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III. THE BIOGENESIS OF ERYTHRONOLIDE, THE C_{21} BRANCHED CHAIN LACTONE IN ERYTHROMYCIN*

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Erythronolide, the macrocyclic lactone which is the aglycon of the complex antibiotic erythromycin (Fig. 1), represents a perfect example of a branched chain lipid entirely made up of repeating 3 carbon subunits (2). Although, quantitatively, branched chain fatty acids are not important lipid constituents, they have been detected in varying amounts in a wide variety of natural products. Methymycin (3), neomethymycin (4), oleandomycin (5), pikromycin (6, 7), and other antibiotics of the macrolide group contain aglycons which are partially branched lactones similar to erythronolide. Mycolipenic and mycoceranic acids, present in large amounts in the lipids of tubercule bacilli (8, 9), are partially branched fatty acids again with the same type of repeating 3 carbon subunit. Other substances which may be considered to have at least one 3 carbon subunit are odd numbered straight chain fatty acids and a wide variety of fatty acids and other substances found to have one or more C-methyl group (*cf.* 10).

Robinson has proposed that 3 carbon subunits like those in mycolipenic and mycoceranic acids could arise from propionate or its "biological equivalent" (11). Evidence exists which indicates that this concept may be true for some of the simpler fatty acids which contain 3 carbon units. For instance, *n*-valeric acid formed in the sheep rumen (12) or by *Clostridium kluyveri* (13) seems to be derived from propionate and acetate as does the β-hydroxyvaleric acid produced by mouse liver slices (14). The perfused cow udder also incorporates propionate-1-C^{14} extensively into the odd numbered and branched fatty acids of the milk fats (15). Of more relevance to the 3 carbon units in erythronolide is the recent observation that α-methylbutyric acid, formed by isolated muscle of *Ascaris lumbricoides*, is made from acetate and propionate with the branch methyl group arising from C-3 of the propionate and the carboxyl group coming from C-1 (16).

Following on the suggestion of Robinson, both Gerzon *et al.* (17) and Woodward (18) have proposed, from the structure of erythronolide, that the repeating 3 carbon subunits in this C_{21} lactone might arise biogenetically from propionate. The perfect regularity of the 3 carbon units in this lactone led Woodward further to propose a *propionate rule* to the effect that similar structures might be predicted to come biologically from the condensation of propionate units, and that this expectation might be used to guide future structural analysis.

A hypothesis differing from that of Gerzon and Woodward has been expressed by Birch *et al.* (19). These authors have stated that the formation of branched 3 carbon polymers such as are found in erythromycin and methymycin may be merely a particular example resulting from the methylation of a preformed acetate polymer. Such methylations are well established (*e.g.* 19–21), at least for the introduction of isolated methyl groups, and the source of the C_1 group could be the methionine—*S*—methyl group, betaine, choline, or some C_1 derivative of tetrahydrofolic acid.

In this communication, which is the result of an investigation to determine the biogenetic origin of the 3 carbon subunits in erythronolide, data are presented which show that the 3 carbon units are derived from intact propionate molecules and that methylation does not play a direct role in their formation.

EXPERIMENTAL PROCEDURE

Materials and Equipment—The culture of *Streptomyces erythreus* (C233.1-Ky-3) and of *Staphylococcus aureus* (Heatley strain, ATCC 9144), as well as the erythromycin used as carrier for isotopic experiments, were generous gifts of Eli Lilly and Company. Radioisotopes were commercial products purchased from the Volk Radiochemical Corporation, and the New England Nuclear Corporation. The propionate-C^{14} samples were used without purification in experiments in which low concentrations at a high specific activity were employed. In addition, similar experiments were carried out with propionate-C^{14} which had been diluted and purified according to the method of Bueding and Yale (22). All of the propionate-C^{14} samples were found to be labeled singly as described, but the propionate-2- and -3-C^{14} samples were contaminated by small amounts of radioactive acetate. Microbiological media, medium components, and fluids were supplied by Difco Laboratories, unless otherwise specified. Tween 80 was a product of the Atlas Powder Company. Versene is the registered trademark for ethylenediaminetetraacetic acid disodium salt. Amberlite resins were supplied by Rohm and Haas, and Dowex resins by the Dow Chemical Company. Melting points were determined on Anschuetz thermometers in a Hershberg apparatus. Infrared analyses were carried out on solutions (0.01 M) in dichloromethane or tetrachloroethylene with a 1-mm cell and a Beckman model IR2a spectrophotometer. Radioactivity measurements were made either on barium carbonate samples and

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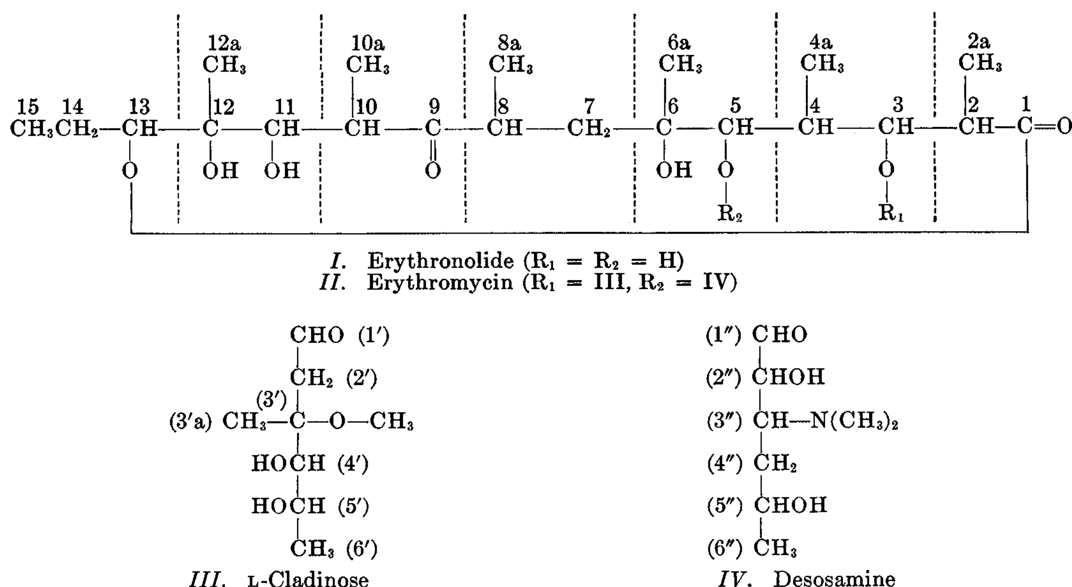

FIG. 1. Structure of erythronolide and of erythromycin

corrected to infinite thinness, or on gaseous CO_2 samples (23). The vegetative growth of *S. erythreus* was carried out on a rotary shaker (300 r.p.m., 1-inch turn radius), and short term incubations were performed with a rotary water bath shaker (200 r.p.m., ½-inch turn radius).

Streptomyces erythreus—An evacuated tube containing a lyophilized culture of *S. erythreus* (Eli Lilly C223.1-Ky-3) was opened, suspended in 5 ml of Difco nutrient broth containing Tween 80 (1%, volume for volume) and used to inoculate (loop) 25 to 50 slant tubes of medium M1-106 (precooked baby oatmeal, 20 g; tomato paste, 20 g; agar, 15 g; and tap water, 1 liter; final pH adjusted to 7.0 to 7.2 with KOH). After incubation in the dark at high relative humidity for 10 days at 30–32°, the resulting slant cultures were suitable for preservation by lyophilization (after suspension in beef serum), for inoculation of liquid media, or for the preparation of second generation slant cultures. The latter, prepared by dry loop inoculation (six slant tubes from one first generation slant culture) followed by incubation as described above, are somewhat more uniform in their behavior than are the first generation cultures. However, if the potency of the first generation culture is low, the second generation culture may be unsuitable for the short term incubations to be described below. The slant and lyophilized cultures are stored at 5° in the dark until used. The slants remain active for several months, whereas the lyophilized preparations remain potent for several years.

Erythromycin Assay and Characterization—A modification of the turbidometric assay for erythromycin reported by Higgens *et al.* (24), with *S. aureus* (Heatley strain) as the test organism, was used throughout this work. The details of the modification will be reported elsewhere. The nature of the antibiotic produced was established by chromatography of a chloroform extract of the fermentation broth (pH 9.8) and bioautography of the developed chromatogram with *Bacillis subtilis* (ATCC 6633) as the test organism (25). The antibiotic substance present in the extract was chiefly erythromycin A plus (possibly) a little erythromycin B (26). No erythromycin C could be detected (25). Essentially all of the antibiotic appears in the mycelial supernatant solution, since neither sonic oscillation nor boiling of the suspensions before centrifugation increased the assay value.

Preparation of Washed Mycelium of S. erythreus—A slant culture of *S. erythreus* was suspended in 5 ml of Difco nutrient broth containing Tween 80 (1%, volume for volume) and 1 ml aliquots of the suspension were added to 500-ml Erlenmeyer flasks with wide mouths, each containing 75 ml of medium M1-102 (glucose, 5 g; commercial brown sucrose, 10 g; tryptone, 5 g; yeast extract, 2.5 g; Versene, 36 mg; tap water, 1000 ml; final pH adjusted to 7.0 to 7.2 with KOH). The flasks were closed with two milk filter disks secured with rubber bands and were incubated at 30° on a rotary shaker. After 2.5 to 3 days of growth, the mycelial volume was 12 to 16% (packed volume after centrifugation for 15 minutes at 1300 × *g*), and the culture was suitable for inoculation of fermentative media or for inoculation of fresh vegetative media (medium M1-102) to prepare large amounts of the mycelium. In the latter case, 20 ml of the culture were used to inoculate 400 ml of medium M1-102 contained in a 2-liter Erlenmeyer flask. After 20 hours of growth, under the same conditions described for the inoculum, the resulting mycelium was collected by centrifugation at 2500 × *g* for 30 minutes and washed twice by suspension in potassium phosphate buffer (0.01 M, pH 7.0) containing Versene (10^{-4} M) followed by recentrifugation. Although the centrifugations were carried out at room temperature, chilled phosphate buffer (5°) was used and the mycelium was kept in an ice bath during the resuspension procedures. The mycelium obtained by this procedure was light brown in color and highly viscous. The preparations which were found to synthesize erythromycin most actively possessed a water-soluble substance that turned light green under anaerobic conditions. Shaking of the mycelium in air caused the pigment to be decolorized. In some cultures, which were of low potency, the addition of betaine (0.5%, weight for weight) and potassium propionate (10^{-3} M) to medium M1-102 caused both the appearance of the green pigment and an increase in the erythromycin formed. The addition of Versene was found to delay or prevent fragmentation (lysis) of the or-

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ganism which was otherwise often observed in the case of long term mycelial growth. Consequently, it was routinely added to all media and washing fluids.

Erythromycin Production by Growing Mycelium of S. erythreus—One hundred milliliters of the Corum medium (27) in a 500-ml Erlenmeyer flask with a wide mouth were inoculated (5%, volume for volume) with a vegetative culture of *S. erythreus* prepared as described above. The resulting suspension was incubated for 7 days at 30° on a rotary shaker. In experiments with radioactive isotopes, sterile solutions of the labeled compound were added at 12-hour intervals starting with the time of inoculation. In a control experiment, several parameters of the fermentation (mycelial weight, pH, utilization of carbohydrate and amino acids, erythromycin production) were measured as a function of time. The results were essentially as reported by Corum *et al.* (27), except that erythromycin production continued in a linear fashion after the third day and mycelial dry weight did not fall after reaching a maximum. This difference was possibly because of the presence of Versene in the medium.

Erythromycin Production during Short Term Incubations of Washed Mycelium of S. erythreus—The packed washed mycelium of *S. erythreus* was diluted with phosphate buffer (0.01 M, pH 7.0)-Versene (10^{-4} M) to a concentration of 100% based on the apparent volume as determined by slow speed centrifugation (1300 $\times$ *g* for 15 minutes). Aliquots of this suspension (2.5 ml) were added to 25-ml Erlenmeyer flasks, each containing glucose (0.3 ml of a 2.5% solution), tryptone (0.3 ml of a 2.5% solution), and inorganic salts (0.5 ml of a solution of K_2HPO_4, 5 g; $NH_4 \cdot NO_3$, 75 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; $ZnSO_4 \cdot 7H_2O$, 0.44 g; $FeSO_4 \cdot 7H_2O$, 0.13 g; in 1 liter of H_2O). In isotope experiments, the C^{14} compound was added in a small volume of water. The final volume of each flask was adjusted to 5.0 ml with water, and the flasks were incubated at 30° on a rotary water bath shaker.

Erythromycin Isolation—After removal of the mycelium from an incubation of *S. erythreus*, carrier erythromycin was added, and the pH of the broth was adjusted to 9.8. The solution was then extracted 5 times with a 2-fold excess (volume for volume) of chloroform. The chloroform extracts were combined, washed once with a small volume of water, and concentrated to dryness. The residue contained erythromycin sufficiently pure for the formation of derivatives. This method of isolation was used after short term incubations.

An alternate means of isolation and purification of erythromycin was by means of adsorption onto an ion exchange resin and elution at constant pH of 7.0 to 7.2 (erythromycin is unstable if kept for long periods above pH 7.5 to 8.0 and it rapidly decomposes to an inactive form below pH 6.5). Amberlite CG-50 (H) Type I resin was made into a slurry with 50% pyridine, washed several times with water, and used to prepare a column (2 cm diameter $\times$ 10 cm height). The column was washed with water until the effluent had a pH (glass electrode) of 7.0 to 7.2 at which time the filtered fermentation broth (pH 7.0 to 7.2) was passed through the column (1 ml per minute). The column was washed with 200 ml of H_2O (pH adjusted to 7.0 to 7.2 with pyridine) and the basic substances adsorbed were then eluted with solutions of 1 to 6 M pyridine-H_2O adjusted to an apparent pH of 7.0 to 7.2 with glacial acetic acid. The erythromycin fraction was eluted with solutions which were 2 to 4 M in pyridine. The erythromycin was detected by bioassay or by a sulfuric acid spot test (28). The tubes containing erythromycin were pooled, diluted with water, and extracted with chloroform at pH 9.8. The washed chloroform extract was concentrated and dried in a vacuum to remove pyridine. An alternate method for recovery was to pass the eluate through a large excess of a weak basic cation exchange resin such as Amberlite CG-45 and then to remove water and pyridine by vacuum distillation. The erythromycin recovered from the chromatographic purification had an infrared absorption spectrum substantially identical with that of pure erythromycin. The method was used after long term incubations in which radioactive neutral and basic substances other than erythromycin might be expected to have been formed in substantial amounts. The procedure is suitable, also, for the purification of dihydroerythromycin and 5-*O*-desosaminyldihydroerythronolide (see below).

Erythromycin-2″-O-benzoate—Preparation of the monobenzoyl derivative of erythromycin was accomplished by the procedure of Murphy (29) or, somewhat more conveniently, by treatment for 12 hours at 25° with a solution of benzoic anhydride (1 equivalent) in acetone. In the latter case, isolation of the derivative was achieved by evaporation of the acetone, brief treatment of the residue in ethanol solution with an excess of Amberlite CG-45 resin, and concentration of the filtered solution. The derivative was crystallized to constant specific activity from acetone or isopropanol. The product was characterized by its optical rotation ($[\alpha]_D^{25}$ −83° (c, 1.4, 95% ethanol)) and absorption spectrum. Both ultraviolet and infrared analysis indicated retention of a nonconjugated ketone group in the derivative, suggesting that C-9 was not involved in hemiketal or ketal linkage (30).

Conversion of Erythromycin into Dihydroerythromycin, Cladinose, and Desosamine—Erythromycin was reduced to dihydroerythromycin as described by Sigal *et al.* (31). Methanolysis of dihydroerythromycin produced methyl cladinoside and 5-*O*-desosaminyldihydroerythronolide. The methyl cladinoside was isolated from the neutral fraction resulting from the methanolysis and purified as the 4-*O*-3,5-dinitrobenzoate (2 isomers; m.p. 166–168° and m.p. 202–203°) as described by Flynn *et al.* (32). The 5-*O*-desosaminyl*dihydroerythronolide* was isolated by adsorption on Amberlite CG-50 (H) Type I resin followed by elution with solutions of pyridine-H_2O containing acetic acid (as described above). The 5-*O*-desosaminyl*dihydroerythronolide* was purified further by crystallization from butanone (m.p. 225–226°, $[\alpha]_D^{29}$ −0.68° (*c*, 2, methanol)) and seemed to be identical with the 5-*O*-desosaminyl*dihydroerythronolide* described by Sigal *et al.* (31).

Cleavage of 5-*O*-desosaminyldihydroerythronolide to desosamine and dihydroerythronolide was accomplished by mixing the 5-*O*-desosaminyldihydroerythronolide (76 mg) with Dowex 50W-X16 (H) resin (5 ml, 20 to 50 mesh) in a dilute H_2SO_4 solution (100 ml, 1 equivalent) and continuously extracting the resulting suspension at 60° with benzene. After 72 hours, the benzene extract was concentrated to dryness, yielding 52 mg (93%) of crystalline residue the infrared absorption spectrum of which was identical with that of an authentic reference sample of dihydroerythronolide. Paper chromatographic examination in the toluene-isooctane-methanol-water system of de Courcy (33) showed that the major component had the same mobility as the reference sample of dihydroerythronolide, and that a minor impurity was present which moved at the same rate as an impurity present in the reference sample of dihydroerythrono-

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lide dehydration product B (31). Preparative purification of the dihydroerythronolide fraction by chromatography on large sheets of Whatman No. 1 paper in the same system gave pure dihydroerythronolide (m.p. 196.1–197.5°, $[\alpha]_D^{31}$ +9.5° (c, 0.5, 95% ethanol)). Melting points of dihydroerythronolide and its dehydration products do not establish purity, because of the tendency for these substances to form mixed crystals (31).

Desosamine was isolated from the Dowex 50 resin beads by elution with 6 M HCl, evaporation to dryness, and crystallization as the hydrochloride (32).

Chemical Degradations—Kuhn-Roth degradations were performed by the procedure of Eisenbraun *et al.* (34) except that the degradation apparatus was swept with a stream of dry CO_2-free nitrogen and the CO_2 produced by the degradation was trapped in CO_2-free alkali. Steam volatile acids produced were separated by partition chromatography on Celite columns by the method of Swim and Krampitz (35). Acetic acid was identified initially by preparation of the *p*-bromphenacyl ester and routinely by its characteristic and constant position in the elution pattern from the Celite columns. Propionic acid was diluted with carrier sodium propionate and rechromatographed on a buffered Celite column (22) which was capable of separating propionate from butyrate, isobutyrate, etc. In all cases, the radioactivity was recovered in the propionate peak. Acetic and propionic acids were degraded as described by Sakami (36).

RESULTS

Erythromycin Biogenesis by Washed Mycelium of S. erythreus—A study of the effect of certain organic substrates on the rate of erythromycin formation was carried out. To minimize complications brought about by randomization of C^{14} from indirect pathways of incorporation, short term incubations of *S. erythreus* were used. This technique also permitted the study of precursors, such as organic acids, which are inhibitory to the long term growth of the mycelium. It was found that the unwashed mycelium of *S. erythreus*, grown on either a chemically defined or crude medium and shaken as a suspension in water, had a high endogenous rate of erythromycin synthesis. After the mycelium was washed with water or dilute salt solution (*e.g.* 0.9% KCl), the rate of endogenous formation of the antibiotic was reduced. However, no stimulation was provided by added glucose. On the other hand, when the mycelium was washed with potassium phosphate buffer (0.01 M, pH 7.0), the endogenous synthesis of erythromycin was reduced to a relatively low rate which was increased by the addition of either glucose or tryptone. Glucose and tryptone had an additive stimulatory effect on erythromycin synthesis during short incubation periods (4 to 16 hours) without permitting a change in dry weight of the mycelium. The addition of yeast extract caused a further increase in erythromycin synthesis, but since this was accompanied by an increase in the dry weight of the organism, it was omitted from the "minimal" medium used in precursor studies.

The washed mycelium of *S. erythreus* was initially used to measure the effect of organic acids on the formation of erythromycin. Salts of several organic acids, particularly propionic, stimulated erythromycin synthesis (Table I). The effect of added acetate and formate was not as constant as that of propionate, but in most experiments, both caused some stimulation.

Incorporation of C^{14} Substrates into Erythromycin—To determine the exact role of propionate and other organic acids in erythromycin biogenesis, C^{14}-labeled acids were incubated for short times with the washed mycelium of *S. erythreus*. The conditions of the incubations were the same as in the experiments performed without radioactive isotopes (Table I), and the antibiotic was isolated at the end of the experiments after the addition of nonradioactive erythromycin. The erythromycin was converted into a monobenzoyl derivative (29) which was purified by crystallization to constant specific activity. When the results with the radioactive substrates were compared (Table II) it was found that propionate-1-, -2-, and -3-C^{14} are all remarkably efficient precursors of erythromycin, and the specific activity of the erythromycin formed was 10 to 30% that of the propionate.

TABLE I

Effect of organic acids on erythromycin production by washed mycelium of S. erythreus

The incubation mixture contained 2.5 ml of mycelium and 0.5 ml of inorganic salts in a final volume of 5 ml. Additions of glucose (42 μmoles), tryptone (7.5 mg), and organic acids (50 μmoles) were made as indicated. The reaction mixture was incubated in a 25-ml Erlenmeyer flask for 4 hours at 30° (see "Experimental Procedure" for details).

Additions	Erythromycin produced		
	Experiment 1	Experiment 2	Experiment 3
	μg/ml/4 hr ± 0.3		
None	2.3	2.0	0.4
Glucose	2.4	2.9	0.7
Glucose + tryptone	6.0	5.7	1.4
Glucose + tryptone + sodium formate	6.7	6.0	0.6
Glucose + tryptone + sodium acetate	6.0	7.7	2.9
Glucose + tryptone + sodium propionate	7.8	8.1	3.9

TABLE II

Incorporation of C^{14} organic acids into erythromycin by washed mycelium of S. erythreus

The reaction mixture contained: mycelium, 2.5 ml; glucose, 42 μmoles; tryptone, 7.5 mg; inorganic salts, 0.5 ml; and organic acids as indicated in a final volume of 5 ml. Incubations were in 25-ml Erlenmeyer flasks for 8 hours at 30° (see "Experimental Procedure" for details). Each experiment represents 5 flasks.

Experiment No.	Additions		Erythromycin formed	$\frac{\text{d.p.m.}}{\text{mmole C}} \times 10^{-6}$		Substrate specific activity / Erythromycin specific activity
	C^{14} substrate	μmoles		Substrate	Erythromycin	
			μg/ml			*fold dilution*
1	Propionate-1-C^{14}	1	8.7	1900	180	10
2	Propionate-1-C^{14}	50	5.7	8.7	3.2	2.7
3	Propionate-2-C^{14}	1	8.7	1500	150	10
4	Propionate-2-C^{14}	50	5.4	7.7	3.0	2.6
5	Propionate-3-C^{14}	9	1.1	157	34	5
6	Succinate-1,4-C^{14}	0.5	3.4	1100	2.0	550
7	Succinate-1,4-C^{14}	1.0	9.0	1100	1.0	1100
8	Succinate-2,3-C^{14}	0.5	5.3	1100	3.0	370
9	Succinate-2,3-C^{14}	1.0	9.0	1100	2.8	390
10	Uniformly labeled succinate-C^{14}	1.0	7.5	1100	3.7	300
11	Acetate-1-C^{14}	0.5	7.9	2200	0.4	5500
12	Acetate-2-C^{14}	0.5	8.3	2200	0.9	2500

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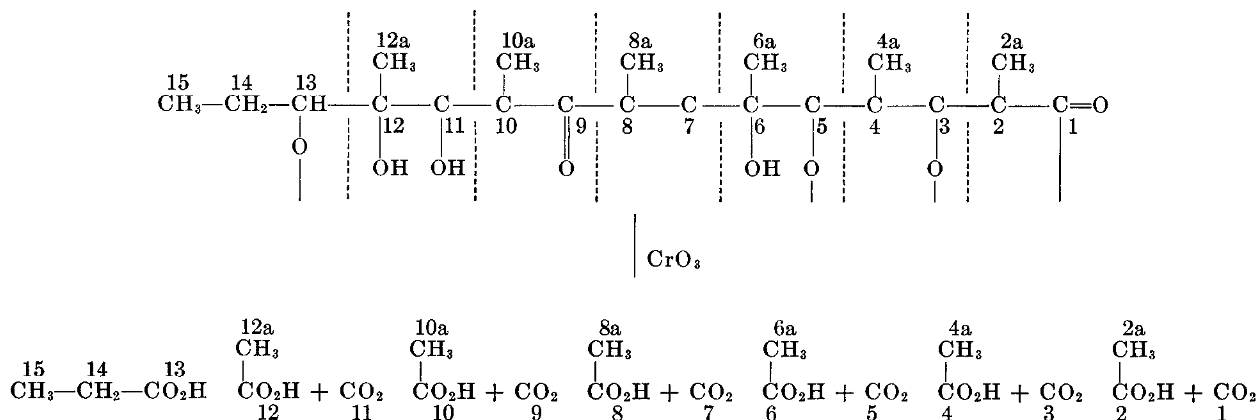

FIG. 2. Kuhn-Roth degradation of the erythronolide moiety of 2″-*O*-benzoyl-erythromycin

TABLE III

*Distribution of C^{14} in propionic acid isolated from Kuhn-Roth degradation of erythromycin produced from propionate-C^{14}**

Substrate	Specific activity					
	Erythromycin	3 Carbon subunit (calculated)	Kuhn-Roth propionate			
			Whole molecule	—COOH (C-13)	—CH_2 (C-14)	—CH_3 (C-15)
	d.p.m./mmole × 10^{-2}					
Propionate-1-C^{14}	20,400	2,914	2,000	2,000	13	6
Propionate-2-C^{14}	19,400	2,770	2,000	15	1,900	12
Propionate-3-C^{14}	3,300	470	460	13	11	480

* Table II, Experiments 1, 3, and 5.

TABLE IV

Distribution of C^{14} in carbon dioxide and acetic acid from Kuhn-Roth degradation of erythromycin

The samples degraded are as listed in Table II.

Experiment No.	Substrate	Specific activity		
		Acetic acid		CO_2
		CH_3	COOH	
		d.p.m./mmole C × 10^{-2}		
1	Propionate-1-C^{14}	0	0	869
2	Propionate-1-C^{14}	0	0	55
3	Propionate-2-C^{14}	9	1560	189
4	Propionate-2-C^{14}	4	94	13
5	Propionate-3-C^{14}	250	4	80
—	Uniformly labeled succinate-C^{14}*	129	133	98

* Long term incubation; 100 μc added in 100 μmoles (see "Experimental Procedure").

As a check on the role of "C_1" donors in erythronolide biogenesis, formate-C^{14} and L-methioninine-$C^{14}H_3$ were studied as precursors of erythromycin. In contrast to the formate-C^{14}, methionine-$C^{14}H_3$ was efficiently incorporated into erythromycin. However, less than 2% of the C^{14} was found in erythronolide with the balance of the radioactivity about equally distributed among the C-3′*a* methyl group of L-cladinose and the *O*- and *N*-methyl groups of L-cladinose and desosamine (37).

Labeling Pattern Produced in Erythronolide by Propionate-C^{14}— Erythromycin synthesized in the presence of propionate-C^{14} is labeled chiefly (>95%) in the erythronolide moiety. Therefore, degradation of the intact erythromycin molecule (*cf.* Fig. 2) will produce radioactive products from erythronolide which are diluted by or mixed with essentially nonlabeled products from the two sugars, L-cladinose and desosamine (Fig. 1, *III* and *IV*).

Although the terminal three carbon subunit (C-13, C-14, C-15) of erythronolide may be similar, biogenetically, to the other six 3 carbon units, it is chemically unique in that it contains a C-ethyl group. On oxidation with chromic acid, according to the Kuhn-Roth procedure, this subunit should be converted into propionic acid (38) and after the oxidation of erythromycin, approximately 1 equivalent of propionic acid was isolated. Radioactivity introduced into the terminal 3 carbon subunit from the differently labeled propionate was measured, and the labeling pattern was determined by stepwise degradation (Table III).

The first six 3 carbon subunits of erythronolide (C_1 to C_{12}) are alike in that, on oxidation by the Kuhn-Roth procedure, carbon dioxide should be produced from the first carbon atom (C_1, C_3, C_5 · · ·) and acetic acid should arise from the second and third carbon atoms (C-2 + C-2*a*, C-4 + C-4*a*, C-6 + C-6*a* · · ·) (Fig. 2). In addition, 3 moles of essentially nonradioactive acetic acid (C-3′ + C-3′*a*, C-5′ + C-6′, C-5″ + C-6″) should be produced from the two sugars in erythromycin benzoate, when propionate-C^{14} has labeled the antibiotic. With these precursors, nonradioactive CO_2 should come from the remaining positions of the sugars, except in the case of the *N*-methyl groups of desosamine which are largely resistant to the oxidation.[1] The benzoyl group in erythromycin benzoate is also partially resistant to the Kuhn-Roth oxidation, giving both CO_2 and benzoic acid. The distribution of C^{14} in the CO_2 and the acetic acid produced by the Kuhn-Roth oxidation is shown in Table IV. The exact specific activity of positions 1, 3, 5, 7, 9, and 11 in erythronolide cannot be calculated since the dilution with nonradioactive CO_2 is not known with accuracy. Accordingly, none of these data are reported in corrected form. The CO_2 is probably also diluted to some extent due to overoxidation of other positions in erythronolide (*cf.* "Discussion"). Although acetic acid and propionic acid are stable under the conditions of the oxidation, lactic acid yields less than 1 equivalent of acetic acid and an excess of CO_2, indicating that overoxidation of oxygenated C-methyl substances can occur.[1]

[1] J. C. Butte and J. W. Corcoran, unpublished data.

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DISCUSSION

The experimental evidence presented in the preceding section demonstrates that propionate carbon is closely related to the 3 carbon subunits of erythronolide. This conclusion is suggested by the stimulatory effect of propionate on erythromycin formation (Table I) and by the high efficiency with which propionate-1-, -2-, and -3-C^{14} are incorporated into erythronolide (Table II). Since erythronolide contains only 21 of the 37 carbon atoms in erythromycin, exclusive utilization of the exogenous propionate for its synthesis would produce erythromycin, for which the specific activity would be 21:37 or about 57% that of the radioactive precursor. This value, which depends upon the sugars of the antibiotic being unlabeled by propionate, is not much different from the dilution observed when the labeled propionate was added in large amounts (5 μmoles per ml).

Proof that propionate carbon enters each of the erythronolide subunits as an intact unit is provided by the approximately equal utilization of propionate-1-, -2-, and -3-C^{14} for erythronolide biosynthesis, and, especially, by the results of the Kuhn-Roth degradation of the resulting erythromycin (*cf.* Fig. 2). The terminal subunit (C-13, C-14, and C-15) was isolated as propionic acid and degraded, and the first six subunits were obtained as acetic acid and carbon dioxide. The degradations (Table III) show that only the first position (C-13) of the terminal subunit is radioactive when propionate-1-C^{14} is the biological precursor, that the second carbon (C-14) of this subunit contains all the C^{14} when propionate-2-C^{14} is the precursor, and that the last carbon (C-15) is exclusively labeled when propionate-3-C^{14} is the precursor of erythronolide. Therefore, propionate-C^{14} is incorporated as an intact unit into C-13, C-14, and C-15 of erythronolide, with the carboxyl carbon becoming C-13. No randomization of the propionate carbon occurs during the incorporation. If the terminal 3 carbon unit is similar to each of the other six subunits of erythronolide it should contain about one-seventh of the total radioactivity in erythronolide (equal to the activity in erythromycin when propionate-C^{14} is the precursor). A calculation of the radioactivity of an individual 3 carbon subunit (Table III, Column 3), based on the assumption that seven equally labeled units are present in erythronolide, is in reasonable agreement with the activities observed in the terminal 3 carbon units (Kuhn-Roth propionic acid). The exact experimental values are somewhat uncertain, since several dilutions with nonlabeled propionate were necessary in the course of purification.

The distribution of C^{14} in the CO_2 and the acetic acid produced by the Kuhn-Roth oxidation (Table IV) show that propionate-C^{14} is incorporated into the first six 3 carbon units of erythronolide in the same way that it is into the terminal subunit. The Kuhn-Roth CO_2 contains all of the radioactivity when erythromycin produced from propionate-1-C^{14} is degraded. The bulk of the C^{14} appears in the carboxyl position of the acetic acid when propionate-2-C^{14} is the precursor, and most of the radioactivity is found in the methyl group of the acetic acid when propionate-3-C^{14} is the biological substrate. The radioactivity found in the CO_2 when erythromycin derived from propionate-2- or -3-C^{14} is degraded is probably the result of some overoxidation in the course of the Kuhn-Roth degradation. The elimination of a methyl group from erythronolide during the Kuhn-Roth oxidation would also explain the discrepancy if the group eliminated is oxidized to CO_2. The postulation of a methyl group shift during the degradation would not explain the observation, since in this case some labeled acetic acid should be produced upon the oxidation of erythromycin derived from propionate-1-C^{14}. In any event, however, the single labeling observed in the Kuhn-Roth acetic acid shows that propionate-C^{14} is incorporated into the first six 3 carbon units of erythronolide without randomization.

These results, coupled with the observed lack of incorporation of C^{14} from formate and the methionine methyl group, lead to the conclusion that the 3 carbon subunits of erythronolide are derived from 3-carbon precursors, as suggested by Gerzon and Woodward.

The proposal of Birch, that methylation of a preformed straight chain lipid derived from acetate units may be an essential step in erythronolide biogenesis, is not supported by these results.

A close relationship of propionate to erythromycin biogenesis has been suggested by the independent work of Musilek and Sevcik (39) and of Vanek *et al.* (40). Confirmation of our finding that propionate is incorporated as an intact unit into the erythronolide subunits has appeared in the work of Griesebach *et al.* (41, 42) and of Vanek (43). A similar role of propionate in the biogenesis of methynolide, a partially branched aglycon similar to erythronolide, has been reported by Birch *et al.* (44).

Propionate, although shown to be in close biogenetic relationship to erythronolide, may not be the immediate precursor of the 3 carbon subunits. By analogy with the synthesis of long chain lipids from malonyl-CoA instead of acetyl-CoA (45), α-methylmalonyl-CoA rather than propionyl-CoA might be the true precursor of erythronolide. This possibility, advanced on a theoretical basis by Lynen (46), is being investigated. Preliminary results indicate that α-methylmalonate-$C^{14}H_3$ is an efficient precursor of erythromycin, and the Kuhn-Roth oxidation products have the same C^{14} distribution as when propionate-3-C^{14} is the precursor (47). α-Methylmalonate also merits consideration as an intermediate in erythronolide formation because it has already been shown to be an intermediate in the interconversion of propionyl-CoA and succinyl-CoA by both the propionibacteria (48–50) and mammalian tissue (51, 52).

The mechanism by which propionate is incorporated into the erythronolide subunits is not clear. Whatever the mechanism may be, obviously the intermediates involved cannot have rotational symmetry (53) with respect to the carbon atoms, for no randomization of propionate carbon occurs during its incorporation into erythronolide.

SUMMARY

The actinomycete *Streptomyces erythreus* incorporates propionate-1-, -2-, and -3-C^{14} as intact units, without randomization of C^{14}, into the 3 carbon subunits of erythronolide, the branched chain C_{21}-lactone in erythromycin. Formate-C^{14} and methionine-$C^{14}H_3$ are not incorporated. These findings support the postulate that propionate or its "biological equivalent" is the precursor of this type of branched lipid, and they rule out methylation of a straight chain lipid as being an obligatory step in the biogenesis. The results are consistent with either propionate or α-methylmalonate being the true precursors of the 3 carbon subunits in erythronolide.

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