product as TAA by paper chromatography, ultraviolet spectrum, and acetate–malonate labeling. However, it has been noted that the evidence presented would describe TAL equally well since the R_F values of TAA and TAL in their solvent systems are very nearly identical. The compound of Brodie *et al.* (1964) is probably the one described by Bressler and Wakil (1962) which had an R_F of 0.35–0.4 in the butanol–ammonia system of Reid and Lederer (1951). In this solvent system we find that TAA has an R_F of 0.11 and TAL an R_F of 0.30–0.36. Thus it appears that the enzymatic product might actually be TAL, although we did not have a sample of it for direct comparison. Brock and Bloch (1966) identified the *E. coli* enzyme product as TAL but suggested that the product may have been TAA which cyclized in the work-up. Our experience indicates that TAA should not have cyclized under their isolation conditions, and so TAL was probably the direct enzymatic product.

The report by Ehrensvärd (1955) that TAL stimulated the production of aromatic compounds in *Penicillium urticae* raised the possibility that TAL might participate as an intermediate in the biosynthesis of MSA. Bu'Lock *et al.* (1965) showed that a change in cellular metabolism of *P. urticae* accompanies the formation of MSA which appears late in the growth of the fungus. One aspect of this metabolic shift involved the switch in glucose metabolism from predominantly phosphogluconate pathway to predominantly glycolysis, a situation that could lead to decreased availability of NADPH. It was attractive to speculate that TAL might be formed by the fatty acid synthetase under these conditions and then metabolized to MSA and other products. [^{14}C]TAL was prepared in order to test this hypothesis.

The levels of incorporation of both [^{14}C]TAL and [^{14}C]TAA into MSA were low and did not vary significantly over the period from 1 to 72 hr. In contrast, the incorporation of [1-^{14}C]acetate into MSA by surface cultures of *P. patulum* increases steadily during the first 10–20 hr, reaching a value of 10% in some cases, and falls to low values between 20 and 50 hours.[7] Incorporation into patulin increases considerably after thirty hours, about the time that radioactivity is disappearing from the MSA.[7] If free TAL or TAA were obligatory intermediates in the pathway of conversion of acetate to MSA, there should be more extensive incorporation of these compounds a few hours after addition than we found. Furthermore the level of incorporation into fatty acids was similar to that for MSA and patulin suggesting a prior degradation to some common precursor of all three compounds. Chemical degradation of the MSA formed both from [^{14}C]TAA and from [^{14}C]TAL substantiated the conclusion that direct incorporation had not occurred. Incorporation of TAL into tropolones by *P. stipitatum* has been studied by Bentley and also appears to take place by degradation and resynthesis (R. Bentley, private communication).

The relationship of TAL formation to aromatic biosynthesis remains unclear. Since TAL appears to be formed by the fatty acid synthetase, it could probably also be a side product of the condensing enzymes of aromatic polyketide biosynthesis. In fact, the possibility that the condensing enzyme leading to MSA might be the same as the enzyme for fatty acid synthesis has not yet been ruled out. Chemical evidence supports the hypothesis that TAL is formed by cyclization of the thiol ester of TAA. Although TAA has been shown not to cyclize significantly in weakly acidic aqueous solutions (see Experimental Procedure), the corresponding thiol acid has been found to be too unstable to be isolated. In neutral or weakly acidic solutions it spontaneously cyclizes to TAL (T. M. Harris and C. M. Harris, unpublished observation). The CoA or other activated ester of TAA would be expected to behave similarly. Consequently, either the rate of synthesis of metabolites *via* polyketo thiol ester intermediates must be fast relative to cyclization or the enzymes involved must provide some stabilization against cyclization.

The inability of TAL to participate directly in MSA formation may reflect insufficient reversibility of the cyclization. Although the thiolate anion is a powerful nucleophile, equilibrium may strongly favor the lactone because of resonance stabilization of its structure. We have found little evidence of cleavage of TAL with several thiols under a variety of conditions.

The question remains of the mode of biological degradation of TAL and TAA. Witter and Stotz (1948b) found that both TAA and TAL were degraded to acetoacetate and acetate by rat liver enzyme systems. Connors and Stotz (1949) were able to achieve partial purification of the TAA cleavage enzyme from beef liver and found it to be inactive toward TAL. Meister (1949) also reported this enzymatic activity in rat liver and kidney and described an enzyme system which cleaved TAL. He presented strong evidence that TAA

[7] Studies of the incorporation of [1-^{14}C]acetate into MSA and patulin by surface cultures of *P. patulum* were carried out by Miss Sharon Arihood and are described in part elsewhere (Arihood and Light, 1966).

was the product. It is possible that similar reactions are responsible for the degradation of TAA and TAL in *P. patulum*.

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