Studies on the Biosynthesis of the Erythromycins. II. Isolation and Structure of a Biosynthetic Intermediate, 6-Deoxyerythronolide B*

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ABSTRACT: A neutral macrocyclic polyhydroxyoxolactone has been isolated from the fermentation beer of a partially blocked mutant of *Streptomyces erythreus*. Recovery and crystallization of the substance can be effected by solvent extraction and column chromatography.

Addition of the lactone to the fermentation medium of a second blocked mutant established that the compound is an intermediate in the erythromycin biogenetic pathway immediately preceding erythronolide B. The structure of the compound has been determined to be 6-deoxyerythronolide B.

The aglycone of the erythromycins, antibiotic glycosides, consists of a macrocyclic polyhydroxyoxolactone (Gerzon *et al.*, 1956; Wiley *et al.*, 1957). Gerzon *et al.* (1956), after noticing the regularity of seven recurring 3-carbon units in the aglycone structure, proposed the "propionate rule" and discussed its possible participation in aglycone biogenesis. Lynen (1959) speculated that seven molecules of 2-methylmalonyl-CoA[1] participated in the biosynthesis and that a 21-carbon polyoxo compound was an intermediate. Grisebach *et al.* (1960) and Kaneda *et al.* (1962) established that propionate was incorporated as a 3-carbon subunit without randomization in erythronolide, the aglycone moiety of the erythromycins. Lynen and Tada (1961) and Vanek *et al.* (1961) suggested that the lactone carbons could be produced from 2-methylmalonyl-CoA with propionyl-CoA as primer. Recently Friedman *et al.* (1964) and Wawszkiewicz and Lynen (1964) have reported results which confirm the proposal of Vanek and that of Lynen and Tada. Although the biogenetic mechanisms remain obscure, the data suggest that a "polyketide" (Birch, 1957) is probably formed during aglycone synthesis.

Recently several erythromycin biogenetic intermediates have been isolated. Tardrew and Nymen (1964) reported the isolation of erythronolide B, the aglycone of erythromycin B, and Hung *et al.* (1965) established that the compound was an intermediate of erythromycin biosynthesis. In an earlier paper of this series (Martin *et al.*, 1966), the isolation of 3-α-L-mycarosylerythronolide B from fermentation beers of a blocked mutant *Streptomyces erythreus* was reported. Evidence was presented that 3-α-L-mycarosylerythronolide B is an intermediate of erythromycin biogenesis following erythronolide B. In this paper we wish to report the isolation and structure of 6-deoxyerythronolide B, a direct biogenetic precursor of erythronolide B.

Experimental and Results Section[2]

Fermentation Organism. The strain employed in this investigation was *S. erythreus* (Abbott 9EI262). This varient was derived by treatment of a high erythromycin-yielding strain with ethylenimine followed by ultraviolet irradiation. The mutant has a partial block in the erythromycin biogenetic pathway and accumulates erythronolide B and 6-deoxyerythronolide B as well as erythromycins A and B in fermentation beers. 6-Deoxyerythronolide B is devoid of antibiotic activity against *Bacillus subtilis*.

Fermentation Procedures. Inoculum cultures of 9EI262 were prepared as previously described (Martin *et al.*, 1966). The inoculum was added at a level of 3–5% (v/v) into 500-ml erlenmyer flasks containing 50 ml of fermentation medium consisting of the following components (in grams): corn starch, 15.0; soybean meal, 20.0; corn steep, 5.0; $CaCO_3$, 1.0; soybean oil (Edsoy), 50.0; and tap water, 1000 ml. The medium was adjusted to pH 6.8 with sodium carbonate before sterilization. The fermentation flasks were incubated at 32° on a rotary shaker (260 rpm) for 168 hr.

Recovery from Fermentation Beer. Fermentation beer (2000 ml) was centrifuged to sediment the mycelium and the supernatant was recovered. The supernatant was clarified and extracted with ethyl acetate as previously described (Martin *et al.*, 1966). The ethyl acetate was washed twice with an equal volume of 0.1 M potassium phosphate buffer of pH 4.5 and the aqueous extract was discarded. The organic extract was washed with water, dried over anhydrous sodium

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[1] Abbreviation used: CoA, coenzyme A.

[2] Nmr spectra were determined with a Varian A-60 spectrometer. Infrared spectra were determined as chloroform solutions.

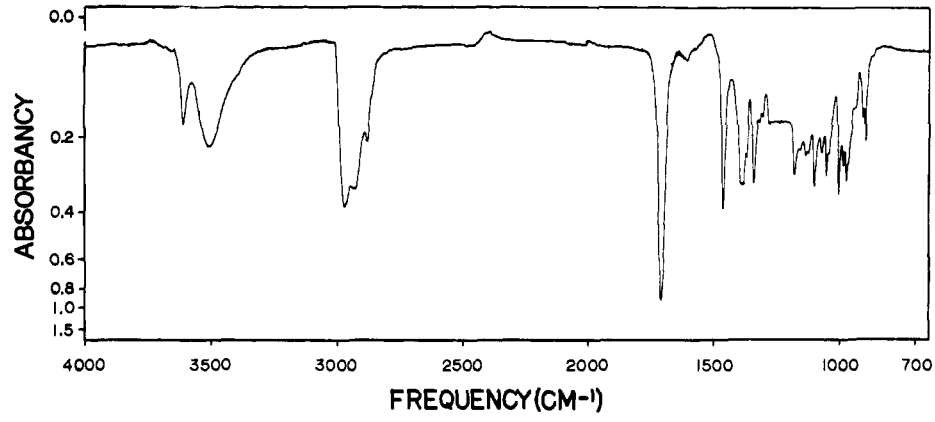
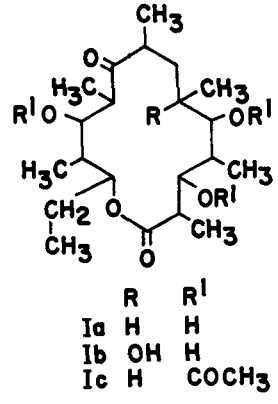


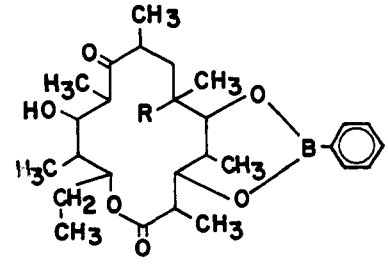
FIGURE 1: Infrared spectrum of 6-deoxyerythronolide B (in chloroform solution).

sulfate, and concentrated under reduced pressure to a viscous yellow oil. The oil was chromatographed on silica gel (Davison Chemical Corp., grade 923, 100–200 mesh) packed in chloroform. Elution was accomplished with increasing concentrations of methanol in chloroform. Fractions containing 6-deoxyerythronolide B were pooled and concentrated under reduced pressure yeilding a light yellow gum. Crystallization from ethyl acetate–hexane afforded 1.79 g of colorless prisms, mp 147–149°, $[\alpha]_D^{23}$ −88° (*c* 0.50, 95% ethanol), λ_{max} 287 mμ ($E_{1\,cm}^{1\%}$ 1.47, 95% ethanol).

Physical Properties of 6-Deoxyerythronolide B (Ia). The infrared spectrum (Figure 1) showed absorption at the following frequencies: 3605, 2967, 2925, 2875, 1712, 1465, 1395, 1385, 1345, 1321, 1308, 1282, 1105, 1078, 1060, 1010, 992, 978, and 905 cm^{-1}. The nuclear magnetic resonance (nmr) spectrum (Figure 2) is similar to that of erythronolide B (Ib).



	R	R¹
Ia	H	H
Ib	OH	H
Ic	H	$COCH_3$



	R
IIa	OH
IIb	H

Anal. Calcd for $C_{21}H_{38}O_6$: C, 65.25; H, 9.91; O, 24.94; mol wt, 386.5. Found: C, 65.54; H, 9.93; O, 24.91.

Acetylation of 6-Deoxyerythronolide B. 6-Deoxyerythronolide B (505 mg) was dissolved in 10 ml of dry pyridine and 5 ml of acetic anhydride and heated on a steam bath under a nitrogen atmosphere for 15 hr. The reaction mixture was cooled to room temperature, then evaporated to dryness under reduced pressure. The residue was dissolved in benzene and washed with water. Concentration of the organic phase gave the crystalline triacetate, Ic (600 mg), which on recrystallization from ethanol–water had mp 153–155°, $[\alpha]_D^{23}$ −16° (*c* 0.75, 95% ethanol), λ_{max} 288 mμ ($E_{1\,cm}^{1\%}$ 0.727, 95% ethanol). The infrared spectrum showed absorption at the following frequencies: 3020, 2963, 2938, 2878, 1742, 1710, 1465, 1388, 1377, 1337, 1245, 1182, 1155, 1130, 1080, 1050, 1030, 1000, 980, 962, and 910 cm^{-1}.

Anal. Calcd for $C_{27}H_{44}O_9$: C, 63.26; H, 8.65; O, 28.09; mol wt, 512.5. Found: C, 63.39; H, 8.61; O, 27.63.

Attempted Periodate Oxidation of 6-Deoxyerythronolide B. An attempt was made to oxidize 6-deoxyerythronolide B with periodate by the method of Dyer (1956). The compound was suspended in 30 ml of 0.00982 M sodium periodate and allowed to stand 24 hr at room temperature; the material did not dissolve. Samples (3 ml) were then diluted with 10 ml of saturated aqueous sodium bicarbonate, 5 ml of 0.0103 M sodium arsenite, and 1 ml of 20% aqueous potassium iodide in saturated sodium bicarbonate. After 10–15 min the solutions were titrated with 0.00818 M aqueous iodine solution (1 ml of soluble starch added). Essentially 100% of the added periodate remained unreacted in all cases. Erythronolide B (0.00748 mmole), treated in a similar manner, dissolved in the sodium periodate solution within 2 hr and consumed 0.00850 mmole of periodic acid.

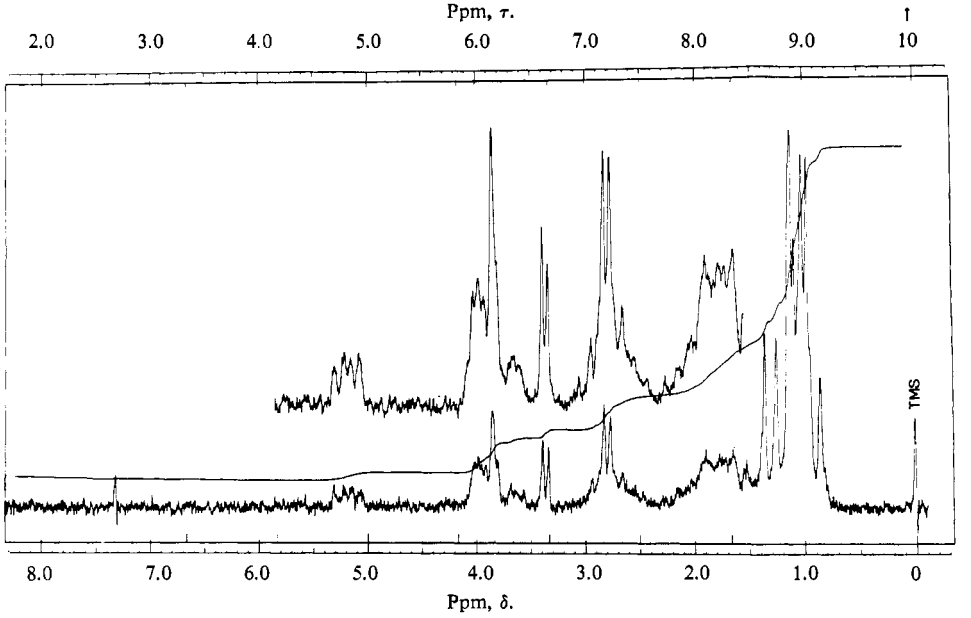


FIGURE 2: Nuclear magnetic resonance spectrum of 6-deoxyerythronolide B (10% solution in deuteriochloroform).

Phenylboronate Ester of 6-Deoxyerythronolide B (IIb). 6-Deoxyerythronolide B (2.02 g) and 0.64 g (an equimolar quantity) of benzeneboronic acid (Aldrich Chemical Co.) were dissolved in 300 ml of dry acetone and refluxed under a nitrogen atmosphere for 4 hr. The reaction mixture was concentrated to 20 ml at 30° under reduced pressure. The solution was heated to boiling, diluted with 7 ml of hot distilled water, and cooled slowly to 4° yielding the crystalline ester (1.8 g). The boronate ester after recrystallization from acetone–water had mp 139–140°. The infrared spectrum showed absorption at the following frequencies: 3505, 3075, 2962, 2938, 2871, 1704, 1600, 1495, 1460, 1440, 1400, 1380, 1330, 1310, 1260, 1170, 1150, 1120, 1070, 1030, 1000, 980, 950, and 900 cm^{-1}.

Anal. Calcd for $C_{27}H_{41}BO_6$: C, 68.64; H, 8.75; B, 2.29; mol wt, 472.4. Found: C, 68.32; H, 8.80; B, 2.46.

Conversion of 6-Deoxyerythronolide B to Erythronolide B and Eyrthromycin by a Blocked Mutant of S. erythreus. 6-Deoxyerythronolide B is readily converted to erythromycin when added to the fermentation medium of a blocked mutant (Abbott 2NU153) of *S. erythreus*. Variant 2NU153, derived from a high erythromycin-producing strain, has a complete block early in the erythromycin pathway and is unable to synthesize erythromycin *de novo*. However, the organism can synthesize erythromycin if certain intermediates, *e.g.*, erythronolide B or 3-α-L-mycarosylerythronolide B, are added to the fermentation. When 25 mg of powdered 6-deoxyerythronolide B was added to 50 ml of a 24-hr culture of 2NU153 in fermentation medium, the beer assayed at 168 hr, by the agar diffusion method, 658 μg/ml (after correction for evaporation) of antibiotic activity against *B. subtilis* using erythromycin A as a standard.

The identity of the antibiotic substance produced was established by paper chromatography and bioautography of the developed chromatogram with *B. subtilis* as the test organism and by isolation and comparison of the substance with authentic erythromycin A. Ascending chromatography of fermentation beers from cultures was performed on Eaton-Dikeman 916 paper with the ammonium hydroxide saturated methyl isobutyl ketone system of Hung *et al.* (1965) and on Whatman No. 3MM paper using 2% potassium phosphate (dibasic) in water as the solvent system. The R_F values, 0.70 and 0.47, respectively, of the anti-

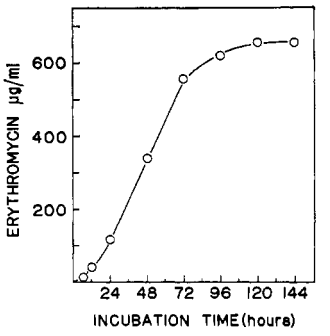


FIGURE 3: Synthesis of erythromycin by a blocked mutant of *S. erythreus* (Abbott 2NU153) after addition of 6-deoxyerythronolide B to the fermentation medium. Powdered 6-deoxyerythronolide B (25 mg) was added to each of several 50 ml of 24-hr cultures of 2NU153 in fermentation medium. The cultures were incubated at 32° on a rotary shaker (260 rpm) and sampled at indicated times. Erythromcyin was determined by the agar diffusion method (after correction for evaporation) using *B. subtilis* as the test organism.

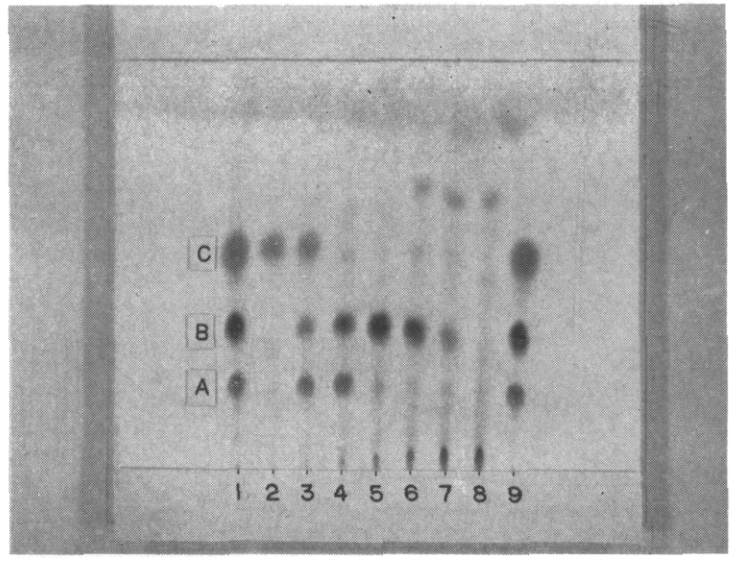


FIGURE 4: Thin layer chromatogram of ethyl acetate extracts of fermentation beers of *S. erythreus* (Abbott 2NU153) after addition of 6-deoxyerythronolide B. Samples (2.0 ml) of the fermentation beer indicated in Figure 3 were clarified and extracted with 1.0 ml of ethyl acetate. The ethyl acetate extract (50 μl) of each sample was spotted on a silica gel G plate and developed in chloroform–95% ethanol 10:1 (v/v); spots were located with the arsenomolybdate reagent of Nelson (1944). The incubation time of the fermentation after addition of 6-deoxyerythronolide B in each lane was as follows: 2, 0 hr; 3, 6 hr; 4, 12 hr; 5, 24 hr; 6, 48 hr; 7, 72 hr; 8, 96 hr. Lanes 1 and 9 contain standards: (A) erythronolide B, (B) 3-α-L-mycarosylerythronolide B, and (C) 6-deoxyerythronolide B. The slow-moving material near the points of origin is erythromycin. The fast-moving trace component in lanes 6–8 is unidentified.

biotic substance were identical in both systems with authentic erythromycin A.

Crystalline erythromycin A was isolated from 168-hr fermentation beer of 2NU153 to which the glycoside has been added at a level of 500 μg/ml at 24 hr. Fermentation beer (800 ml) was filtered and the crude erythromycin was isolated as previously described (Martin *et al.*, 1966). The isolated material (478 mg) afforded 194 mg of antibiotic after crystallization from methylene chloride. The infrared spectrum of the isolated antibiotic was identical with that of erythromycin A. Figure 3 shows the production of erythromycin during the course of the fermentation.

Thin layer chromatography of ethyl acetate extracts of clarified fermentation beers 6 hr after addition of 6-deoxyerythronolide B showed that as the added compound disappears, erythronolide B and 3-α-L-mycarosylerythronolide B appear (Figure 4). Erythronolide B had disappeared at 24 hr but 3-α-L-mycarosylerythronolide B was evident until 96 hr. These data suggest that 6-deoxyerythronolide B is a biogenetic precursor of erythromycin immediately preceding erythronolide B.

Discussion

A neutral crystalline compound, an erythromycin biogenetic intermediate, was recovered from fermentation beer of a partially blocked mutant of *S. erythreus* by solvent extraction and column chromatography. The material was demonstrated to be an intermediate preceding erythronolide B (Ib). Thus, erythronolide B, 3-α-L-mycarosylerythronolide B, and erythromycin A were produced when the powdered compound was added to fermentation beers of a mutant *S. erythreus* which has a complete block early in the erythromycin pathway and is unable to synthesize erythromycin unless an intermediate is added. Thin layer chromatography of ethyl acetate extracts of fermentation beer (Figure 4) indicated that the compound is an intermediate immediately preceding erythronolide B suggesting a macrocyclic polyhydroxyoxolactone structure very similar to the structure of erythronolide B.

The unknown compound analyzed for $C_{21}H_{38}O_6$ or an oxygen atom less than the molecular formula ($C_{21}H_{38}O_7$) for erythronolide B and the ultraviolet and infrared absorption spectra were nearly identical with those of erythronolide B (Tardrew and Nyman, 1964). Of particular significance was the presence of a single nonbonded hydroxyl absorption band at 3605 cm^{-1} in the infrared spectrum (Figure 1), whereas the erythronolide B spectrum exhibited two sharp absorptions of nearly equal intensity at 3575 and 3605 cm^{-1}. The infrared spectra of these compounds exhibit only a single absorption band in the carbonyl region at 1712 cm^{-1}. These data suggested that the compound was a deoxyerythronolide B.

The nmr spectrum of the erythronolide B precursor is shown in Figure 2. Comparison of the nmr spectra of the compound and of erythronolide B shows that these materials have several common structural features. However, the nmr spectrum of erythronolide B

(10% in perdeuteriomethanol) gave a singlet at τ 8.70 which can be assigned to the protons of the methyl group at C-6. This singlet was replaced by a doublet at τ 8.99 (J = 6.8 cycles/sec) in the nmr spectrum of the erythronolide B precursor recorded under identical conditions. These data indicate that the erythronolide B precursor is 6-deoxyerythronolide B (Ia).

Supporting evidence for the structure was obtained by acetylation of the compound with acetic anhydride. The presence of three hydroxyl groups was indicated by the formation of a triacetate (Ic) analyzing for $C_{27}H_{44}O_9$ and possessing an infrared spectrum devoid of hydroxyl absorption. The nmr spectrum (10% in deuteriochloroform) of the triacetate showed a complex multiplet for the C-3, C-5, C-11, and C-13 protons at an average τ value of 4.98 which integrated for 4.1 protons. The three acetyl methyl groups gave a multiplet at an average τ value of 7.94 and integrated for 8.7 protons. The C-6 tertiary hydroxyl group of erythronolide B is resistant to acetylation and a tetraacetate cannot be derived (personal communication, T. J. Perun). Complete acetylation of the compound, which would not be expected if the C-6 tertiary hydroxyl group were present, and the identification of 4 protons at τ 4.98 corroborates the assigned structure.

The C-5 and C-6 hydroxyl groups of erythronolide B are typical of 1,2-diols and readily undergo oxidation by periodate as evidenced by the consumption of 1 equiv of the reagent. The erythronolide B precursor, however, does not submit to periodate oxidation. This observation indicates the absence of a 1,2-diol in the compound and is consistent with the assigned structure.

Recently Harris *et al.* (1965) defined the relative and absolute stereochemistry of erythromycin A hydroiodide. These investigators found that the aglycone moiety of erythromycin A has the following relative configurations: C-3:C-5 *cis*, C-3:C-6 *trans*, and C-5:C-6 *trans*. Although the stereochemistry of 6-deoxyerythronolide B is unknown, it is reasonable to assume that centers at carbons 3 and 5 have the same configuration as the antibiotic aglycone.

Additional evidence for the assigned structure, although not unequivocal owing to conformational uncertainties in macrocycle systems (Sicher, 1962), can be obtained from consideration of the phenylboronate ester derivative. Like erythronolide B, the compound reacts with benzeneboronic acid to form a phenylboronate ester. The erythronolide B boronate ester (IIa) is formed from the *cis* related C-3 and C-5 hydroxyl groups and forms the preferred six-membered ring (personal communication, T. J. Perun). The alternate five- and seven-membered ring boronate esters, formed from the C-5 and C-6 and C-3 and C-6 hydroxyl groups, respectively, are less common (Sugihara and Bowman, 1958), and each would require the unlikely esterification of the tertiary hydroxyl group which is also *trans* to the C-3 and C-5 hydroxyl groups.

The infrared spectrum of the 6-deoxyerythronolide B phenylboronate ester exhibits a single bonded hydroxyl absorption at 3505 cm^{-1} (C-11 hydroxyl) and absorption bands characteristic of the phenylboronate ester at 1600, 1440, and 1310 cm^{-1}. The nmr spectrum (10% in deuteriochloroform) exhibits complex multiplets for the aromatic protons at approximately τ 2.1 and 2.6 which integrate for 2.0 and 3.0 protons, respectively. These chemical shifts and splitting patterns are nearly identical with those exhibited by the 3,5-phenylboronate ester of erythronolide B. These data are fully consistent with a 3,5-phenylboronate ester of the precursor and further support the assigned structure.

Combination of the information derived from nmr spectra, analytical, and infrared data and biological conversion studies suggest 6-deoxyerythronolide B as the structure of the erythromycin intermediate. The positions of the ketone function and the three hydroxyl groups were not conclusively established; but since the compound is an efficient direct biological precursor of erythronolide B differing by a single oxygen atom, it follows from biosynthetic considerations that the compound must be 6-deoxyerythronolide B.

The isolation and establishment of 6-deoxyerythronolide B as an intermediate preceding erythronolide B allows further insight into erythromycin biosynthesis. Woodward (1957) speculated that the insertion of "extra" oxygen in macrolide algycone biosynthesis was probably a late biochemical event. Certainly, in the case of erythromycin, the suggestion of Woodward is confirmed.

Acknowledgments

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Studies on the Interaction of *p*-Mercuribenzoate with Turnip Yellow Mosaic Virus. IV. Conformational Change, Exposure of Buried Prototropic Groups, and pH-Induced Degradation*

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ABSTRACT: The unstable derivative of turnip yellow mosaic virus (TYMV), resulting from its reaction with *p*-mercuribenzoate (PMB) at pH 4.6, disintegrates in neutral media of finite ionic strength. This reaction was investigated by means of potentiometric and ultracentrifugal methods. Upon mixing the mercurial-TYMV derivative with KCl, an immediate exchange of protons for K^+ ions took place, causing a relatively large acid pH shift. Approximately 3600 protons were released per particle under the experimental conditions. No exchange occurred with untreated TYMV.

This indicated that the incorporation of PMB into the virus structure, *via* the protein sulfhydryl groups, had induced a conformational change sufficient to expose previously buried prototropic groups of the protein, or the ribonucleic acid (RNA), or both. Subsequent upscale titration of PMB-substituted TYMV caused the majority of the virus particles to collapse in the relatively narrow range of pH 6–7. The potentiometric titration curves of PMB-substituted TYMV and untreated TYMV allowed for the construction of a difference titration curve in the pH range above 3.8; this represented the titration properties of the newly exposed prototropic groups. Since the removal of PMB with mercaptoethanol restabilized most of the virus preparation, and also restored its titration properties, these phenomena appeared to be interrelated, justifying attempts to correlate the titration curves and the degradation curves. The protein capsids of TYMV did not retain their structural integrity upon reaction with PMB at pH 4.6, indicating an involvement of the RNA in stabilizing TYMV's structure. Such a possible involvement is discussed with respect to the correlation between titration and degradation properties of PMB-substituted TYMV.

Chemical studies of biologically significant proteins have frequently established a link between enzymatic properties and the reactivity of protein sulfhydryl groups. A number of excellent review articles have been written on this subject (Boyer, 1959; Cecil and McPhee, 1959; Cecil, 1963). In recent years, more and more reports have appeared in which sulfhydryl groups were assigned a strategic role in maintaining conformational stability of proteins. This provided another, though less direct, link between sulfhydryl groups and biological activity. Such a spatially more distant involvement of SH in the enyzmatic activity is reminiscent of an allosteric site (Gerhart and Schachman, 1965).

Among the pioneer studies which concerned the relationship of sulfhydryl reactivity, protein conformation, and enzymatic activity were those of Madsen and Cori (1956), Madsen (1956), and Madsen and Gurd (1956). They found a reversible inactivation of muscle phosphorylase as a result of its reaction with the mercurial *p*-mercuribenzoate (PMB),[1] which

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[1] Abbreviations used: TYMV, turnip yellow mosaic virus; ATC, artificial top component (empty protein shells or capsids); PMB, *p*-mercuribenzoate; ME, mercaptoethanol.