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†

W. Thomas Shier, Phillip C. Schaefer, David Gottlieb, and Kenneth L. Rinehart, Jr.*

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and C2, analogs of paromomycins I and II, respectively, in which the only modification is that 2-deoxystreptamine has been replaced by streptamine.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

† From the School of Chemical Sciences (W.T.S., P.C.S., K.L.R.) and the Department of Plant Pathology (D.G.), University of Illinois, Urbana, Illinois 61801. *Received June 20, 1974.* This work was supported by research grants (AI 01278 and AI 04769) from the National Institute of Allergy and Infectious Diseases.

of the producing organisms of paromomycin and kanamycin, *S. rimosus* forma *paromycinus* and *S. kanamyceticus*) in accepting deoxystreptamine analogs. We report here the use of these three mutants in studies of the biosynthesis of the respective antibiotics and report the preparation of analogs of paromomycins I and II and paromamine.

Experimental Procedure

Materials. Deoxystreptamine dihydrobromide was prepared from neomycin (The Upjohn Co.) by the method of Dyer (1954). Neamine was provided by The Upjohn Co. Paromamine was prepared from paromomycin sulfate (Humatin, Parke, Davis & Co.) by the method of Frohardt *et al.* (1959). Kanosaminidodeoxystreptamine was prepared from kanamycin sulfate (Kantrex, Bristol Laboratories) by the method of Maeda *et al.* (1958). Streptamine hydrosulfate was prepared from dihydrostreptomycin (Eli Lilly and Co.) by the method of Peck *et al.* (1946). Ribostamycin (Akita *et al.*, 1970) was obtained from Dr. E. Akita, Meija Seika Kaisha, Ltd.

Methods. Nuclear magnetic resonance spectra were obtained on a Varian Associates spectrometer, Model HA-100, optical rotations on a Zeiss polarimeter or on a JASCO spectropolarimeter, ORD/UV-5, and mass spectra on an Atlas mass spectrometer, Model CH4, with an oven inlet lock. Vapor phase chromatography was carried out on pertrimsylated (Millington *et al.*, 1974) derivatives of antibiotics (Tsuji and Robertson, 1969, 1970). Paper chromatography was carried out in a descending direction using Whatman 3MM paper for preparative procedures and Whatman No. 1 for analytical procedures.

Isolation of D⁻ Mutant of S. kanamyceticus (ATCC 12853). A nearly monoconidial preparation of 5-day-old *S. kanamyceticus* spores grown on V-8 agar (Diener, 1953) was prepared, and treated for 6 hr, as described for *S. fradiae*, with 2 mg of nitrosoguanidine in 100 ml of a medium prepared by dissolving 1.75 g of dehydrated Antibiotic Assay Broth (Baltimore Biological Laboratory) in 100 ml of water. After incubation, 1-ml aliquots were diluted tenfold in sterile water, and 1-ml aliquots of the diluted suspension were transferred to V-8 agar slants and incubated at 26° for at least 5 days. The growth from one of these slants was transferred to 100 ml of sterile water in a Waring Blendor, and blended for 8 min. One-milliliter aliquots were subjected to serial dilution through three tubes containing 9 ml of sterile water, V-8 plates were inoculated with 1-ml aliquots of the final dilution, and treated as described for *S. fradiae* (Shier *et al.*, 1969) except that replica plates were incubated at 26° for 48 hr before they were overlaid with seeded agar.

Isolation of D⁻ Mutant of S. rimosus forma paramomycinus (ATCC 14827). A nearly monoconidial suspension of *S. rimosus* spores grown on V-8 agar slants was prepared, treated for 12–14 hr with 3 mg of nitrosoguanidine in 100 ml of Antibiotic Assay Broth medium, and treated subsequently as described for *S. kanamyceticus*, except that serial dilution of the final spore suspension through four tubes of 9 ml of sterile water was required, and that replica plates were incubated 50–52 hr before they were overlaid with seeded agar.

Production of Hybrimycin C Complex. Hybrimycin C complex for structure proof and biological testing was produced and isolated as follows. A suspension of spores of one of the deoxystreptamine-negative mutants was used to inoculate a 500-ml flask containing 100 ml of a medium of the following composition: cerelose, 1 g; soybean meal, 1 g; casein, 0.25 g; sodium chloride, 0.5 g; calcium carbonate, 0.5 g; ammonium chloride, 0.167 g; distilled water to volume. The medium was adjusted to pH 7.5 prior to sterilization. The inoculated medium was incubated 72 hr at 26° with reciprocal shaking and 7.5-ml aliquots were used to inoculate 500-ml production flasks containing 100 ml of the same medium supplemented with 40 mg of streptamine hydrosulfate. The production flasks were incubated 120 hr at 26° with reciprocal shaking and then the contents were pooled and filtered. The antibiotic was isolated from the filtered broth by adsorption on Amberlite IRC 50 resin in the ammonium form followed by elution with 2 N ammonium hydroxide solution. Evaporation of the eluates yielded crude hybrimycin C, which was purified by preparative paper chromatography in the solvent system methanol–concentrated ammonium hydroxide (4:1). Repeated preparative paper chromatography in the same system separated the antibiotic complex into two components, hybrimycins C1 and C2.

Preparation of Hybrimycin C3. Hybrimycin C3 was prepared from hybrimycin C complex by selective hydrolysis in 0.5 N hydrochloric acid (Haskell *et al.*, 1959). The product was purified by preparative paper chromatography in the solvent system methanol–concentrated ammonium hydroxide (4:1). The antibacterial activity of the hybrimycins was assessed by the broth dilution technique. The antibiotic preparations were dissolved in brain heart infusion broth and twofold decrements were made from 50 μg/ml. An 18-hr culture of each test organism was diluted 1:2000 and 1 drop of the diluted suspension was added to 1 ml of broth containing the antibiotic. The test system was estimated to contain 10^5 organisms/ml. All tubes were incubated at 37° for 24 hr. The minimum inhibitory concentration (MIC) is the lowest concentration of antibiotic which prevented growth.

Evaluation of Plausible Biosynthetic Precursors of Antibiotics. The plausible biosynthetic precursors except ribostamycin were tested for bioconversion to active antibiotics by addition of a solution containing the required amount of sample to a 1-day-old streak colony of the 2-deoxystreptamine-negative mutant of *S. fradiae* on nutrient agar in a petri plate. The plates were incubated 2 days at 26°, overlaid with Streptomycin Bioassay agar (Difco), seeded with *Bacillus subtilis*, stored briefly at 0–4°, and incubated at 26° for 14 hr. A zone of inhibition of growth of *B. subtilis* around the colony of the 2-deoxystreptamine-negative mutant indicated antibiotic production. As a control, 200 μg of deoxystreptamine was included with the plausible precursor at each concentration tested. Under these conditions antibiotic production was observed in each case, indicating that the added plausible precursor did not repress antibiotic production in the original experiment.

Ribostamycin was tested for bioconversion to neomycin by the 2-deoxystreptamine-negative mutant in submerged culture employing standard neomycin production conditions (Shier *et al.*, 1969, 1972) except that after 48 hr of incubation 20 mg of ribostamycin dissolved in sterile water was added to each of three 100-ml production flasks and incubation was continued an additional 72 hr. The antibiotics in the filtered culture broths were extracted by the standard neomycin isolation procedure, *i.e.*, by absorption of the antibiotic from the filtered culture broth on Amberlite IRC 50 resin in the ammonium form followed by elution with 2 N ammonium hydroxide and lyophilization. The resultant ma-

TABLE I: Antibiotic Production by Mutant Organisms in the Presence of Added Plausible Intermediates.

2-Deoxystreptamine-Negative Mutant	Plausible Intermediate Added	Antibiotic Production from Amount Indicated Added to 10 ml of Nutrient Agar			
		50 μg	100 μg	150 μg	200 μg
S. fradiae	2-Deoxystreptamine[a]	+	+	+	+
	Neamine	−	*b*	*b*	*b*
	Paromamine	−	−	−	−
	6-Kanosaminido-2-deoxystreptamine	−	−	−	−
S. kanamyceticus	2-Deoxystreptamine[a]	+	+	+	+
	Neamine	−	*b*	*b*	*b*
	Paromamine	−	−	−	−
	6-Kanosaminido-2-deoxystreptamine	−	−	−	−
S. rimosus forma *paromomycinus*	2-Deoxystreptamine[a]	+	+	+	+
	Neamine	−	*b*	*b*	*b*
	Paromamine	−	−	−	−
	6-Kanosaminido-2-deoxystreptamine	−	−	−	−

[a] Added as the dihydrobromide. [b] Neamine added at this concentration killed all the test organisms.

terial was analyzed by chromatography on a column of Dowex 1-X2 resin, 200–400 mesh, in the hydroxide form. Elution with water yielded four ninhydrin positive peaks which were analyzed by vapor phase chromatography of the trimsyl derivatives or by paper chromatography in the solvent system methyl ethyl ketone–*tert*-butyl alcohol–methanol–6.5 N ammonium hydroxide (80:15:5:30).

Results

Isolation of 2-Deoxystreptamine-Negative Mutants. 2-Deoxystreptamine-negative mutants of *S. rimosus* forma *paromomycinus* ATCC 14827 and of *S. kanamyceticus* ATCC 12853 were isolated in a screening program similar to that described for the isolation of the corresponding mutant of *S. fradiae* (Shier *et al.*, 1969). A nearly monoconidial preparation of spores in nutrient broth was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to give a 99.9% kill, and the survivors were grown on a sporulating medium. Spores from the second sporulation were screened as described previously (Shier *et al.*, 1969) for a mutant showing bacterial antagonism only in the presence of exogenous 2-deoxystreptamine. Two 2-deoxystreptamine-negative mutants of *S. rimosus* forma *paromomycinus* were isolated from approximately 5000 clones screened in this manner. One of these, a sporulating revertant of a nonsporulating strain, produced substantially less antibiotic in the presence of the same concentration of deoxystreptamine than did the other, which was morphologically indistinguishable from the parent strain. One 2-deoxystreptamine-negative mutant of *S. kanamyceticus* was isolated from approximately 1800 clones screened.

Conversion of Plausible Biosynthetic Intermediates to Antibiotics. A series of plausible intermediates in the biosynthesis of neomycin, kanamycin, and paromomycin containing one, two, or three of the subunits of the intact antibiotics (see Figure 1) was obtained by selective degradation of the parent antibiotics or, in the case of ribostamycin, from another antibiotic producing organism. The plausible mono- and disaccharide intermediates listed in Table I were presented to the mutant organisms growing on nutrient agar. 2-Deoxystreptamine was bioconverted to active antibiotics by all of the mutants under these conditions but none of the disaccharide fragments was bioconverted to an active antibiotic by any of the mutants. The trisaccharide antibiotic ribostamycin was tested for bioconversion to neomycin by the 2-deoxystreptamine negative mutant of *S. fradiae* in submerged culture. The antibiotics in the culture broth were extracted by the same procedure used to isolate the neomycins (Hessler *et al.*, 1970). This procedure yielded only ribostamycin as the major component and three minor components did not correspond to 2-deoxystreptamine, neamine, neomycin B, or neomycin C.

Structure Determination of Hybrimycins C1, C2, and C3. The structures of hybrimycins C1 and C2 were established by comparison of the mass spectral, nuclear magnetic resonance (nmr), optical rotatory, and paper chromatographic properties of appropriate derivatives with the corresponding derivatives of paromomycins I and II. The mass spectra of penta-*N*-acetyl-nona-*O*-trimsylhybrimycins C1 and C2, prepared in the usual manner (Shier *et al.*, 1969), were identical (establishing that they differ only in stereochemistry) and similar to the mass spectrum of the corre-

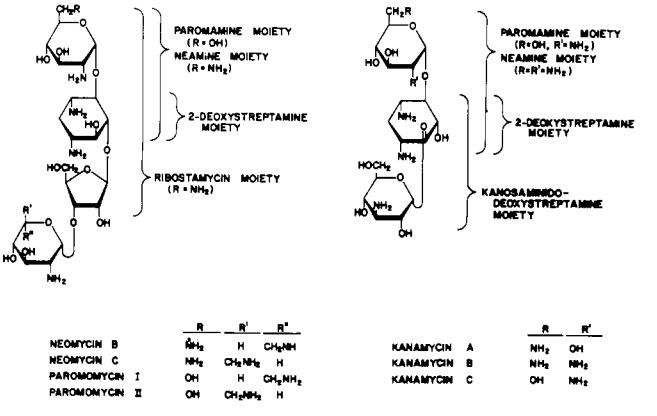

FIGURE 1: Structures of the 2-deoxystreptamine antibiotics studied, with the subunits tested as intermediates indicated.

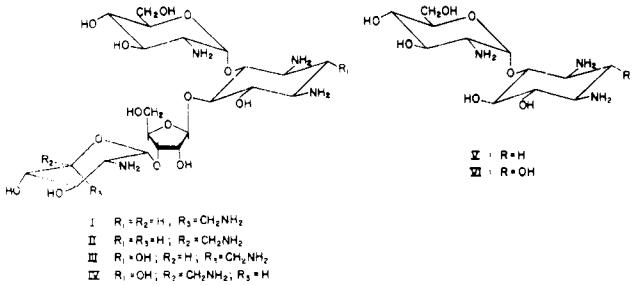

FIGURE 2: Structures of paromomycin I (I), paromomycin II (II), hybrimycin C1 (III), hybrimycin C2 (IV), paromamine (V), and hybrimycin C3 (VI).

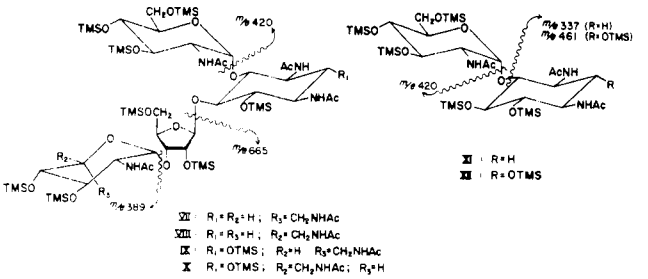

FIGURE 3: Diagnostic mass spectral fragmentations of the *N*-acetyl-*O*-trimsyl derivatives of the antibiotics paromomycin I (VII), paromomycin II (VIII), hybrimycin C1 (IX), hybrimycin C2 (X), paromamine (XI), and hybrimycin C3 (XII).

sponding derivative of paromomycin I (De Jongh *et al.*, 1967) except that numerous peaks were shifted 88 mass units higher (corresponding to an additional trimsyloxy group), including the molecular ion M (*m/e* 1489 *vs.* 1401), and the ions M − 15 (loss of methyl, *m/e* 1474 *vs.* 1386) and M − 90 (loss of trimethylsilanol, *m/e* 1399 *vs.* 1311). However, peaks at *m/e* 665, 420, and 389, produced by the fragmentations indicated in Figure 3, were not shifted in spectra of the derivatives of hybrimycins C1 and C2 as compared to derivatives of paromomycins I and II, thereby locating the extra trimsyloxy group in the aminocyclitol moiety. The nmr spectrum of the hybrimycin C complex showed no absorption by methylene protons (no ABX_2 multiplet in the region δ 1.75–2.40) characteristic of paromomycin and other 2-deoxystreptamine derivatives (Lemieux and Cushley, 1963), conclusively locating the extra hydroxyl group on the carbon between the two amino groups of the aminocyclitol moiety. The specific rotations and R_f values in the solvent system 1-butanol–pyridine–water (6:4:3) link penta-*N*-acetylhybrimycin C1, $[\alpha]_D^{19.5}$ +60.6° (*c* 0.01, water), R_f 0.36, to penta-*N*-acetylparomomycin I, $[\alpha]_D^{19.5}$ +64.5° (*c* 0.01, water), R_f 0.33, and penta-*N*-acetylhybrimycin C2, $[\alpha]_D^{19.5}$ +81.9° (*c* 0.01, water), R_f 0.24, to penta-*N*-acetylparomomycin II, $[\alpha]_D^{19.5}$ +87.8° (*c* 0.01, water), R_f 0.21. The specific rotations of the hybrimycin C1 and C2 derivatives are less dextrorotatory by 3.9 and 5.9°, respectively, than those of the corresponding paromomycin I and II derivatives, paralleling the levorotatory contribution of the additional hydroxyl group in hybrimycins A1, A2, and A3 relative to neomycins B and C and neamine, respectively, and in satisfactory agreement with the theoretical contribution of −5.4° calculated by Wiffen's method (Wiffen, 1956) and the contribution of −5.0 ± 0.5° calculated by Brewster's method (Brewster, 1959) for the C-2 hydroxyl in the configuration of streptamine. Hence, the antibiotics can be assigned the structures given in Figure 2.

The structure of hybrimycin C3 was established in a similar manner by comparing the spectral properties of appropriate derivatives with those of the corresponding derivatives of paromamine. Comparison of the mass spectra of the *N*-acetyl-*O*-trimsyl derivatives localized the additional hydroxyl group on the aminocyclitol moiety (see Figure 3), and the nmr spectra of the underivatized antibiotics further localized it to the carbon between the amino groups. The specific rotation of tri-*N*-acetylhybrimycin C3, $[\alpha]_D^{19}$ +85.7° (*c* 0.01, water), is in satisfactory agreement with the calculated value for the additional hydroxyl group in the same configuration as found in hybrimycins C1 and C2 and streptamine. Hence, hybrimycin C3 possesses the structure indicated in Figure 2.

Antibacterial Activities of Hybrimycins C1, C2, and C3. The antibacterial activities of the antibiotics of the hybrimycin C complex were compared with those of the paromomycin complex and paromamine for several pathogenic bacteria using a broth dilution assay (see Table II).

Discussion

The 2-deoxystreptamine-negative mutants of *S. fradiae*, *S. kanamyceticus*, and *S. rimosus* provide a convenient tool for studying the mechanism of assembly of the antibiotics produced by the unmutated organisms. A mutant that requires 2-deoxystreptamine to complete the synthesis of an antibiotic may also accept a deoxystreptamine derivative further down the biosynthetic pathway providing certain conditions are met. If, for example, neamine were to appear

TABLE II: Antibacterial Activities of Hybrimycins C1, C2, and C3.

Test Organism[a]	Minimum Inhibitory Concentration (μg/ml)				
	Paromomycin[b]	Hybrimycin C1	Hybrimycin C2	Paromamine	Hybrimycin C3
E. coli ATCC 26	17.8	25.0	50.0	>50	>50
S. aureus UC80	2.1	6.2	12.5	>50	>50
K. pneumoniae ATCC 10031	4.4	6.2	12.5	>50	>50
P. vulgaris ATCC 8427	8.9	25.0	50.0	>50	>50
Sarcina lutea UC 130	>35.7	>50.0	>50.0	>50	>50
Ps. fluorescens UC 3049	0.6	1.6	6.2	0.8	50
St. pyogenes UC 146	17.8	25.0	25.0	>50	>50
B. subtilis UC 564	1.1	3.1	3.1	>50	>50

[a] UC, The Upjohn Co.; ATCC, American Type Culture Collection. [b] Principally paromomycin I.

underivatized on the biosynthetic pathway to neomycin, then a D^- mutant is also a neamine negative mutant and exogenous neamine should allow production of antibiotic provided it can enter the cell. The failure to obtain bioconversion of the plausible disaccharide precursors to antibiotics in the present study might be explained in several ways.

(1) The order of subunit assembly in neomycin and paromomycin is one in which the ribose or neobiosamine moiety is linked to deoxystreptamine before neosamine or glucosamine. This explanation cannot be extended to the biosynthesis of kanamycin, however.

(2) Deoxystreptamine is converted to a derivative before the next subunit is attached, giving disaccharide intermediates that are also derivatized. No enzymes exist to convert the added disaccharides to the appropriate derivatives.

(3) The disaccharide stage of assembly is an enzyme-bound intermediate.

(4) The plausible disaccharide precursors did not enter the producing cells. This explanation is difficult to accept for the case of neamine, since at higher concentrations it is toxic to all three organisms.

Attempts to induce D^- mutants to produce antibiotics other than those produced by the parent organisms by use of appropriate substitutes (*i.e.*, neomycins B and C by *S. rimosus* in the presence of neamine, paromomycins I and II by *S. fradiae* in the presence of paromamine, and kanamycins B and C by *S. fradiae* and *S. rimosus*, respectively, in the presence of kanosaminidodeoxystreptamine) were uniformly unsuccessful (see Table I).

Although our mutants did not incorporate any of the appropriate disaccharide precursors, this does not seem to be a universal result for all deoxystreptamine producing organisms. Kojima and Satoh (1973) very recently reported that, using our procedure, a D^- mutant of *Streptomyces ribosidificus* incorporates neamine to give ribostamycin, 4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-5-*O*-(β-D-ribofuranosyl)-2-deoxystreptamine (neomycin B minus neosamine B; see Figure 1), and that it incorporates 3′,4′-dideoxyneamine to give the corresponding ribostamycin lacking hydroxyl substituents at C-3 and C-4 of neosamine C. Thus, the *S. ribosidificus* mutant appears to accept larger subunits than our *S. fradiae*, *S. rimosus*, and *S. kanamyceticus* mutants. To the extent that the biosyntheses of neomycin, paromomycin, and kanamycin are related to that of ribostamycin the results of Kojima and Satoh (1973) suggest that explanation 4 may be the most plausible.

A comparison of the bioactivities of hybrimycins C1, C2, and C3 (Table II) with other aminoglycoside antibiotics against some of the same microorganisms—*E. coli* ATCC 26, *K. pneumoniae* ATCC 10031, *P. vulgaris* ATCC 8427, *B. subtilis* UC564 (Shier *et al.*, 1969, 1970)—confirms the existence of certain structure-activity relationships among the aminoglycoside antibiotics (Davies, 1968) and suggests additional relationships. An important initial observation is that the presence of a neobiosamine (the disaccharide unit at the bottom of the neomycin and paromomycin structures in Figure 1) is very important since the neomycins, paromomycins, hybrimycins A_1 (or A_2), B_1 (or B_2), and C_1 (or C_2) are all far more potent antibiotics than their counterparts lacking a neobiosamine unit—neamine, paromamine, hybrimycins A_3, B_3, and C_3. An amino group on C-6 of neosamine C (the uppermost sugar in Figure 1) must give much higher activity than a hydroxyl group, since the neomycins are more active than the paromomycins, the hybrimycin A antibiotics are more active than the hybrimycin C antibiotics, and neamine is more active than paromamine. Neosamines B and C (2,6-diamino-2,6-dideoxy-L-idose and 2,6-diamino-2,6-dideoxy-D-glucose, respectively) differ only in their configuration at C-5. The neosamine B configuration in the bottom sugar in Figure 1 (found in neomycin B, paromomycin I, and hybrimycins A1, B1, and C1) confers much higher antibacterial activity on the antibiotics containing it than the neosamine C configuration (the opposite enantiomer at C-5) confers on the otherwise identical antibiotics containing it in place of neosamine B (neomycin C, paromomycin II, and hybrimycins A2, B2, and C2, respectively). Finally, in the neomycins and neamine as well as in the paromomycins and paromamine the introduction of an additional hydroxyl group at C-2 of the 2-deoxystreptamine moiety to give corresponding hybrimycins results in a reduction in activity. This reduction in activity is greater when the hydroxyl group introduced is axial (hybrimycins B1, B2, and B3) than when it is equatorial (hybrimycins A1, A2, and A3) (Shier *et al.*, 1969, 1970). However, the majority of the activity is retained in the hybrimycins, establishing that the methylene group is not essential for activity and suggesting that further modification in this region of the molecule may prove fruitful.

Acknowledgment

We thank Dr. W. T. Sokolski, The Upjohn Co., Kalamazoo, Mich., for obtaining the minimum inhibitor concentration data reported, and Mr. J. Wrona, University of Illinois, for obtaining the mass spectra.

References

Akita, E., Tsuruoka, T., Ezaki, N., and Niida, T. (1970), *J. Antibiot. 23,* 173.

Benveniste, R., and Davies, J. (1973), *Annu. Rev. Biochem. 42,* 471.

Brewster, J. H. (1959), *J. Amer. Chem. Soc. 81,* 5483.

Davies, J. (1968), *Antimicrob. Ag. Chemother. 1967,* 297.

De Jongh, D. C., Hribar, J. D., Hanessian, S., and Woo, P. W. K. (1967), *J. Amer. Chem. Soc. 89,* 3364.

Demain, L., and Inamine, E. (1970), *Bacteriol. Rev. 34,* 1.

Diener, U. L. (1953), Ph.D. Thesis, University of North Carolina.

Dyer, J. R. (1954), Ph.D. Thesis, University of Illinois.

Falkner, F. C. (1969), Ph.D. Thesis, University of Illinois.

Frohardt, R. P., Haskell, T. H., Ehrlich, J., and Knudsen, M. P. (1959), U. S. Patent 2,916,485; *Chem. Abst. 54,* 8673i, 11389d.

Haskell, T. H., French, J. C., and Bartz, Q. R. (1959), *J. Amer. Chem. Soc. 81,* 3482.

Hessler, E. J., Jahnke, H. K., Robertson, J. H., Tsuji, K., Rinehart, K. L., Jr., and Shier, W. T. (1970), *J. Antibiot. 23,* 464.

Horner, W. H. (1967), *in* Antibiotics, Vol. II, Gottlieb, D., and Shaw, P. D., Ed., Berlin, Springer-Verlag, p 373.

Hunter, G. D., and Hockenhull, D. J. D. (1955), *Biochem. J. 59,* 268.

Kojima, M., and Satoh, A. (1973), *J. Antibiot. 26,* 784.

Lemieux, R. U., and Cushley, R. J. (1963), *Can. J. Chem. 41,* 858.

Maeda, K., Marase, M., Mawatari, K., and Umezawa, H. (1958), *J. Antibiot. 11,* 163.

Millington, D. S., Steinman, D. H., and Rinehart, K. L., Jr. (1974), *J. Amer. Chem. Soc. 96,* 1909.

Peck, R. L., Graber, R. P., Walti, A., Peel, E. W., Hoff-

hine, C. E., Jr., and Folkers, K. (1946), *J. Amer. Chem. Soc. 68,* 29.
Rinehart, K. L., Jr. (1964), The Neomycins and Related Antibiotics, New York, N. Y., Wiley.
Rinehart, K. L., Jr. (1969), *J. Infect. Dis. 119,* 345.
Rinehart, K. L., Jr., Malik, J. M., Nystrom, R. S., Stroshane, R. M., Truitt, S. T., Taniguchi, M., Rolls, J. P., Haak, W. J., and Ruff, B. A. (1974), *J. Amer. Chem. Soc. 96,* 2263.
Shier, W. T. (1970), Ph.D. Thesis, University of Illinois.
Shier, W. T., Ogawa, S., Hichens, M., and Rinehart, K. L., Jr. (1973), *J. Antibiot. 26,* 551.
Shier, W. T., Rinehart, K. L., Jr., and Gottlieb, D. (1969), *Proc. Nat. Acad. Sci. U. S. 63,* 198.
Shier, W. T., Rinehart, K. L., Jr., and Gottlieb, D. (1970), *J. Antibiot. 23,* 51.
Shier, W. T., Rinehart, K. L., Jr., and Gottlieb, D. (1972), U. S. Patent 3,669,838; *Chem. Abstr. 77,* 150584x.
Tsuji, K., and Robertson, J. H. (1969), *Anal. Chem. 41,* 1332.
Tsuji, K., and Robertson, J. H. (1970), *Anal. Chem. 42,* 1661.
Walker, J. B. (1971), *Lloydia 34,* 363.
Walker, J. B., and Walker, M. S. (1967), *Biochemistry 6,* 3821.
Wiffen, D. H. (1956), *Chem. Ind. (London),* 964.

A Comparative Electron Spin Resonance Study of the Erythrocyte Membrane in Myotonic Muscular Dystrophy†

D. Allan Butterfield, Allen D. Roses, Michael L. Cooper, Stanley H. Appel, and Donald B. Chesnut*

ABSTRACT : Stearic acid methyl ester spin-labels with the paramagnetic center located at the 5, 12, and 16 positions on the fatty acid alkyl chain have been used to study the erythrocyte membrane from patients with myotonic muscular dystrophy, a systemic disorder inherited as an autosomal dominant trait. It has been demonstrated that the physical state of the erythrocyte membrane from patients with this disease is different from that of normal controls. At all levels of penetration into the membrane probed by the spin-label, myotonic membranes were more fluid and less polar than control membranes. The overall average values of the order parameter, S, and the nitrogen isotropic coupling constant, a_N, obtained with the 5, 12, and 16 labels were respectively (0.65, 15.9 G), (0.59, 15.5 G), and (0.26, 13.9 G) in normal membranes while the parameters were respectively (0.61, 15.8 G), (0.58, 15.3 G), and (0.25, 13.8 G) for the three labels in myotonic erythrocytes. The fluidity difference between normal and myotonic membranes is most apparent near the surface of the membrane while the polarity difference is approximately constant at various depths within the membrane. These results support the concept of myotonic muscular dystrophy as a diffuse membrane disease.

Myotonic muscular dystrophy is a systemic disorder inherited as an autosomal dominant trait. Physiological investigations have localized the functional defect to muscle membranes (Lindsley and Curren, 1936; Denny-Brown and Nevin, 1941; Landau, 1952) although recent studies have suggested that muscle changes are secondary to neuropathic disturbances (Gallup and Dubowitz, 1973; McComas *et al.*, 1971). Biochemical data from our own laboratories have supported a more diffuse membrane involvement rather than a defect limited to the muscle or nerve. Endogenous membrane protein kinase was decreased in aged frozen erythrocyte ghosts as well as in freshly prepared nonfrozen ghosts (Roses and Appel, 1973, 1974). Similar alterations in membrane bound protein kinase activity have also been demonstrated in carefully controlled muscle membrane experiments (Roses and Appel, 1974b). These studies not only document the wide-spread membrane involvement in this disorder, but also support the usefulness of red blood cells as a readily available membrane source with which to assess the metabolic error.

Our more recent study employed electron spin resonance (esr) spectroscopy to substantiate the presence of a membrane defect in myotonic erythrocytes (Butterfield *et al.*, 1974). The spin-labeling technique is well documented (McConnell and McFarland, 1970; Jost *et al.*, 1971; Keith and Mehlhorn, 1972; Schreier-Muccillo and Smith, 1973) and has been particulary successful in understanding both model (Mukai *et al.*, 1972) and biological (Hubbell and McConnell, 1968, 1969a,b, 1971, Hubbell *et al.*, 1970; Kaplan *et al.*, 1973) membranes. The spectra of spin-labeled erythrocytes from patients with myotonic dystrophy were found to be recognizably different from those with normal erythrocytes. Using the methyl ester of stearic acid substituted with a nitroxide-containing oxazolidine ring at the 5 position (5-NMS)[1] we noted incorporation of the spin-label

† From the Paul M. Gross Chemical Laboratory, Duke University, Durham, North Carolina 27706 (D.A.B., M.L.C., and D.B.C.), and the School of Medicine, Division of Neurology, Duke University, Durham, North Carolina 27710 (A.D.R. and S.H.A.). *Received July 25, 1974.* This work was supported in part by the National Science Foundation (Grant GP-22546, D.A.B. and D.B.C.), the Duke University National Institutes of Health Biomedical Support Grant (D.A.B. and D.B.C.), a Research Award from the Graduate School of Duke University (D.A.B.), the National Institutes of Health (Grant NS07872, A.D.R. and S.H.A.), and the Multiple Sclerosis Society (Grant 923-A-2, A.D.R.).

[1] Abbreviations used are: 5-NMS, 5-nitroxide methyl stearate; 12-NMS, 12-nitroxide methyl stearate; 16-NMS, 16-nitroxide methyl stearate.