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The Chloroperoxidase-Catalyzed Oxidation of Thiols and Disulfides to Sulfenyl Chlorides†

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ABSTRACT: The reactions of the heme-containing enzyme chloroperoxidase with aromatic thiols and disulfides were studied. In a reaction requiring inorganic chloride and hydrogen peroxide, the enzyme rapidly oxidized 2-nitro-5-thiobenzoic acid (TNB) or 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to 3-carboxy-4-nitrobenzenesulfenic acid chloride. This latter compound decomposed nonenzymatically to the corresponding sulfonic acid, 3-carboxy-4-nitrobenzenesulfonic acid. The enzyme first oxidized TNB to DTNB before formation of the sulfenyl chloride. To identify the above compounds, the authentic sulfenyl chloride and sulfonic acid were prepared and characterized. The synthesis of the proposed sulfenyl chloride by this enzyme was established by comparing the ultraviolet absorbance spectra of the enzyme-generated compound with the authentic compound. In addition, kinetic studies indicated that the authentic sulfenyl chloride and the enzymatic product were equivalent. Both sulfenyl chlorides hydrolyzed to the sulfonic acid at equal rates.

Much of the interest in chloroperoxidase is due to its functional similarity with the thyroglobulin-iodinating enzyme found in the mammalian thyroid (Morris and Hager, 1966a). Both enzymes catalyze the iodination of tyrosine (Thomas and Hager, 1969) and are inhibited by antithyroid agents (Morris and Hager, 1966a). One type of antithyroid agent contains the thiourylene group and includes such compounds as thiourea, thiouracil, and 5-vinyl-2-oxazolidinethione. A previous study (Morris and Hager, 1966a) showed that chloroperoxidase oxidized thiourea and thiouracil to the corresponding disulfides in a reaction requiring enzyme, hydrogen peroxide, and inorganic chloride. To account for the halide requirement of the reaction, a sulfenyl chloride (RSCl) was proposed to be the product of the enzymatic reaction. The proposed intermediate then reacted with another substrate molecule to form the disulfide. However, the proposed sulfenyl chloride intermediates were not isolated or trapped. This was probably due to the instability of the sulfenyl chlorides derived from the thiourylene group and an insufficient concentration of the trapping reagent.

In the present study, the reaction of chloroperoxidase with thiols was reexamined in the hope of demonstrating the formation of the sulfenyl chlorides. This in turn could provide information about the mode of inhibition and the reactive halide species generated by the enzyme. Because the previous study used substrates that probably produced unstable sulfenyl chlorides, we sought other compounds that might yield stable sulfenyl chlorides. Compounds that appeared likely to satisfy this requirement included aromatic sulfenyl halides with either nitro- or azobenzene substituents (Scoffone *et al.*, 1968). Thus, TNB was selected for further study because the nitro group imparts favorable spectral properties facilitating identification of the various species and stabilizes the sulfenyl chloride, the carboxy group gives good solubility, and the disulfide, a convenient precursor of the thiol and the sulfenyl chloride, is readily available. We found that chloroperoxidase did react with this thiol to give a product which was identified as the corresponding sulfenyl chloride.

Experimental Section

Materials. DTNB[1] was obtained from Sigma and East-

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[1] Abbreviations used are: CPO, chloroperoxidase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoic acid; TNB-Cl, 3-carboxy-4-nitrobenzenesulfenic acid chloride; TNB-SO_3H, 3-carboxy-4-nitrobenzenesulfonic acid.

man. Enzyme used in these experiments had A_{403}/A_{280} = 1.2 (Morris and Hager, 1966b). Reagent grade salts and glass-distilled water were used throughout. Commercial bleach (Chlorox) was used as the source of HOCl. Its concentration was determined by titration with iodide as described below.

Synthesis of TNB. Four grams each of DTNB and cysteine hydrochloride were dissolved in about 30 ml of water and neutralized to about pH 6 with 1 N NaOH. After the solution became a deep red, it was acidified to about pH 3 with 1 N HCl and the water was removed on a rotary evaporator. The product was extracted with hot ether which was evaporated. The product was crystallized from hot ether and *n*-pentane and dried *in vacuo* at room temperature. The reddish-orange needles melted at 143.5–146.5° (lit. 137–8°, Degani and Patchornik, 1971). The mass spectrum showed a parent peak at *m/e* 199. The product contained more than 96% thiol as determined by its absorbance at 412 nm pH 6.1) assuming a molar extinction coefficient of 13,600 $A_{412}M^{-1}$ cm^{-1} (Ellman, 1959).

Synthesis of TNB-Cl. The material used for analytical purposes was synthesized in the following manner; 1 g of DTNB was dissolved in 25 ml of dry ethylene chloride and heated to reflux. A trace of iodine was added and a gentle stream of chlorine was introduced. Refluxing was continued for about 10 min after which time most of the starting material was dissolved giving the solution a deep brown color. The solution was cooled and the solvent containing the product was decanted. Excess chlorine, iodine, and solvent were removed using the rotary evaporator until the product was odorless. The product was a dark oil and stored *in vacuo* over P_2O_5 or was frozen. The purity of the sulfenyl chloride was determined by its reduction of iodide to triiodide followed by the titration of the triiodide with a standard sodium thiosulfate solution (eq 1). The triiodide was

$$2RSCl + 3I^- \longrightarrow RSSR + I_3^- + 2Cl^- \quad (1)$$

also determined spectrally by the following method. About 2 μl of the oil was weighed into 1 or 3 ml of dry acetonitrile or ethylene chloride. An aliquot of 50 μl of this solution was added to 3 ml of 10 mM HCl and 10 mM KI. The absorbance at 350 nm was determined from which the triiodide concentration was calculated using a molar extinction coefficient of 25,470 A_{350} M^{-1} cm^{-1}. This value was determined using a standard triiodide solution prepared from recrystallized reagent grade $NaIO_3$. A blank in which the KI was omitted was also measured. The purity of the TNB-Cl based upon this method was 90%. The parent peak in the mass spectrum occurred at *m/e* 233. *Anal.* Calcd for $C_7H_4ClNO_4S$: C, 35.98; H, 1.73; N, 5.97; Cl, 15,18: Found: C, 32.43; H, 1.69; N, 5.21: Cl, 14.83.

Synthesis of TNB-SO_3H. The corresponding sulfonic acid was prepared by the hydrolysis of the sulfenyl chloride in dilute NaOH. After several hours, the mixture was acidified with HCl, the solvent was evaporated, and the product was extracted with hot acetonitrile. Tan crystals were obtained from ether and were dried in a drying pistol over refluxing methanol. The product decomposed at 215°. The mass spectrum showed a parent peak at *m/e* 247. *Anal.* Calcd for $C_7H_5NO_7S$: C, 34.03; H, 2.02; N, 5.67; S, 12.95. Found: C, 33.94; H, 1.85; N 6.01; S, 12.47.

Methods. Kinetic and spectral measurements were performed at 25.0 ± 0.3° on a Cary 15 spectrophotometer or a stopped-flow spectrophotometer (Hager *et al.*, 1971). Rate measurements in the Cary 15 were performed by placing aliquots of the various substrates and enzyme (2–100 μl) on the flattened end of a glass rod and stirring them into 3 ml of 0.1 M potassium buffer (pH 2.75).

The reaction of CPO and TNB or DTNB was performed in the presence of 15.2 mM KCl, 2.62 mM H_2O_2, 0.103 mM TNB or DTNB, and 1.5 nM CPO in 3.32 ml of 0.1 M potassium phosphate buffer, (pH 2.75). The initial reaction was observed for about 1 min at 340 nm. The wavelength was then changed to 245 nm to follow the slow phase of the reaction.

In order to observe the synthesis of TNB-Cl directly from TNB, the following concentrations of reactants were used: 5.16 μM TNB, 81.7 μM KCl, 1.12 mM H_2O_2, and 0.13 μM CPO. The rate of the fast phase of the enzyme reaction was observed using a stopped-flow spectrophotometer. The concentration of reactants for these studies were 26.3 μM TNB, 83.3 μM KCl, 1.14 mM H_2O_2, and 0.27 μM CPO. Both reactions were followed at 340 nm and 25°. In other kinetic experiments on the Cary, hypochlorous acid (2.3 mM) was substituted for enzyme, KCl, and H_2O_2. The concentrations of TNB and DTNB were 63.4 and 34.3 μM, respectively.

Results

Product Characterization. The identity of TNB was established by mass and absorbance spectroscopies as described in the Experimental Section. Since TNB is a relatively stable product, its characterization did not present any special problems. However, the instability of the corresponding sulfenyl chloride presented unusual difficulties in characterization. The presence of the sulfenyl chloride could be confirmed by showing that the product of the synthesis gave the expected parent peak in the mass spectrum and was 90% pure as determined by iodometry. The poor agreement of the theoretical and observed elemental analyses was undoubtedly due to the instability of the TNB-Cl. Despite repeated attempts under a wide variety of conditions, the TNB-sulfenyl chloride could not be crystallized. In those instances when a solid was obtained, it consisted of the disulfide and/or sulfonic acid. Thin-layer chromatography on silica was also unsatisfactory. All of these observations illustrate the instability of the TNB-Cl toward hydrolysis and oxidation.

Other synthetic routes known to yield sulfenyl chlorides were used to produce TNB-Cl. These methods included the chlorination of TNB or DTNB in CCl_4, the *in situ* reaction of TNB or DTNB with HOCl, and the *in situ* reaction of TNB or DTNB with *N*-chlorosuccinimide. In all these cases, the absorbances of the products obtained were identical with each other and with that of the material prepared for elemental and mass spectroscopic analyses (see the Experimental Section). Finally, all of the synthetic TNB-Cl's hydrolyzed to yield a compound with the spectrum of TNB-SO_3H. The rates of decomposition were identical and were first-order for at least 5 half-lives (k = 3.05 ± 0.13 × 10^{-3} sec^{-1}).

The homologous sulfonic acid (TNB-SO_3H) gave the expected results in mass spectroscopy and satisfactory elemental analysis could be obtained for this derivative.

The Enzymatic Synthesis of TNB-Cl. When TNB, H_2O_2, and KCl were mixed together in a cuvet placed in the Cary 15 spectrophotometer, no spectral change was observed at 340 nm (Figure 1). When chloroperoxidase was added to the reaction mixture, the absorbance decreased

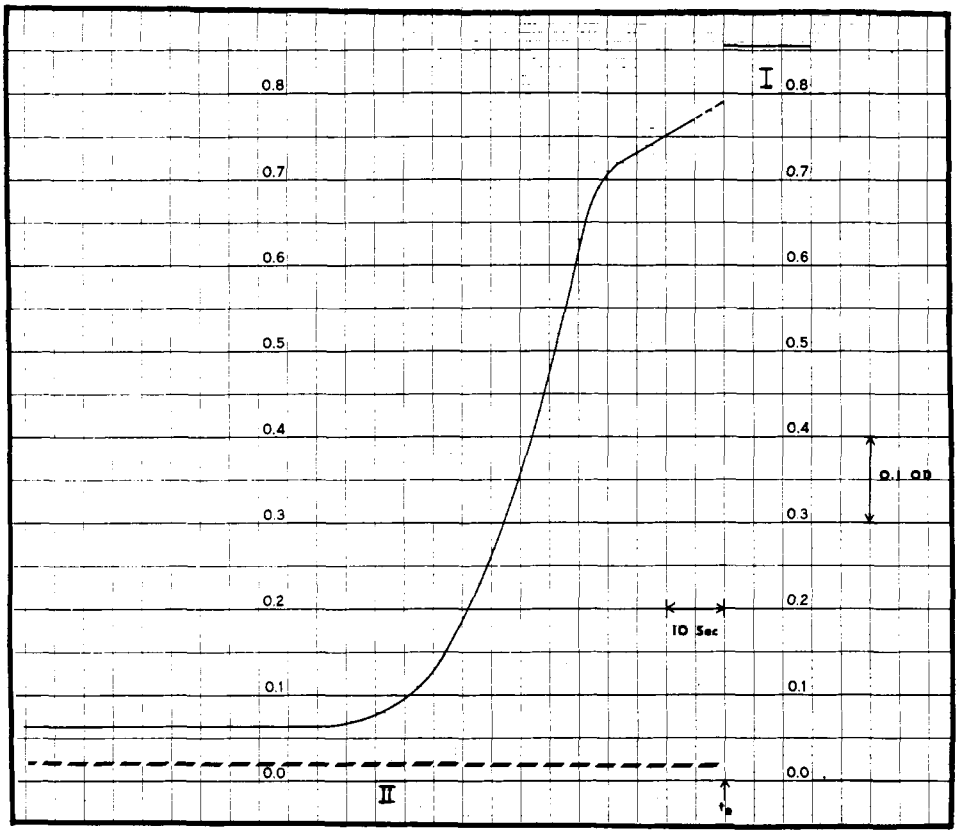

FIGURE 1: The time course of the reaction of CPO, TNB, KCl, and H_2O_2. The experimental conditions were 15 mM KCl, 2.6 mM H_2O_2, 0.103 mM TNB, and 1.5 nM CPO in a total volume of 3.32 ml of 0.1 M potassium phosphate buffer (pH 2.75). The dashed line near phase I represents the extrapolated absorbance from when the recording began to zero time. The heavy broken line denotes phase II. The reaction was monitored at 340 nm and the direction of the time scale is right to left.

within the first 5 sec. (phase I). This fast decrease could be revealed by extrapolation of the slope of the initial rate to zero time as shown in Figure 1. A second decrease in absorbance at 340 nm could then be measured (Figure 1, phase II) and was completed within 70 sec. There were no further detectable absorbance changes for at least 50 sec. The spectrum of the reaction mixture at the end of phase II was recorded between 230 and 350 nm, then the wavelength was set to 245 nm and a much slower spectral change was recorded as a function of time (phase III). The observed kinetics of the phase III spectral change were first order for at least 5 half-lives ($k = 2.9 \times 10^{-3}$ sec^{-1}, phase III not shown in Figure 1). The absorbance spectrum between 230 and 350 nm was again recorded at the end of phase III. In order to interpret the chemical changes taking place in the enzymatic reaction mixture, the spectra recorded at the ends of phases II and III (Figure 2) were compared with the spectra of the synthetic TNB-Cl and TNB-SO_3H. This comparison indicated that TNB-Cl and TNB-SO_3H were the products of the phase II and III reactions, respectively. It was possible to record an accurate spectrum at the end of phase II because only 50 sec were required for the scan. Since the kinetics of phase III were slow with respect to phase II, the 50-sec scan only allowed about one-quarter of the first half-life of decomposition of TNB-Cl to occur. The product of phase III was established to the sulfonic acid, TNB-SO_3H. The final spectrum of the enzymatic product was identical with that of the authentic sulfonic acid. Additional support for the identities of TNB-Cl and TNB-SO_3H came from the rate of hydrolysis of the sulfenyl chloride to the sulfonic acid. The observed first-order rate constants for the hydrolysis of TNB-Cl, both enzymatically generated and chemically generated TNB-Cl, were equal and were independent of enzyme, peroxide, and chloride concentrations.

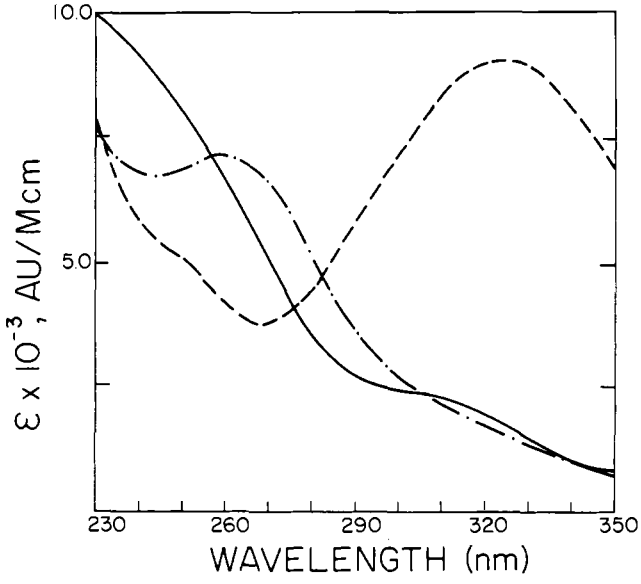

FIGURE 2: Spectra of compounds observed during the enzymatic oxidation of TNB. The experimental conditions were the same as in Figure 1 except the concentration of TNB was 57.7 μM. After the initial spectral change as in Figure 1 (phase II), the spectrum was rapidly scanned and the wavelength reset to 245 nm to observe the decomposition of TNB-Cl. Symbols: (- - -) TNB; (—) TNB-Cl; (- · - ·) TNB-SO_3H.

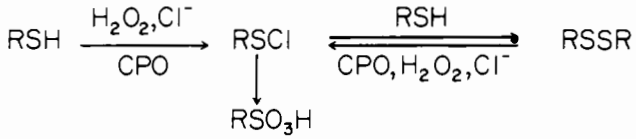

FIGURE 3: Proposed mechanism of oxidation of bivalent sulfur compound by chloroperoxidase. The results with TNB and DTNB are summarized in the above scheme where R denotes TNB.

Intermediate Formation of DTNB. Although phase I was too rapid to be observed on the Cary 15 spectrophotometer, it could be observed on a stopped-flow instrument. When the reaction was studied in this way, a linear decrease in absorbance at 340 nm lasting about 1 sec occurred. No time lag could be detected. The size of the absorbance decrease indicated the oxidation of TNB to DTNB. To reconfirm this, an experiment was conducted on the Cary 15 using less enzyme than in the stopped-flow spectrophotometer experiment. At the end of the reaction, the spectrum of the reaction mixture was recorded. The absorption spectrum at the end of phase I was identical with that of authentic DTNB. Finally, cysteine was added to the cuvet. Cysteine would be expected to react with DTNB and convert it back to TNB. The spectral changes induced by adding cysteine to the reaction mixture were consistent with the presence of DTNB. After cysteine addition, there was increased absorbance at 412 nm and the spectrum of the reaction mixture was identical with that of authentic TNB. Lastly, the enzyme reaction was quenched at the end of phase I by adjusting to pH 8.8. CPO is inactive at this pH. The spectrum of DTNB was recorded in the quenched reaction.

If DTNB was indeed the product of phase I, then the sudden decrease observed in Figure 1 should be eliminated by using DTNB rather than TNB in the enzymatic reaction. Furthermore, phases II and III should still produce TNB-Cl and TNB-SO_3H as their respective products and the kinetics of phases II and III should be unchanged. These predictions were confirmed when DTNB was substituted for TNB in an experiment similar to the one carried out in Figure 1. When DTNB was the substrate, the early linear portion of phase II could be extrapolated to zero time and yielded the absorbance value which was observed prior to adding chloroperoxidase. The recorder tracing for phase II of the DTNB reaction was superimposable over that of phase II with TNB. Phase III was also observed to be kinetically identical with phase III of the TNB reaction. Lastly, TNB-Cl and TNB-SO_3H were identified spectrally. Therefore, these experiments show that chloroperoxidase can catalyze the oxidation of TNB to DTNB. In addition, these experiments also show that DTNB is the precursor of TNB-Cl.

In some experiments, hypochlorous acid was substituted for the enzymatic oxidation system. At hypochlorous acid, TNB, and DTNB concentrations comparable to those used in the CPO reaction mixtures, TNB-Cl was formed stoichiometrically in less than 10 sec. Hence, the oxidation of DTNB must be the rate-determining step in the enzymatic reaction.

Discussion

These results show that the chloroperoxidase-catalyzed reaction with bivalent sulfur compounds is complex. The overall stoichiometry is shown in eq. 2. However, the overall

$$\text{TNB} + H_2O_2 + O_2 \longrightarrow \text{TNB-}SO_3H + H_2O \qquad (2)$$

reaction may be resolved into several distinct steps. When TNB is the substrate, the first step is its oxidation to TNB-Cl catalyzed by CPO (eq 3). The sulfenyl chloride could not

$$\text{TNB} + H_2O_2 + Cl^- \xrightarrow{\text{CPO}} \text{TNB-Cl} + H_2O + OH^- \qquad (3)$$

$$\text{TNB-Cl} + \text{TNB} \longrightarrow \text{DTNB} + HCl \qquad (4)$$

$$\text{DTNB} + H_2O_2 + 2Cl^- \xrightarrow{\text{CPO}} 2\text{TNB-Cl} + 2OH^- \qquad (5)$$

$$\text{TNB-Cl} + OH^- + O_2 \longrightarrow \text{TNB-}SO_3H + Cl^- \qquad (6)$$

be observed in the presence of excess TNB which rapidly and nonenzymatically reacts to give DTNB (eq 4). CPO then oxidizes the DTNB to give TNB-Cl as in eq 5. In the absence of strong nucleophiles, this decomposes nonenzymatically to give TNB-SO_3H (eq 6). Figure 3 summarizes the various steps in this entire system.

Although the initial purpose of this study was to identify the intermediates in the chloroperoxidase-catalyzed reaction of bivalent sulfur compounds, the elucidation of the various steps in the overall reaction did not explain the role of the enzyme in greater detail. The only steps catalyzed by chloroperoxidase are the oxidations of TNB and DTNB to TNB-Cl (eq 3 and 5) and knowledge of this fact can allow one to focus attention on this simple oxidation rather than the more complicated reaction to the sulfonic acid. Based upon this information and the previous understanding of the mechanism of action of this enzyme, one may reasonably speculate that chloroperoxidase oxidizes a chloride ion to a chloronium ion or its equivalent using hydrogen peroxide as the oxidant. Whether the TNB is then oxidized on the surface of the enzyme by this chloronium species or in free solution by hypochlorous acid produced by a rapidly diffusing chloronium species is an intriguing question yet to be answered. Further experiments will thus be necessary to describe the oxidation of these bivalent sulfur compounds by chloroperoxidase.

In conclusion, the reaction of chloroperoxidase and bivalent sulfur compounds involves several distinct steps (Figure 3). Extrapolating these results to the mammalian thyroglobulin-iodinating enzyme, these compounds possibly inhibit physiological iodination in two ways. First, the inhibitors consume the enzymatically generated halogenating species faster than do the substrate proteins. Second, the inhibitors also consume the oxidizing agents required to produce the biological halogenating species.

References

Brown, F. S., and Hager, L. P. (1967), *J. Amer. Chem. Soc. 89,* 719.

Degani, Y., and Patchornik, A. (1971), *J. Org. Chem. 36,* 2727.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70.

Hager, L. P., Doubek, D. L., Silverstein, R. M., Harges, J. H., and Martin, J. C. (1972), *J. Amer. Chem. Soc. 94,* 4364.

Hager, L. P., Doubek, D. L., Silverstein, R. M., Lee, T. T., Thomas, J. A., Harges, H. H., and Martin, J. C., (1971), The Mechanism of Oxygen Evolution from Peroxy Acid by Chloroperoxidase: A Contribution to the Structure of

Compound I, The Second International Conference on Oxidation and Reduction, Memphis, Tenn., June 1971.
Morris, D. R., and Hager, L. P. (1966a), *J. Biol. Chem. 241* 3582.
Morris, D. R., and Hager, L. P. (1966b), *J. Biol. Chem. 241,* 1763.
Scoffone, E., Fontana, A., and Focchi, R. (1968), *Biochemistry 7,* 971.
Silverstein, R. M. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31,* 906.
Thomas, J. A., and Hager, L. P. (1969), *Biochem. Biophys. Res. Commun. 35,* 444.

Use of Mutants in the Study of Aminocyclitol Antibiotic Biosynthesis and the Preparation of the Hybrimycin C Complex†

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ABSTRACT: A technique previously used to isolate a mutant of *Streptomyces fradiae* capable of synthesizing the antibiotic neomycin only in the presence of the subunit 2-deoxystreptamine has been applied to *S. rimosus* forma *paromomycinus* and *S. kanamyceticus* to produce mutants capable of synthesizing paromomycin and kanamycin, respectively, only in the presence of added 2-deoxystreptamine subunit. Neamine, paromamine, and 6-kanosaminido-2-deoxystreptamine—2-deoxystreptamine-containing glycosides that are plausible intermediates in the biosynthesis of the three antibiotics—were also tested as substrates. The mutants studied did not convert the glycosides to active antibiotics. The mutant of *S. rimosus* forma *paromomycinus* converts streptamine, an analog of 2-deoxystreptamine, to two new antibiotics, hybrimycins C1 and C2, analogs of paromomycins I and II, respectively, in which the only modification is that 2-deoxystreptamine has been replaced by streptamine. Selective hydrolysis yielded a third new antibiotic, hybrimycin C3, an analog of paromamine.

While considerable effort has been devoted to the study of the biosynthesis of streptomycin (Horner, 1967; Demain and Inamine, 1970; Walker, 1971), relatively little attention has been directed toward the study of the biosynthesis of another important group of aminoglycoside antibiotics, the deoxystreptamine antibiotics, despite the clinical importance of nearly every member of the group. This group has been assigned over 25 members to date (Rinehart, 1969; Benveniste and Davies, 1973), including the neomycins, the kanamycins, and the paromomycins. The close structural similarity of the latter three antibiotics has prompted the suggestion that a corresponding similarity prevails in their biosynthesis (Rinehart, 1964). The studies on the biosynthesis of neomycin carried out by Rinehart and coworkers, using the technique of microbiological incorporation of radioactively labeled precursors followed by degradation of the labeled antibiotic to determine the distribution of label (Rinehart, 1964) and the technique of carbon-13 incorporation and carbon magnetic resonance spectroscopy (Rinehart *et al.,* 1974), established that certain parallels exist between the biosynthesis of streptomycin and neomycin, most notably the ability of D-glucose to provide the entire carbon skeleton of both antibiotics (Rinehart *et al.,* 1974; Horner, 1967). The biosynthesis of the aminocyclitol moiety of the two antibiotics appears to be distinctly different, however, even though streptamine (the aminocyclitol moiety of streptomycin) differs from deoxystreptamine (the aminocyclitol moiety of neomycin) only in that the former bears an additional hydroxyl group. Labeled streptamine was not incorporated either effectively or specifically into the streptidine moiety of streptomycin by *Streptomyces griseus* (Hunter and Hockenhull, 1955), consistent with the subsequent (Walker and Walker, 1967) finding that the streptidine moiety is biosynthesized without employing underivatized streptamine as an intermediate; in contrast, ^{14}C-labeled deoxystreptamine was incorporated specifically, and with a high per cent incorporation into the deoxystreptamine moiety of neomycin by *S. fradiae,* suggesting that deoxystreptamine appears underivatized on the biosynthetic pathway (Falkner, 1969).

We have reported (Shier *et al.,* 1969) the isolation of a mutant of *S. fradiae* capable of synthesizing neomycin only in the presence of exogenous 2-deoxystreptamine, and the use (Shier *et al.,* 1972) of the mutant to incorporate two synthetic analogs of the 2-deoxystreptamine subunit, streptamine and 2-epistreptamine, into four new antibiotics, hybrimycins A1 and A2 (from streptamine, analogs of neomycins B and C, respectively) and hybrimycins B1 and B2 (from 2-epistreptamine, further analogs of neomycins B and C, respectively). Selective hydrolysis of the hybrimycin A and B complexes yielded two more antibiotics (Shier *et al.,* 1970), hybrimycins A3 and B3, respectively (analogs of another neomycin component, neamine). The existence of this mutant supports the conclusion that 2-deoxystreptamine appears underivatized on the biosynthetic pathway of neomycin. We have also reported (Shier *et al.,* 1973) the specificity of the *S. fradiae* mutant and of the mutants described in the present report (2-deoxystreptamine-negative mutants

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