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Polynucleotide Analogs: Acrylic Acid and Maleic Acid Copolymers of 1-Vinyluracil and 9-Vinyladenine†

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ABSTRACT: Radical-induced copolymerization of 1-vinyluracil and maleic anhydride gave, after hydrolysis, a polymer containing a 1:1 monomer ratio of 1-vinyluracil–maleic acid. γ-Ray-induced copolymerization of 1-vinyluracil with acrylic acid gave a polymer with a ratio of 1:1.7. Similar treatment of 9-vinyladenine and acrylic acid resulted in a polymer with a 1:3.2 ratio. These three compounds are potent stimulants of poly(uridylic acid) coded polyphenylalanine synthesis in an in vitro cell free system purified from *Escherichia coli* MRE 600. The double-stranded polymer, poly(inosinic acid)-poly(cytidylic acid), also stimulates polyphenylalanine synthesis in this assay.

A novel approach to the control of disease would be a class of agents that do not act by affecting the rate of catalysis of an enzyme but, rather, exert their action by controlling the level of enzymes. Such agents, to be effective, must act selectively to either decrease or increase the formation of the target enzyme. If the primary lesion in a particular disease is identified as an excess of an enzyme, the ideal agent would be one acting specifically to decrease the formation of that enzyme. To achieve this selectivity such an agent must alter genetic expression at the level of transcription or translation.

While this approach is premature with the current state of knowledge in molecular biology, the enormous effort directed to elucidating the control mechanisms of gene expression implies a great deal of confidence, among molecular biologists, that the goal is attainable. As one example, the sequence of bases in the *Escherichia coli lac* operator is known (Gilbert and Maxam, 1973; Maizels, 1973).

Assuming the primary information for the control of gene expression ultimately resides in unique sequences of bases in nucleic acids, therapeutic agents that compliment and have high affinity for a control sequence should inhibit the expression of that gene product—a particular enzyme. In contrast, a therapeutic agent that mimics the control sequence also should have affinity for the natural repressor. The result would be derepression of the gene and formation of that particular enzyme.

Preliminary studies leading to the distant goal of medicinal agents acting by the control of gene expression have been directed to identifying structural requirements in analogs of nucleic acids that have affinity for or mimic natural nucleic acids. To this end copolymers of 1-vinyluracil with maleic anhydride or acrylic acid and the copolymer of 9-vinyladenine with acrylic acid were prepared. These agents stimulate, in vitro, the poly(uridylic acid) coded synthesis of polyphenylalanine using *Escherichia coli* MRE 600. The double-stranded polynucleotide, poly(I)-poly(C)[1], a potent interferon inducer (Field et al., 1967), also has been found to stimulate protein synthesis in this system.

Experimental Section

Poly(U), poly(A), and poly(I)-poly(C) were purchased from Miles Laboratories. [^{14}C]- and [^{3}H]Phenylalanine were purchased from Schwarz/Mann. *Escherichia coli* MRE 600 cells were purchased as frozen packed 3/4 log cells from General Biochemicals; *Escherichia coli* tRNA was from Plenum Scientific. Poly(acrylic acid) was obtained from Aldrich Chemicals. ATP, GTP, DNase, and other common reagents were products of Sigma Chemicals.

Copolymerization of 1-Vinyluracil and Maleic Anhydride. A solution of 1-vinyluracil (Ueda et al., 1968) (250 mg, 1.8 mmol), maleic anhydride (355 mg, 3.6 mmol), and

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[1] Abbreviations used are: poly(U), poly(uridylic acid); poly(A), poly(adenylic acid); poly(I)-poly(C), poly(inosinic acid)-poly(cytidylic acid); poly(vU-MA), poly(vinyluracil–maleic acid); poly(vU-AA), poly(vinyluracil–acrylic acid); poly(vA-AA), poly(vinyladenine–acrylic acid).

α,α'-azodiisobutyronitrile (0.9 mg, 0.0055 mmol) in 10 ml of anhydrous acetonitrile was refluxed under nitrogen for 8 hr. A precipitate developed from the clear solution during the first several minutes. After the solution stood overnight at 25° the pink solid was collected and twice resuspended in hot acetonitrile. The insoluble material (**1**, 96 mg, 23%) had a strong anhydride band in the ir at 1850 and 1780 cm^{-1} in addition to the broad uracil carbonyl absorption. Anal. Calcd for $(C_{10}H_8N_2O_5)_n$: N, 11.86. Found: N, 11.40.

The polymeric anhydride (**1**, 40 mg) was refluxed in 1.5 ml of water for 1 hr and the cooled solution filtered to give poly(vU-MA) (**2**) as a tan solid which did not have absorption in the ir at 1850 or 1780 cm^{-1}. Uv λ_{max} (H_2O) 263 mμ (ϵ 4800). Anal. Calcd $(C_{10}H_{10}N_2O_6)_n$: C, 47.25; H, 3.96; N, 11.02. Found: C, 47.61; H, 3.99; N, 11.10.

This and all other synthetic polymers in this study were fractionated by gel filtration on Sephadex G-75 using 0.1 *M* triethylamine. The void volume containing the higher molecular weight fractions was used in in vitro biological testing.

Copolymerization of 1-Vinyluracil with Acrylic Acid (**3**). The procedure of Hoffmann and coworkers (Hoffmann et al., 1974) was found to be the most convenient for this polymerization. 1-Vinyluracil (222 mg, 1.6 mmol) and freshly distilled acrylic acid (139 mg, 1.9 mmol) were dissolved in 1 ml of 7 *M* sodium hydroxide. The vial was evacuated rapidly to 0.2 mm and sealed. The vial was irradiated at 39,000 rads/hr for 4.5 hr (~178 krads total) using a ^{60}Co source. The viscous solution was treated with methanol to precipitate the product. Solution in water and acidification to pH 5 with hydrochloric acid gave the solid product poly(vU-AA) (**3**, 230 mg, 68%) which was washed with methanol and dried. Ir showed the expected absorption: uv λ_{max} (H_2O) 266 mμ (ϵ 7250 based on a 1:1 monomeric unit). Successive elemental analyses did not show any change after reprecipitation, methanol washing, and drying. The results based on nitrogen analysis (Found: 10.69%) indicate that the polymer ratio is [1-vinyluracil (1.0)-acrylic acid (1.7)]$_n$. On this basis (monomer mol wt 344) the molar extinction coefficient is 9970, in good agreement with the value of 9800 for 1-methyluracil (Shugar and Fox, 1952).

Copolymerization of 9-Vinyladenine and Acrylic Acid (**4**). A solution of 9-vinyladenine (Ueda et al., 1968) (200 mg, 1.24 mmol) and acrylic acid (111 mg, 1.54 mmol) in 4.5 ml of water was acidified to pH 2 with hydrochloric acid. The degassed solution was sealed in an ampoule and irradiated for 4.5 hr at room temperature with approximately 178 krads of γ radiation from a ^{60}Co source. The solution was treated with 2 *M* sodium hydroxide to give a pH of 6; the solid, poly(vA-AA) (**4**), was collected, washed repeatedly with methanol, and dried; the ir spectrum was as expected; uv λ_{max} (H_2O) 258 mμ (ϵ 6100 calculated for the 1:1 monomeric unit).

Nitrogen microanalysis was constant for several samples and found to be 18.0%. Theoretical nitrogen for a 1:1 ratio of the monomers $(C_{10}H_{11}N_5O_2)_n$ is 30.0%. These results indicate a polymer composition of [9-vinyladenine (1.0)-acrylic acid (3.2)]$_n$, mol wt ~390; on this basis the molar extinction coefficient is 13,400 which is in reasonable agreement with the value of 14,600 reported for adenosine.

Preparation of E. coli Ribosomes and S-100. All operations were carried out at 2°. *E. coli* MRE 600 cells were washed in a buffer containing 50 m*M* Tris-HCl (pH 7.8), 10 m*M* $MgCl_2$, 6 m*M* 2-mercaptoethanol, and 60 m*M* NH_4Cl and packed by centrifugation at 6000*g* for 10 min; 30 g of packed cells was ground in a mortar with 45 g of sand, suspended in 20 ml of buffer, and centrifuged at 10,000*g* for 15 min. The supernatant was treated with DNase (5 μg/ml) and 2-mercaptoethanol (6 μmol/ml) for 15 min and the solution was clarified by centrifugation at 30,000*g* for 20 min. Ribosomes were collected by centrifugation of the S-30 supernatant at 105,000*g* for 2 hr. The supernatant, S-100, was stored at −80° in small aliquots. Ribosomes were washed again with buffer and centrifuged as above; the pellet was resuspended in a small amount of buffer and stored at −80° in small aliquots.

Assay of Poly(U)-Dependent Polyphenylalanine Synthesis. The reaction mixture contained in 0.1 ml: 50 m*M* Tris-HCl (pH 7.8), 56 m*M* NH_4Cl, 6 m*M* 2-mercaptoethanol, 5 m*M* ATP, 0.5 m*M* GTP, 15 m*M* $MgCl_2$, 120 μg of *E. coli* B tRNA, 5 μl of S-100 fraction, approximately 1 A_{260} of ribosomes, poly(U) as indicated, 30 μM [^{14}C]phenylalanine (10 Ci/mol) or [^{3}H]phenylalanine (500 Ci/mol), and other additions as indicated. After incubation for 10 min (unless noted as a different time) at 37° the reaction was terminated by adding 2 ml of 10% trichloroacetic acid. The samples were heated 10 min at 95°, cooled, filtered on glass pads, washed with 5% trichloroacetic acid, dried, and counted in a scintillation counter.

Results and Discussion

Pitha and coworkers (Reynolds et al., 1972; Pitha et al., 1973) reported the synthesis and biological activity of polyvinyl compounds containing uracil or adenine and the copolymer poly(vinyluracil-vinyladenine). These compounds induced interferon and were found to be inhibitors of in vitro protein synthesis. Kaye and Chang (1973) have studied the physicochemical properties of the interaction of polyvinyluracil with poly(adenylic acid) and describe a 2:1 complex.

Space-filling models suggest that the optimum distance between bases that allows for Watson-Crick type hydrogen bonding to a natural nucleic acid is satisfied by a four carbon atom chain. Two research groups have reported the formation of copolymers of this type. Kondo and coworkers (1969) studied copolymers of 9-vinyladenine or 1-vinyluracil with acrylamide or maleic anhydride. While the work in this report was in progress Hoffmann and coworkers (1974) published the synthesis of the copolymer of 1-vinyluracil and acrylic acid.

Using a 1:2 mole ratio, 1-vinyluracil (Kaye and Chang, 1973) and maleic anhydride were copolymerized in anhydrous acetonitrile using 0.003 mol equiv (relative to 1-vinyluracil) of α,α'-azodiisobutyronitrile as the polymerization catalyst. The solid obtained after cooling was a mixture of the polymer and 1-vinyluracil. Trituration of this solid with hot acetonitrile gave the product **1** as a 1:1 copolymer. Hydrolysis of the anhydride in water gave the diacid **2** (Figure 1); no anhydride absorption was found in the ir spectrum.

Although the elemental analyses (C, H, N) of **2** are within ± 0.4% of theory the ultraviolet analysis did not confirm a 1:1 ratio of maleic acid-vinyluracil in the polymer. The expected maximum for a 1-alkyluracil of 263 mμ in water was observed. However, the observed molar extinction coefficient of 4800 is only 49% of theory when compared to the reported ϵ of 9800 at 267 mμ for 1-methyluracil (Shugar and Fox, 1952). Using essentially the same method of preparation Kondo and coworkers (Kondo et al., 1969) reported a 1:1 ratio of each of the monomers in the polymer, how-

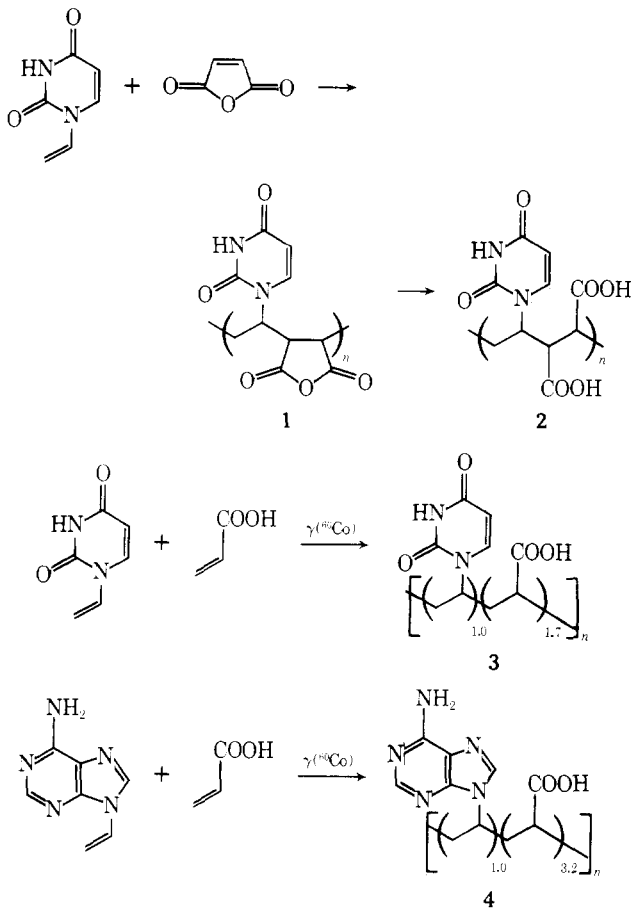

FIGURE 1: Copolymer synthesis.

Table I: Stimulation of [^{3}H]- or [^{14}C]Phe Incorporation by Copolymers of Vinyl Bases.[a]

	Poly-(U) (μg)	Additions	Concn (μg)	[^{3}H]- or [^{14}C]Phe Incorporated % control
1.	1.2	Poly(vU–MA)	0.41	266
2.	3.0	Poly(vU–MA)	0.41	162
3.	3.0	Poly(vU–MA)	17.4	290
4.	3.0	Poly(vU–AA)	16.8	350
5.	3.0	Poly(vA–AA)	15.3	270
6.	1.2	EDTA	0.38	89
7.	1.2	EDTA, poly(vU–MA)	0.38; 0.41	208
8.	1.2	Succinic acid	0.59	98
9.	3.0	Poly(I)-poly(C)	3.5	134
10.	3;0	Poly(acrylic acid)	5.1	97

[a] Assays were run for 10 min as described in the Experimental Section. Control levels of Phe incorporated were 68 pmol using 1.2 μg of poly(U) and 118 pmol using 3.0 μg of poly(U). The results are corrected for blanks.

ever, no details of this determination are included in their report. The low molar extinction coefficient found for **2** is not due to a hypochromic effect of base stacking since there was no change in the absorption when the cuvet was heated to 70°, a temperature sufficient to melt or denature an ordered structure of this type.

Alternatively, the low extinction coefficient may be caused by polymerizations that involve the 5–6 double bond of uracil giving dihydrouracil residues in the mixed polymer. Kaye (1971) has found that the free radical polymerization of 1-vinyluracil gave polyvinyluracil that had an extinction coefficient of only 11% of theoretical. This was attributed to the presence of dihydrouracil residues in the polymer.

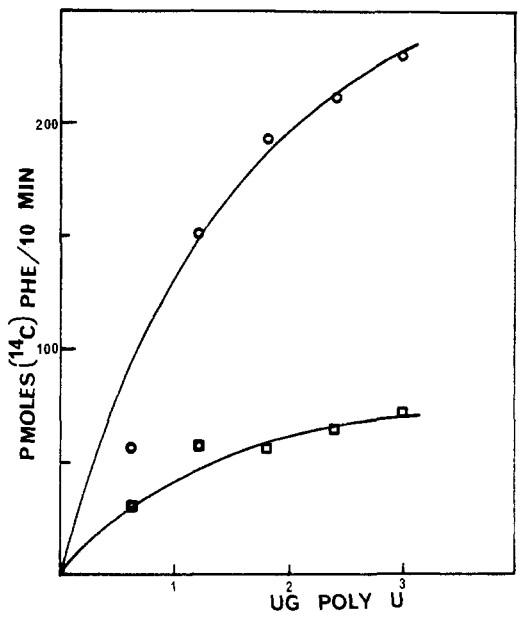

FIGURE 2: Assays for the effect of message concentration on poly(vU–MA) stimulation are described in the Experimental Section; 12 μg of poly(vU–MA) was added to each assay (○); control (□). The incorporated [^{14}C]Phe is corrected for the blank lacking message.

Maleic acid is reported to give alternating copolymers with styrene leading to a polymer with a 1:1 ratio of monomers (Sorenson and Campbell, 1968). However, acrylic acid copolymerization with other vinyl polymers is more likely to give random polymers.

The copolymerization of vinyl bases and acrylic acid was done by γ irradiation (Figure 1). Hoffmann (Hoffman et al., 1974) reported this procedure gave a copolymer with a 1:1 ratio using 1-vinyluracil and acrylic acid. Using the same procedure of irradiation at 25° instead of the reported 13° we obtained a polymer that was not a 1:1 ratio, but, a 1.0:1.7 ratio with acrylic acid predominating. Evidence in support of this ratio is derived from the nitrogen elemental analysis and the extinction coefficient. The latter value calculated on a 1:1 ratio of the monomers is 7250, however, a 1.0:1.7 ratio gives a molar extinction coefficient of 9970, which agrees with the reported value of 9800 for 1-methyluracil ($\lambda_{max}(H_2O)$ 267 mμ) (Shugar and Fox, 1952).

Copolymerization of 9-vinyladenine (Ueda et al., 1968) with acrylic acid was done in acid (pH 2) using γ irradiation. The product obtained, poly(vA–AA) (**4**), was found, by nitrogen analysis, to be composed of a 1:3.2 ratio, again, with acrylic acid as the predominant unit. The molar extinction coefficient based on a 1:3.2 ratio (mol wt 389) was found to be 13,400.

For biological testing all polymers were fractionated according to size by gel filtration using 0.1 *M* triethylamine on Sephadex G-75. The void volume containing excluded molecules was collected and used as such.

Preliminary biological studies on poly(U) coded polyphenylalanine synthesis was done using an in vitro protein synthesis assay purified from *Escherichia coli* MRE 600. Poly(vU–MA) (**2**) stimulated protein synthesis in this system over a wide concentration range using limiting amounts of poly(U) as the message (Table I). Highest stimulation,

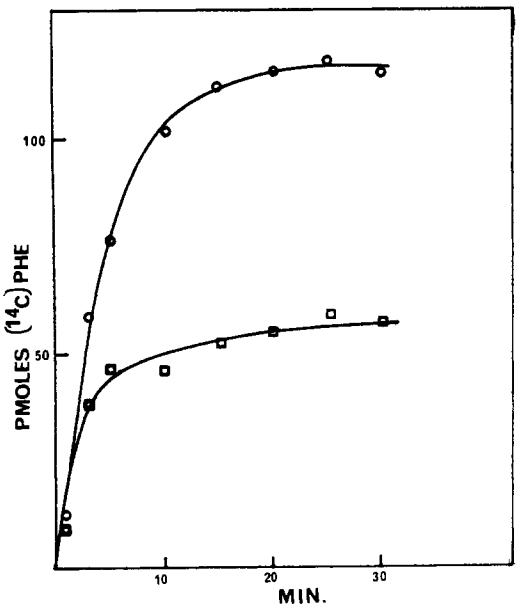

FIGURE 3: Assays for the time course of poly(vU-MA) stimulation of poly(U) coded polyphenylalanine synthesis are described in the Experimental Section. Poly(U) concentration in both assays was 1.2 μg. Poly(vU-MA) (2.9 μg) was added to the upper curve (○); control (□). The blank, without message, was subtracted from the determinations.

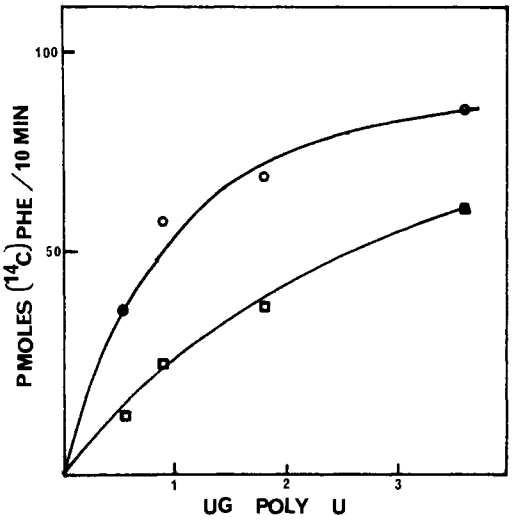

FIGURE 4: Assays for the effect of message concentration on poly(I)-poly(C) stimulation of poly(U) coded phenylalanine incorporation are described in the Experimental Section. Poly(I)-poly(C) (17.4 μg) was added to each point on the upper curve (○); control (□). The values are corrected for blank cpm.

290% of the control, was obtained using 17.4 μg of the polymer **2** in the assay. At the same message concentration 0.4 μg of poly(vU-MA) gave 162% stimulation. Poly(vU-AA) (**3**) and poly(vA-AA) (**4**), also at high concentrations, greatly stimulated phenylalanine incorporation. That this was not a chelation effect was shown by the lack of stimulation with EDTA. Neither was any effect observed with EDTA on the poly(vU-MA) stimulation (assay 7). Succinic acid was tested in the search for a nonspecific effect due to carboxylate anions either directly or indirectly (ionic strength); there was no change.

If the stimulation observed with **2**, **3**, and **4** is an effect of a poly(carboxylic acid) then poly(acrylic acid) should show this effect, however, no stimulation was observed. Poly(I)-poly(C), a unique interferon inducer, was tested and found to give stimulation, however, the increase was not as dramatic as that obtained from the vinyl polymers.

In Figure 2 a study of the effect of varying message concentration using high levels of poly(vU-MA) showed message-dependent stimulation. At low poly(U) concentrations, 177% of control synthesis was observed while at the highest poly(U) concentration 327% was found.

The effect of poly(vU-MA) on the rate of phenylalanine incorporation is shown in Figure 3. While there is little stimulation during the first several minutes the control synthesis was over at 5 min while the stimulated synthesis continued for 15 min.

In Figure 4 the stimulation caused by poly(I)-poly(C), again, as with poly(vU-MA), is message concentration dependent. However, with this polymer greatest stimulation (296%) is observed at the low message concentration.

The stimulatory effect is clearly dependent on message concentration and the results of the time course synthesis study strongly suggest that the stimulation observed is due either to prolonging the message life (RNase inhibition) or to stabilizing some other limiting component of the system from degradation (Miller et al., 1974). The polymers used were not messages since there was no phenylalanine incorporation in the absence of poly(U).

The structural features common in the stimulant polymers are (1) a high molecular weight polyanion backbone, and (2) a heterocyclic base (adenine, uracil, cytosine, inosine) attached to the polymer.

There does not appear to be any tendency for these polymers to form double-stranded complexes with potentially complimentary polymers. Of the polymers examined (poly(vU-MA), poly(vU-AA)) there was no hypochromic effect when heated to 70° or when allowed to equilibrate (2°) with poly(adenylic acid).

Further studies are in progress to elucidate the mechanism whereby poly(vU-MA), poly(vU-AA), poly(vA-AA), and poly(I)-poly(C) stimulate in vitro poly(U) coded polyphenylalanine synthesis.

Acknowledgments

The authors acknowledge Cheryl Ristig, Marilyn Lang, and Roxane Barfknecht for technical assistance.

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Surface and Solution Properties of Steroid Antibiotics: 3-Acetoxylfusidic Acid, Cephalosporin P_1 and Helvolic Acid†

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ABSTRACT: The colloid/chemical properties of the fusidane antibiotics, 3-acetoxylfusidic acid, cephalosporin P_1, and helvolic acid, and their sodium salts, were investigated. The sodium salts of 3-acetoxylfusidic acid and cephalosporin P_1 were found to be detergent-like molecules with micellar properties comparable to the parent compound sodium fusidate and the bile salt sodium cholate. Critical micellar temperatures (cmt) were less than 0°C except for sodium helvolate which being sparingly soluble did not form micelles between 0 and 50°C. Potentiometric titrations of dilute solutions gave apparent pK values (5.2–6.5) in the range expected for carboxylated steroid detergents. The apparent pK values increased significantly once the detergent concentration exceeded the critical micellar concentration (cmc). Micellar properties were determined by surface tension, titration with a water-soluble dye (Rhodamine 6G), light scattering, and solubilization of lecithin and cholesterol. Cmc's, in the range of 1.5 to 5.6 mM, were found which varied slightly depending on the method employed and in all cases fell slightly in the presence of added NaCl. The number of monomers per micelle (aggregation number) in concentrations well above the cmc was extrapolated from Debye light scattering plots in 0.15 M NaCl. The values varied from 6 for fusidate to 14 for 3-acetoxylfusidate with sodium cephalosporin P_1 having an intermediate value. Each detergent readily solubilized the phospholipid lecithin. The maximum solubility of cholesterol in lecithin–detergent mixed micelles varied from 6 to 10 mol %. The counterion binding of micelles in water and in 0.15 M Na^+ was calculated from the log cmc–log Na^+ concentration curves and from Debye light scattering plots. The percent counterions bound to fusidane micelles was similar to that bound to sodium cholate micelles, but was significantly less than that found with typical straight-chain detergent micelles. Pressure–area isotherms were determined on a Langmuir-Pockels surface balance for each compound on an aqueous subphase containing 5 M NaCl at pH 2. Each of the isotherms was distinctive and the area of each antibiotic at its collapse point varied from 95 to 124 $Å^2$ per molecule, all significantly larger than cholic acid (90 $Å^2$ per molecule). These areas correlated well with estimates of the areas of the salts from surface tension measurements (107–115 $Å^2$) and with areas calculated from Stuart-Briegleb molecular models of the salts lying flat (103–118 $Å^2$). It is suggested that the unique physical chemical characteristics of these amphiphilic antibiotics may be important in their antibiotic activity and in their ability to mimic many of the physiological properties of the bile salts. Owing to their close chemical and biophysical similarity to bile salts, these drugs may serve as model compounds for detergent replacement in bile salt deficiency syndromes.

A wide variety of drugs are surface active and aggregate to form micelles in aqueous solution (Florence, 1968). This is of considerable importance in drug action because the structural requirements for surface activity and micelle formation are often similar to those for interaction of a drug with receptor sites, serum proteins, or membrane components (Tanford, 1973). Steroids of the fusidane family represent a unique class of soluble amphiphiles in that they structurally resemble the bile salts, the alimentary biodetergents of vertebrates (Godtfredsen, 1967; Florence, 1968; Carey and Small, 1971). The aggregation properties of sodium fusidate and some of its derivatives including their glycine and taurine conjugates were previously studied, and a marked similarity with the micellar properties of the common vertebrate bile salts was found (Carey and Small, 1971, 1973). One of these derivatives, taurodihydrofusidate, was actively secreted into bile of primates without appreciable metabolism and significantly influenced the biliary secretion of bile salts, phospholipids, and cholesterol (Beaudoin et al., 1973).

In this paper we evaluate the colloidal properties of 3-acetoxylfusidic acid (Godtfredsen, 1967), shown previously

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