interact at sites on the separate transport carriers which may be essential for transport regulation. Such regulatory sites could be sufficiently similar so that each site would exhibit the same affinity for colchicine. Furthermore, similar regulatory sites for the separate transport systems would provide a mechanism for the coordinated control of nucleoside uptake under different environmental conditions.

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Nucleoside Antibiotics. Asymmetric Incorporation of Glutamic Acid and Acetate into the Maleimide Ring of Showdomycin by *Streptomyces showdoensis*†

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ABSTRACT: Carbon-14 from [1-^{14}C]acetate, [2- and 5-^{14}C]-glutamate, and [5-^{14}C]α-ketoglutarate, but not [1-^{14}C]α-ketoglutarate, resides in the carbonyl carbons of the maleimide ring. Degradation of the maleimide ring of showdomycin showed that all of the ^{14}C from the [1-^{14}C]acetate or [5-^{14}C]-glutamate resides in C-5 of showdomycin while all of the ^{14}C from [2-^{14}C]glutamate resides in C-2 of showdomycin. Radioactivity from [2,3-^{14}C]succinate, [2,3-^{14}C]fumarate, and [2-^{14}C]acetate was found in all four carbons of the maleimide ring of showdomycin. Apparently the Krebs cycle and the malic enzyme in *Streptomyces showdoensis* function in the conversion of fumarate and succinate to showdomycin. Further proof that C-4 of glutamate or α-ketoglutarate forms the carbon–carbon bond with D-ribose was obtained in an experiment with [5-^{14}C,4-^{3}H]glutamate. While ^{14}C is incorporated into the maleimide ring all of the tritium is lost. Therefore, the four-carbon maleimide ring arising from C-2 to C-5 of α-ketoglutarate must be an asymmetrical unit when condensation occurs with D-ribose to form showdomycin.

Recent studies on the biosynthesis of showdomycin by *Streptomyces showdoensis* have shown that C-2, -3, -4, and -5 of α-ketoglutarate serve as the four-carbon precursor for the maleimide ring of this antibiotic (Elstner and Suhadolnik, 1971a). All of the ^{14}C in showdomycin from [1-^{14}C]acetate, [2-^{14}C]glutamate, and [5-^{14}C]glutamate resided in the car-

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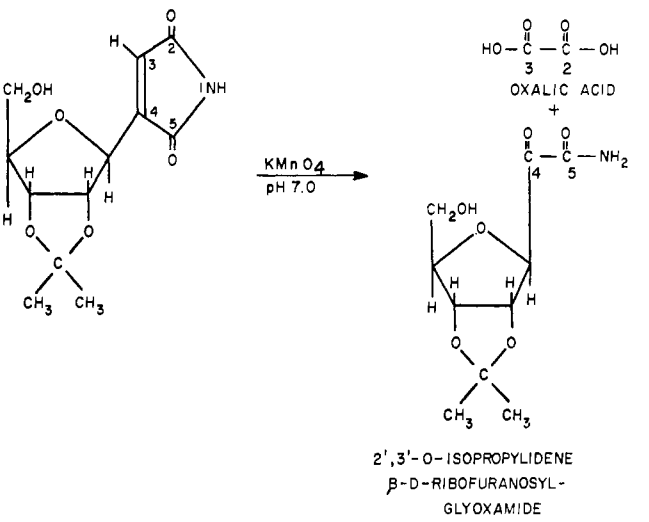
bonyl carbons. It was not possible to determine if the ^{14}C was located in one or both of the carbonyl carbons of the maleimide ring since the Hofmann degradation method was used to convert the carbonyl carbons to carbon dioxide. Thus, the location of the ^{14}C in the maleimide ring following the incorporation of [2-^{14}C]- or [5-^{14}C]glutamate and the nature of the four-carbon intermediate is not known. To determine if this four-carbon unit that comprises the maleimide ring is a symmetrical or asymmetrical intermediate prior to condensation with D-ribose, the maleimide ring was oxidized with permanganate (Scheme I). C-2 and -3 are isolated as oxalic acid; C-4 and -5 are isolated as ribosylglyoxylamide.[1] The data presented here show that the C_4 maleimide unit of showdomycin, arising from ^{14}C- or ^{3}H-labeled acetate or glutamate, condenses as an asymmetrical unit with D-ribose.

SCHEME I



SCHEME I: Degradation of 2′, 3′-*O*-Isopropylideneshowdomycin by $KMnO_4$ to Oxalic Acid and 2′,3′-*O*-Isopropylidene-β-D-ribofuranosylglyoxylamide.

Experimental Section

Melting points were taken with the Thomas-Hoover silicone bath apparatus and are uncorrected. The ultraviolet spectra were recorded on Gilford Model 2400 and Beckman Model DB spectrophotometers. Elemental analyses were performed by Baron Consultants, Orange, Conn. Nuclear magnetic resonance spectra were determined on a Varian XL-100-15 spectrometer. Samples were dissolved in deuterated Me_2SO or chloroform using Me_4Si as the internal standard. Radiochemical measurements were made on Packard liquid scintillation spectrometers Models 314E and 3320 using Bray's (1960) scintillation solution. Gas-flow counting was done with the Nuclear-Chicago Counter Model D-47. Paper electrophoresis was carried out on a Gelman Model 51170 chamber for 1.5 hr at 500 V, 4°, 0.05 N phosphate buffer (pH 8.0). Maintenance of *S. showdoensis* cultures and isolation of showdomycin were as described (Elstner and Suhadolnik, 1971a). DE52 (exchange capacity 1.0 mequiv/g) was obtained from Reeves-Angel. Thin-layer chromatography was done on silica gel thin-layer plates (Merck, F254). The solvents used for chromatography were solvent A, benzene–ethyl acetate–ethanol (5:1:1); solvent B, water–saturated 1-butanol; solvent C, 1-propanol–water (7:3).

[1-^{14}C]Acetate, [2-^{14}C]acetate, and [2,3-^{14}C]succinate were obtained from Schwarz-Mann; D,L-[2-^{14}C]glutamate, and D,L-[5-^{14}C]glutamate were obtained from New England Nuclear Corp. [4-^{3}H]Glutamate was a generous gift of Dr. J. Katz, Cedars of Lebanon Hospital. All other chemicals used were of the highest purity available.

Synthesis of 2′,3′-O-Isopropylideneshowdomycin. Crystalline showdomycin (100 mg) was stirred with 500 mg of anhydrous copper sulfate in 20 ml of freshly distilled acetone. The copper sulfate used was dried for 3 hr at 190° over phosphorus pentoxide under vacuum. Isopropylidene formation was determined by thin-layer chromatography on silica gel plates using solvent A (R_F showdomycin, 0.35; R_F acetonide, 0.85). After 48 hr, the reaction mixture showed only one ultraviolet-absorbing compound (R_F 0.85). The copper sulfate was removed from the reaction mixture by filtration. The isopropylidene in the filtrate was concentrated to dryness. The dried residue was extracted with 50 ml of chloroform, filtered, and evaporated to dryness. The acetonide was dissolved in acetone and crystallized from acetone–water (1:7): mp 123°, lit. (Nakagawa *et al.*, 1967) mp 140.5–141°. The ultraviolet absorption spectra was λ_{max} (pH 6.4, water), 218 mμ. *Anal.* Calcd for $C_{12}H_{15}NO_6$: C, 53.30; H, 5.20; N, 5.30. Found: C, 53.28; H, 5.32; N, 5.46. The nmr spectra in deuteriochloroform, (δ) 8.0 (1 broad proton signal, imide proton), 6.6 (1 vinylic proton signal, 3H), 3.70–4.75 (a broad multiplet for D-ribofuranose, 5 protons; the anomeric proton signal is split and centered at 4.76 (J = 3 cps)), 1.55 (one sharp signal, CH_3), 1.35 (one sharp signal, CH_3)) (literature for showdomycin: Darnall *et al.*, 1967; Nakagawa *et al.*, 1967). The specific activities of the crystalline showdomycin and the isopropylidene derivative were the same. The crystalline isopropylidene derivative of showdomycin was hydrolyzed to showdomycin by heating 1 ml of a solution in 0.05 M acetic acid for 20 min on a steam bath. The thin-layer chromatograms showed only one ultraviolet-absorbing compound with the same R_F as authentic showdomycin in either solvents A, B, or C.

Oxidation of 2′,3′-O-Isopropylideneshowdomycin with Potassium Permanganate. The acetonide of showdomycin (0.3 mmole) was oxidized by the addition of a 2.5% solution of permanganate to an aqueous solution of the acetonide of showdomycin for 90 min with stirring at pH 7.0 at room temperature. Acidic conditions were avoided since the oxalic acid formed would be oxidized to carbon dioxide. Similarly, alkaline conditions were avoided since alkali converts showdomycin to the cyclic derivative (Nakawawa *et al.*, 1967). For each mmole of the isopropylidene derivative of showdomycin oxidized, 1.9–2 mmoles of permanganate was utilized. After standing 15 min, the brown precipitate was removed by centrifugation and the slightly yellow supernatant was added to the DE52 column (1.6 × 15 cm). The column was equilibrated with 0.4 M triethylammonium carbonate buffer (pH 7.6). Oxidation of authentic maleimide in the same manner followed by treatment of the supernatant with 5 N hydrochloric acid for 1 hr at 120° resulted in the isolation of pure crystalline oxalic acid.

Separation of the Products of 2′,3′-O-Isopropylideneshowdomycin Following Oxidation with Permanganate. The degradation products were eluted from the DE52 column with a lin-

[1] For convenience in referring to the location of ^{14}C in the maleimide ring of showdomycin from the [2- or 5-^{14}C]glutamate incorporation, the maleimide ring of showdomycin has been numbered as shown in Scheme I or Figure 1. Therefore, C-2 of glutamate is equivalent to C-2 of the maleimide ring and carbon-5 of glutamate is equivalent to C-5 of the maleimide ring of showdomycin.

ear gradient from 0.005 to 0.4 M triethylammonium carbonate buffer (pH 7.6) (200 ml each). Two compounds were isolated and fully characterized following the oxidation of 0.3 mmole of 2′,3′-*O*-isopropylideneshowdomycin. The first compound is 2′,3′-*O*-isopropylidene-β-D-ribofuranosylglyoxylamide (Figure 1, II). This product represents C-4 and -5 of showdomycin. Compound II (7 mg) was isolated following crystallization from water and water–ethanol (1:1): mp 177°; nmr spectra in Me_2SO-d_6, (δ) 6.87 and 7.1–7.5 (integrated for two amide protons), 3.45–5.10 (a broad multiplet for D-ribofuranose integrated for 6 protons, 1.45 (one sharp signal, CH_3), 1.35 (one sharp signal, CH_3). The second compound isolated was oxalic acid (Figure 1, compound VII). The oxalic acid represents C-2 and -3 of the maleimide ring of showdomycin.

Compound II was further characterized by the formation of the 2,4-dinitrophenylhydrazone. A solution of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid was added to an aqueous solution of II. After 12 hr, the yellow precipitate of the 2,4-dinitrophenylhydrazone derivatives of β-D-ribofuranosylglyoxylamide and the acetone (due to the hydrolysis of the acetonide) was centrifuged, washed twice with 2 N hydrochloric acid, water, and finally chloroform to remove the 2,4-dinitrophenylhydrazone of acetone. The remaining insoluble yellow precipitate was crystallized from ethanol and recrystallized from water: yield, 3.5 mg; mp 235°. *Anal.* Calcd for $C_{13}H_{15}N_5O_9$: C, 41.40; H, 4.00; N, 18.70. Found: C, 40.83; H, 4.10; N, 19.09; mnr spectra in Me_2SO-d_6 (δ) 8.3, 8.4, and 9.15 (3 aromatic protons), a broad multiplet for D-ribofuranose (6 protons in the region 3.6–5.2).

Oxalic acid eluted from the DE52 column (Figure 1) was precipitated as calcium oxalate with calcium chloride (pH 4.5) (Lewis and Weinhouse, 1957). The calcium oxalate was washed with water and dried under vacuum; yield, 4.5 mg. Free oxalic acid was obtained by treatment of the calcium oxalate with Dowex (H^+) 50-X8. The resin was removed by filtration. The solution was lyophilized to 0.25 ml. Oxalic acid crystallized over night at 4°. The crystalline oxalic acid was removed by filtration and dried under vacuum: mp 188°, mmp 188°. The crystalline oxalic acid gave the blue color on treatment with diphenylamine (Feigl and Fredhen, 1935; Lewis and Weinhouse, 1957). The oxalic acid was determined quantitatively by weighing or by titration with a 0.01 N potassium permanganate solution in 5 N sulfuric acid at 60° (Lewis and Weinhouse, 1957). Finally, the radioactive oxalic acid isolated following the permanganate oxidation of the isopropylidene derivative of showdomycin was shown to be pure by the addition of authentic unlabeled oxalic acid to the radioactive oxalic acid. Following crystallization and radiochemical measurements, the specific activity of the diluted crystalline oxalic acid (when corrected for dilution) was the same as before dilution.

Determination of α-Keto Acids. Compounds II was converted to β-D-ribofuranosylglyoxic acid by refluxing with 5 N hydrochloric acid for 45 min. The α-keto acid was decarboxylated with ceric sulfate (Meister, 1952; Krebs and Johnson, 1937; Fromageot and Desnuelle, 1935). A microdistillation apparatus was used with nitrogen gas bubbled through the reaction mixture into a 1-ml solution of 1 N Hyamine to trap the carbon dioxide. Qualitative tests for the presence of α-keto acids were performed as described (Lu, 1939; Katsuki *et al.*, 1971).

Decarboxylation of Malate by Malic Acid Dehydrogenase. Crude extracts were prepared as described (Elstner and Suhadolnik, 1971b) except that 5×10^{-3} M cysteine was added to the homogenate. The enzyme assay was as follows: crude extract (7 mg of protein), Tris-HCl buffer (pH 8.0, 0.1 M), 1×10^{-5} M $MnCl_2$, 1×10^{-3} M cysteine, 1×10^{-2} M $NADP^+$ or NAD^+, and 1×10^{-3} M L-[U-^{14}C]malic acid (0.1 Ci/mole); 20 min, 32° in a microdistillation apparatus. The reaction mixture was stopped by injecting 1 ml of 2 N HCl and bubbling a stream of nitrogen gas for 10 min. The carbon dioxide was trapped in 1 ml of 1 N Hyamine. An aliquot was counted by liquid scintillation counting. The acidic solution was centrifuged and the 2,4-dinitrophenylhydrazine derivative of the pyruvate was prepared (mp 214°; lit. (Allen, 1930) mp 213–214°).

This system decarboxylated 50 nmoles of malic acid to stoichiometric amounts of carbon dioxide and pyruvate per mg of protein per hr. The reaction is dependent upon NAD^+ or $NADP^+$, Mn^{2+}, and protein. When the incubation mixture contained $NADP^+$ instead of NAD^+, the decarboxylation of the malic acid decreased by 50%; when Mn^{2+} was omitted only 20% decarboxylation occurred. There is no decarboxylation of malic acid in control experiments (minus NAD^+ or $NADP^+$ or with denatured protein).

Results

Oxidation of 2′,3′-O-Isopropylideneshowdomycin by Potassium Permanganate and Separation of the Products on DEAE-cellulose. The uncharged molecules, mono-, and dicarboxylic acids that are formed following the oxidation of the acetonide derivative of showdomycin by permanganate can be separated by gradient elution on DE52. In addition to the separation of products based on charge, identification was also made by detection of the α-keto acids and oxalic acid. Oxidation of the acetonide derivative of showdomycin labeled with ^{14}C from [2-^{14}C]acetate and the distribution of radioactivity in the eluent from the DE52 column was followed. The theoretical products, radioactive peaks, α-keto acids, and oxalic acid formation are shown in Figure 1. Three radioactive peaks were observed. The first peak represents the neutral fraction; the second, the monocarboxylic acids; the third, the dicarboxylic acids. Authentic pyruvic acid and α-ketoglutaric acid were found in fractions 9–14 (peak two) and fractions 15–18 (peak three). The α-keto acids were detected in all three fractions. Free oxalic acid was detected in the third peak. The possible structures following the oxidation of the acetonide derivative of showdomycin (I) are shown in Figure 1. Of the eight possible oxidation products (Figure 1) II, III, and VII (peaks 1, 2, and 3) have been isolated and rigorously identified. The elution pattern from DEAE-cellulose of the products of permanganate oxidation of the isopropylidene derivative of showdomycin following the incorporation of [2-^{14}C]glutamate or [5-^{14}C]glutamate showed a marked difference in the ^{14}C in 2′,3′-*O*-isopropylidene-β-D-ribofuranosylglyoxylamide (II) (Figure 2). While there is essentially no ^{14}C in II from the [2-^{14}C]glutamate, there was a distinct radioactive peak in II following the incorporation of [5-^{14}C]glutamate. The elution profile of the oxidation products from the isopropylidene derivative of showdomycin following the incorporation of [5-^{14}C]glutamate was identical with the elution pattern when either ^{14}C from [1-^{14}C]acetate, [2-^{14}C]acetate, [2,3-^{14}C]succinate, or [2,3-^{14}C]fumarate were incorporated into showdomycin. Although the elution pattern of the oxidation products of I from the [2-^{14}C]glutamate experiments did not have any radioactivity in peak one, these fractions gave a positive test for α-keto acids. The marked difference in the amount of radioactivity in II from the incorporation of either [2-^{14}C]-glutamate or [5-^{14}C]glutamate into showdomycin provides

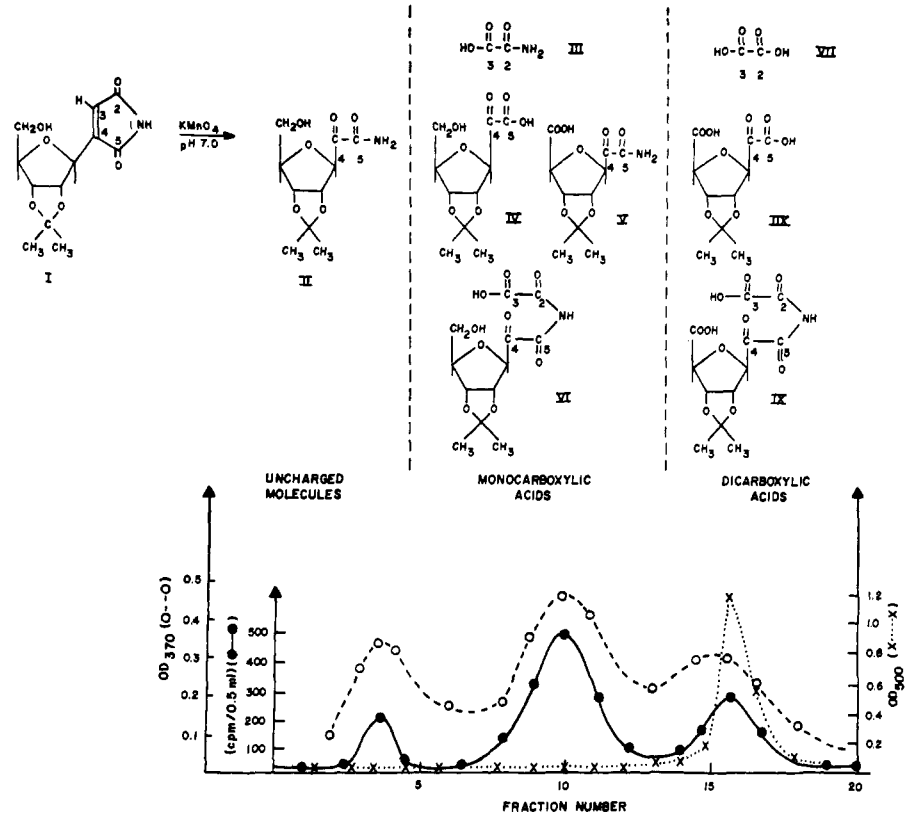


FIGURE 1: The postulated uncharged molecules and the mono- and dicarboxylic acids formed following oxidation of 2′,3′-*O*-isopropylideneshowdomycin with $KMnO_4$ at pH 7.0 and their separation on DEAE-cellulose. Ten-milliliter fractions; (●) radioactivity; (○) color test for α-keto acids; (×) turbidimetric measurement of calcium oxalate.

strong evidence that the four carbon precursor for the maleimide ring of showdomycin is an asymmetrical intermediate.

Distribution of ^{14}C in 2′,3′-O-Isopropylidene-β-D-ribofuranosylglyoxylamide (II) Following the Incorporation of ^{14}C from Glutamate, Acetate, Fumarate, and Succinate. Compound II (Figure 1) was eluted with the void volume from the DE52 column. It gave a positive test for α-keto acids. These fractions were combined and the 2,4-dinitrophenylhydrazone derivative was isolated and characterized (see Experimental Section). The specific activities and per cent distribution of ^{14}C are shown in Table I. As reported earlier (Elstner and Suhadolnik, 1971a), ^{14}C from [5-^{14}C]α-ketoglutarate and [2- or 5-^{14}C]glutamate but not ^{14}C from [1-^{14}C]α-ketoglutarate was incorporated into the maleimide ring of showdomycin. The distribution of ^{14}C in the maleimide ring was not determined. The permanganate oxidation described here provides the first experimental evidence on the distribution of ^{14}C in the maleimide ring formed from the ^{14}C compounds described in Table I.

Further Separation and Identification of Compounds from Peak Two. The fractions (peak two) from the ^{14}C-labeled glutamate and acetate experiments, were concentrated, spotted on a Whatman No. 3MM paper chromatogram, and subjected to paper electrophoresis. All of the ^{14}C migrated toward the anode (Figure 3). While the ^{14}C profile of the oxidized acetonide of showdomycin from the [2-^{14}C]glutamate experiment shows two radioactive peaks (3 × 10 cm migration), the profile from [5-^{14}C]glutamate showed only one peak (3-cm migration). Both peaks were cut out and eluted. The compound with an R_F of 3 cm gave a positive color test for α-keto acids. The α-keto acids were not further identified. However, from the possible structures shown in Figure 1 (monocarboxylic acids) it is likely that the compound migrating 3 cm from the origin are IV, V, or VI. The radioactivity in the area 10 cm from the origin was eluted. It formed a precipitate upon the addition of calcium chloride (pH 4.7) but only after the eluate has been hydrolyzed by refluxing for 45 min with hydrochloric acid. The insoluble calcium oxalate was converted to oxalic acid by treatment with Dowex (H^+) 50-X8. The specific activities of the oxalic acid are shown in Table I. Similar treatment of the acetonide derivative of showdomycin from the [1-^{14}C]acetate and [2-^{14}C]acetate experiments produced the data shown in Figure 4. Electrophoresis of the eluate in peak two from the [2-^{14}C]acetate experiment gave

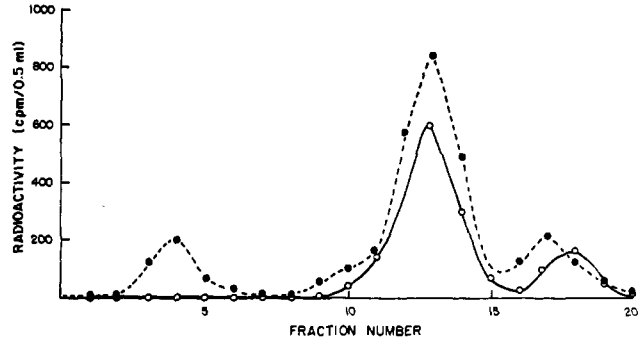


FIGURE 2: ^{14}C elution profile resulting from the oxidation of 2′,3′-*O*-isopropylideneshowdomycin following the incorporation of D,L-[5-^{14}C]-glutamate (●) or D,L-[2-^{14}C]glutamate (○).

TABLE I: Distribution of ^{14}C in the Maleimide Ring of Showdomycin Following Incorporation of ^{14}C-Labeled Glutamate, Acetate, Succinate, and Fumarate.

Compound Incorpd into Showdomycin	2′,3′-*O*-Isopropylidene-showdomycin Oxidized (nCi/μmole)[a]	Compound II		Osazone Derivative[d]		Oxalic Acid			
		Sp Act. (nCi/μmole)	^{14}C in C-4 and -5 (%)	Sp Act. (nCi/μmole)	^{14}C in C-4 and -5 (%)	Isolated from Peak 2 (nCi/μmole)[b]	^{14}C in C-2 and -3 (%)	Isolated from Peak 3 (nCi/μmole)[c]	^{14}C in C-2 and -3 (%)
D,L-[2-^{14}C]Glutamate	0.19			0.01	5	0.19	100	0.20	105
D,L-[5-^{14}C]Glutamate	0.10			0.10	100	0.00	0	0.01	10
[1-^{14}C]Acetate	0.13	0.12	93					0.009	7
[2-^{14}C]Acetate	0.15			0.091	61			0.065	40
[2,3-^{14}C]Succinate	0.32	0.16	50	0.18	55			0.17	52
[2,3-^{14}C]Fumarate	0.09			0.043	47			0.047	51

[a] The specific activities of showdomycin and the isopropylidene derivative were the same. [b] Isolated by paper electrophoresis and hydrolysis of oxalamide (III, Figure 1). [c] Isolated as crystalline oxalic acid from peak three (Figure 1). [d] This is the osazone derivative of II.

rise to two compounds (R_F 3 × 10 cm), while only one radioactive compound was observed from the [1-^{14}C]acetate experiments. Therefore, these data also show that all of the ^{14}C in the maleimide ring of showdomycin from both [5-^{14}C]glutamate and [1-^{14}C]acetate is in C-4, C-5, or both. No ^{14}C is located in C-2 or -3 (Table I and Figures 3 and 4).

Isolation of Oxalic Acid from Peak 3. Addition of calcium chloride to the radioactive fractions of peak 3 gives a white precipitate. Isolation of the precipitate and treatment with Dowex (H^+) 50-X8 gave rise to free oxalic acid. The specific activities of the oxalic acid isolated following hydrolysis of oxalamide (Figure 2, peak 2) or the free oxalic acid isolated from peak 3 are the same (Table I).

Isolation of C-5 of Showdomycin by Decarboxylation of β-D-Ribofuranosylglyoxylate, To determine the distribution of ^{14}C in C-4 and -5 of the maleimide ring of showdomycin following the incorporation of ^{14}C from glutamate, acetate, and fumarate, the crystalline β-D-ribofuranosylglyoxylamide was decarboxylated (Table II). Essentially all of the ^{14}C in the maleimide ring of showdomycin from the incorporation of [5-^{14}C]glutamate and [1-^{14}C]acetate resides in C-5. Only 22% of the ^{14}C in the β-D-ribofuranosylglyoxylamide from [2-^{14}C]-acetate resides in C-5 while 37% of the radioactivity resides in C-4 and 40% resides in C-2 and -3 (Tables I and II). Similarly, 27% of the ^{14}C from [2,3-^{14}C]fumarate resides in C-5 and 23% resides in C-4 of showdomycin.

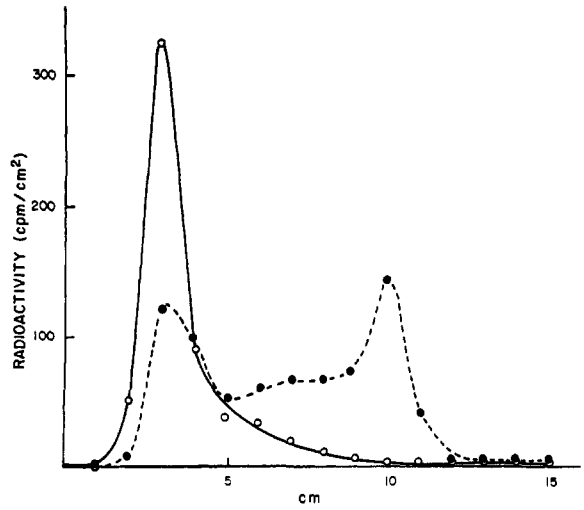


FIGURE 3: Migration and isolation of oxalamide (R_F 10 cm) by paper electrophoresis. The radioactive compounds separated by electrophoresis represent the combined fractions from peak two from the DEAE-cellulose column (see Figure 1 and Experimental Section). (○) ^{14}C migration profile following the incorporation of [5-^{14}C]glutamate into showdomycin; (●) ^{14}C migration profile following incorporation of [2-^{14}C]glutamate into showdomycin.

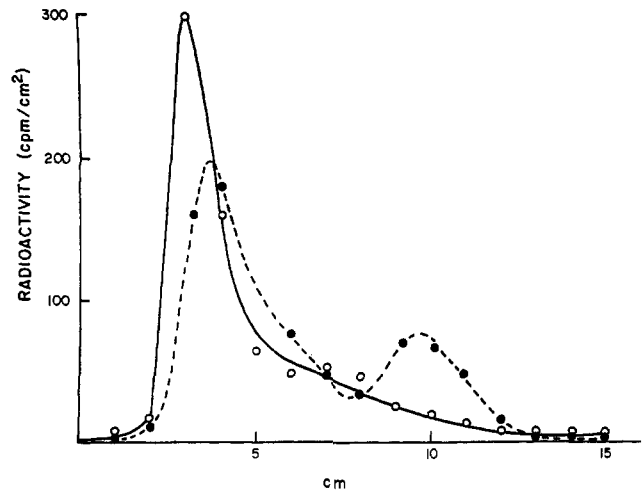


FIGURE 4: Migration and isolation of oxalamide (R_F 10 cm) by paper electrophoresis. The radioactive compounds separated by electrophoresis represent the combined fractions from peak two from the DEAE-cellulose column (see Figure 1 and Experimental Section). (○)^{14}C migration profile following the incorporation of [1-^{14}C]-acetate into showdomycin; (●) ^{14}C migration profile following the incorporation of [2-^{14}C]acetate into showdomycin.

TABLE II: Decarboxylation of β-D-Ribofuranosylglyoxylic Acid by Ceric Sulfate.

Compound Incorpd into Showdomycin	β-D-Ribofuranosylglyoxylate			^{14}C in	
	Amt Oxidized (nCi)	C-5		C-5 of Showdomycin (%)	C-4 of Showdomycin[a] (%)
		Amt of CO_2 Released (nCi)	^{14}C (%)		
[5-^{14}C]Glutamate	0.73	0.73	100	100	0
[1-^{14}C]Acetate	0.38	0.35	93	93	7
[2-^{14}C]Acetate	0.55	0.21	38	22	37
[2,3-^{14}C]Fumarate	0.19	0.10	53	27	23

[a] Per cent ^{14}C was calculated by difference for the ^{14}C in C-2, -3, and -5 of showdomycin.

Incorporation of [5-^{14}C,4-^{3}H]Glutamate and [2-^{14}C,2-^{3}H]-Acetate into Showdomycin. The purpose of these double-labeled experiments was to support the notion that C-4 of glutamate becomes C-4 of the maleimide ring of showdomycin. While the ^{14}C was incorporated into showdomycin, tritium was not incorporated (Table III).

Discussion

Earlier studies on the biosynthesis of showdomycin showed that C2- to -5 of either glutamate or α-ketoglutarate served as the four-carbon precursor for the maleimide ring (Elstner and Suhadolnik, 1971a). It was not known if this four-carbon unit, following decarboxylation of α-ketoglutarate, exists as a symmetrical or asymmetrical unit when it condenses with D-ribose to form showdomycin. By ^{14}C and ^{3}H experiments and oxidation of 2′,3′-*O*-isopropylideneshowdomycin to oxalate (C-2 and -3) and 2′,3′-*O*-isopropylidene-β-D-ribofuranosylglyoxylamide (C-4 and -5), it is now possible to show that C-2 and -5 of glutamate become C-2 and -5 of the maleimide ring, respectively (Scheme I). The carbon–carbon bond formed between the anomeric carbon of D-ribose and the maleimide ring takes place at C-4 of the asymmetric four-carbon unit arising from α-ketoglutarate. Therefore, in a double-labeled experiment with [5-^{14}C,4-^{3}H]glutamate, ^{14}C is incorporated into C-5 of showdomycin, but the tritium is lost. All of the ^{14}C from [1-^{14}C]acetate is located in C-5 of showdomycin. These findings are consistent with the formation of [5-^{14}C]-glutamate from [1-^{14}C]acetate once acetate enters the Krebs cycle (Wilcox *et al.*, 1950; Hoare, 1963).

The showdomycin from [2-^{14}C]acetate had ^{14}C in C-2 to C-5 of the maleimide ring. If only the Krebs cycle were operating in the utilization of [2-^{14}C]acetate, ^{14}C should be located in carbons-1–4 of α-ketoglutarate which in turn would give rise to ^{14}C in C-2, -3, and -4 of showdomycin. Experimentally, it was observed that ^{14}C resides in all four carbons of the maleimide ring with most of the ^{14}C in C-4. This can be explained by the fact that in addition to the Krebs cycle, the malic enzyme is operating in *S. showdoensis*. Similarly, if only the Krebs cycle were functioning in *S. showdoensis*, the [2,3-^{14}C]fumarate and [2,3-^{14}C]succinate should produce ^{14}C in glutamate in C-1, -2, and -3. This labeling pattern in glutamate would yield ^{14}C in C-2 and -3 of the maleimide ring. Experimentally, it was observed that ^{14}C was uniformly located in all four carbons of the maleimide ring. The conversion of malate to pyruvate is substantiated by the following. First, malate is decarboxylated by cell-free extracts of *S. showdoensis* to pyruvate and carbon dioxide. Second, *S. showdoensis* can grow and produce showdomycin on malate as the sole carbon source (Mayama *et al.*, 1968).

The conversion of α-ketoglutarate to an asymmetrical four-carbon unit in the biosynthesis of the maleimide ring of showdomycin and the degradation scheme described here makes this end product of metabolism by *S. showdoensis* a most useful biochemical tool to follow the distribution of carbons from those compounds that enter the Krebs cycle. Experiments are currently underway with [1-^{13}C]acetate in which ^{13}C nmr will be used to further prove that the carboxyl group of acetate resides exclusively in C-5 of the maleimide ring of showdomycin. Similar studies will be done with [2-^{13}C]acetate in an attempt to substantiate the distribution of ^{14}C in the maleimide ring of showdomycin.

With the advent of nmr and mass spectrometry in combination with other physicochemical techniques, the structure of showdomycin was elucidated without the usual chemical degradations. The only degradations reported for showdomycin were those described by Darnell *et al.* (1967) in which D-ribose was isolated following treatment of showdomycin with hydrazine. The study reported here shows that the maleimide ring of showdomycin can be split into oxalic acid and ribofuranosylglyoxylic acid. It is of interest to note

TABLE III: Distribution of ^{14}C and ^{3}H in Showdomycin from Double-Labeled Experiments with [5-^{14}C,4-^{3}H]Glutamate and [2-^{14}C,2-^{3}H]Acetate.

Compounds Added		Amt Added/ Flask (μmoles)	Sp Act. (Ci/ mole)	Showdomycin Isolated[b] (nCi/ μmole)
Expt I[a]	D,L-[5-^{14}C]-Glutamate	0.05	15	0.15
	D,L-[4-^{3}H]-Glutamate	0.013	80	0.00
Expt II[a]	[2-^{14}C]Acetate	3.25	2.8	1.9
	[2-^{3}H]Acetate	1.42	10	0.00

[a] Each double-labeled experiment was performed with ten flasks containing 300 ml of medium. [b] The yield of crystalline showdomycin was 10 mg/flask.

that ribofuranosylglyoxylic acid was the key C-7 α-keto acid used in the chemical synthesis of showdomycin (Kalvoda *et al.*, 1970).

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Chemical Modification of Carboxypeptidase A Crystals. Azo Coupling with Tyrosine-248†

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ABSTRACT: Coupling of bovine carboxypeptidase A crystals with diazotized *p*-arsanilic acid modifies one tyrosyl residue. Cyanogen bromide cleavage of the enzyme and separation of the resultant fragments demonstrates virtually complete incorporation of the arsanilazo label into segment F_I containing residues 104–301. This fragment was solubilized by succinylation, digested with chymotrypsin, and the arsanilazotyrosyl-containing peptide was isolated by affinity chromatography using an antibody–Sepharose conjugate specific for the arsanilazotyrosyl moiety. The arsanilazotyrosyl peptide was purified by subsequent ion-exchange chromatography and recovered in 90% overall yield. Its amino acid composition, N-terminal threonine, and tryptophan content are uniquely compatible with the sequence of carboxypeptidase A containing residues 246–257 (Thr-Ile-Tyr-Gln-Ala-Ser-Gly-Gly-Ser-Ile-Asp-Trp). Tyrosine-248 is the residue labeled specifically by diazotized *p*-arsanilic acid in carboxypeptidase A crystals.

Diazonium salts have proven to be particularly useful reagents to introduce environmentally sensitive chromophores into proteins for the investigation of structure–function relationships (Fairclough and Vallee, 1970, 1971; Vallee *et al.*, 1971). In this regard diazotized *p*-arsanilic acid has been especially advantageous in studies of carboxypeptidase A. When crystals of the enzyme are treated with this reagent, one tyrosyl residue is labeled specifically (Johansen and Vallee, 1971a,b; J. T. Johansen and B. L. Vallee, in preparation). The resulting azotyrosyl chromophore has served to monitor changes in conformation as the enzyme changes from the solution to the crystalline state and *vice versa* (Johansen and Vallee, 1971b; J. T. Johansen and B. L. Vallee, in preparation). It has also been a valuable probe in assessing local conformational changes coincident with alterations in enzyme activity by limited proteolysis (Riordan and Livingston, 1971; D. M. Livingston and J. F. Riordan, in preparation).

Localization of the structural changes to a particular region of the protein required quantitative isolation and identification of the tyrosyl residue modified. Since conventional methods of peptide isolation usually involve many steps with consequent loss of material, we have employed affinity chromatography (Cuatrecasas *et al.*, 1968; Cuatrecasas and Anfinsen, 1971; Givol *et al.*, 1970) with specific antiarsanilazotyrosyl antibodies. By this procedure an arsanilazotyrosyl peptide is isolated in more than 90% yield. Its sequence demonstrates the fact that tyrosine-248 is the residue modified.

Materials

Carboxypeptidase A crystals prepared according to the procedure of Anson (1937) (Worthington Biochemical Corp.) were labeled with diazotized *p*-arsanilic acid and characterized

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