[^{35}S]Uridine 3′-Phosphorothioate. [^{35}S]Uridine 2′,3′-cyclic phosphorothioate, crystalline isomer (750 mμmol, 7 mμmol = 7951 cpm), was dissolved in 0.3 ml of Tris–acetate buffer (0.05 *M*, pH 7.4) and pancreatic ribonuclease (159 μg in 10 μl of buffer) added. After 12 hr at room temperature, the solution was chromatographed in system A and the radioactivity of the uv-active spots counted as described under General Procedures. A total of 60 mμmol of [^{35}S]uridine 2′,3′-O,O-cyclic phosphorothioate (1.2 mμmol = 1226 cpm) as well as 600 mμmol of [^{35}S]uridine 3′-phosphorothioate (12 mμmol = 12,807 cpm) was isolated. The latter traveled with uridine 2′(3′)-phosphate in electrophoresis at pH 7.5.

Hydrolysis of [^{35}S]Uridine 3′-Phosphorothioate by Alkaline Phosphatase. [^{35}S]Uridine 3′-phosphorothioate (0.3 μmol) was dissolved in 0.1 ml of 0.1 *M* Tris–acetate (pM 8.0) and 200 μg of alkaline phosphatase was added. After 12 hr at 37°, the reaction solution was analyzed by electrophoresis. Only uridine (0.28 μmol) could be recovered. A control experiment without enzyme indicated no change in the starting material.

Adenosine 3,′5′-Cyclic Phosphorothioate (11). Adenosine 5′-phosphorothioate (Na^+ salt, 2.4 g, 6 mmol) was dissolved in dry dimethylformamide (100 ml), dimethylformamide dimethyl acetal (10 ml) added, and the reaction mixture stirred overnight. Approximately 0.6 mmol had not gone into solution after this period. The solution was decanted, dried by codistillation with pyridine, and evaporated to dryness, and the residue taken up in dimethylformamide (800 ml). This solution was added dropwise over 5 hr under stirring to a solution of triisopropylbenzenesulfonyl chloride (8 g) in pyridine (600 ml). After another 5 hr water (10 ml) was added and the solution evaporated to dryness after 30 min. The residue was treated for 2 hr with concentrated ammonia (100 ml) and again evaporated to dryness. The reaction mixture was purified by chromatography on DEAE-cellulose. The resulting triethylammonium salt was converted to the K^+ salt by passage over a column of Merck I ion exchanger (K^+ form) and lyophilized: yield 1.2 g (50 %) of a white powder; $\lambda_{max}^{H_2O}$ 259 mμ (ε 15,000); ^{31}P nmr (H_2O) −18.7. In electrophoresis at pH 7.5 the material had a relative mobility of 0.83 to adenosine 3′,5′-cyclophosphate.

Anal. Calcd for $C_{10}H_{17}N_5O_5PSK \cdot 4H_2O$: N, 15.30; P, 6.80; S, 7.01. Found: N, 15.56; P, 5.90; S, 6.57.

Uridine 2′,3′-Cyclic Phosphorodithioate (12). 5′-Acetyluridine (960 mg, 3.3 mmol) and P_2S_5 (448 mg, 4 mmol) were dissolved in pyridine (8 ml) and left for 1 hr at room temperature and for a further hour at 60°. The yellow solution was evaporated and the residue treated with concentrated ammonia (50 ml) for 2 hr. After evaporation of the solvent, the residue was chromatographed on DEAE-cellulose. The reaction products obtained were uridine 2′,3′-cyclic phosphorothioate (2000 OD_{260} units, 0.2 mmol), identical by electrophoresis and paper chromatography with an authentic sample, a compound with the same mobility in electrophoresis as uridine 2′(3′)-phosphate, presumably uridine 2′(3′)-phosphorodithioate (2000 OD_{260} units), and uridine 2′,3′-cyclic phosphorodithioate (10,000 OD_{260} units) as a thick oil which crystallized on standing. Recrystallization from EtOH yielded 0.40 mg (28%) of white needles: mp 164–166°; $\lambda_{max}^{H_2O}$ 260 mμ (ε 10,000); ^{31}P nmr (H_2O) −126; in electrophoresis at pH 7.5 the material had a relative mobility of 0.90 to uridine 2′,3′-cyclic phosphate.

Anal. Calcd for $C_{15}H_{25}N_3O_6PS_2$ (as a monotriethylammonium salt): N, 9.59; P, 7.08; S, 14.64. Found: N, 9.36; P, 6.97; S, 13.94.

[^{35}S]Uridine 2′,3′-cyclic phosphorodithioate was synthesized in the same way using [^{35}S]P_2S_5.

[^{35}S]Uridine 3′-Phosphorodithioate. [^{35}S]Uridine 2′,3′-cyclic phosphorodithioate (**12**) (1 μmol, 12 mμmol = 4884 cpm) was dissolved in 0.3 ml of Tris–acetate buffer (0.05 *M*, pH 7.4) and pancreatic ribonuclease (450 μg in 30 μl of buffer) added. After 18 hr at room temperature the solution was chromatographed in system A and the radioactivity counted as described under General Procedures. [^{35}S]Uridine 2′,3′-cyclic phosphorodithioate (120 mμmol, 3.4 mμmol = 1156 cpm) and 350 mμmol of [^{35}S]uridine 3′-phosphorodithioate (7 mμmol = 2821 cpm), which was identical in electrophoresis at pH 7.5 with uridine 2′(3′)-phosphate, were isolated.

Hydrolysis of Uridine Cyclic Phosphates by Pancreatic Ribonuclease (Table II). To a 1-ml solution of dimethylglutaric acid buffer (pH 7.0, 0.1 *M*) and NaCl (0.1 *M*) in a thermostated cuvette, 2–15 μl of substrate solution was added. The reaction was started by addition of 10–30 μl of enzyme solution. The concentration of enzyme was determined by measuring the extinction at 277 mμ assuming ε = 9800. The reaction was followed at 283 mμ at 25° in a Cary 15 spectrophotometer. Initial velocities were determined by a first-order rate plot, K_m and k_{+2} values by a Lineweaver–Burk plot.

Inhibition of Diesterases. Inhibition experiments with snake venom phosphodiesterase were performed at 25° in a Gilford Model 2000 recorder connected with a Beckman Model DUR spectrophotometer. The liberation of *p*-nitrophenol was followed at 400 mμ assuming ε = 17,200. The reaction solution (3 ml) contained Tris–HCl buffer (pH 8.7, 0.3 mmol), protein (1 μg), and substrate and inhibitor as indicated in Figure 1.

In experiments with spleen phosphodiesterase, the reaction solution (300 μl) contained succinate buffer (pH 5.6, 50 μmol), 1% Tween 80 (10 μl), protein (0.68 μg), and substrate and inhibitor as indicated in Figure 2. The liberation of *p*-nitrophenol after 4 min of incubation at 25° was determined by transfer of 50 μl of the incubation solution to 1.0 ml of 0.1 *N* NaOH and measurement of the optical density of the resulting solution at 400 mμ.

Communications to the Editor

Detection of a "Michaelis" Complex by Spin Labeling in a Model Enzyme System

Sir:

The detection and characterization of enzyme–substrate intermediate complexes is critical to the elucidation of enzymatic reaction mechanisms. Although numerous studies on proteolytic enzymes have been concerned with the direct detection of covalent acyl enzyme intermediates, the techniques employed in these investigations have not been generally suitable for the direct observation of noncovalent "Michaelis" enzyme–substrate complexes.[1] Frequently, evidence for the postulation of the formation of intermediate Michaelis complexes has come from the kinetic dependence of the enzymatic reactions on either the substrate or enzyme concentration (*i.e.*, saturation effects). We wish to report the use of the spin-labeling technique[2] to detect directly a noncovalent "Michaelis" complex as well as a covalent complex in a model enzyme catalyzed hydrolysis of an ester. The model enzyme employed is cycloheptaamylose, one of the cyclodextrins, which are toroidal polysaccharides known to catalyze the hydrolysis of phosphate[3] and carboxylic[4]

(1) M. L. Bender and F. J. Kézdy, *Annu. Rev. Biochem.*, **34**, 49 (1965).

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(4) M. L. Bender, R. L. Van Etten, G. A. Clowes, and J. F. Sebastian, *ibid.*, **88**, 2318 (1966); M. L. Bender, R. L. Etten, and G. A. Clowes, *ibid.*, **88**, 2319 (1966); M. L. Bender, R. L. Van Etten, G. A. Clowes, and J. F. Sebastian, *ibid.*, **89**, 3242 (1967); **89**, 3253 (1967).

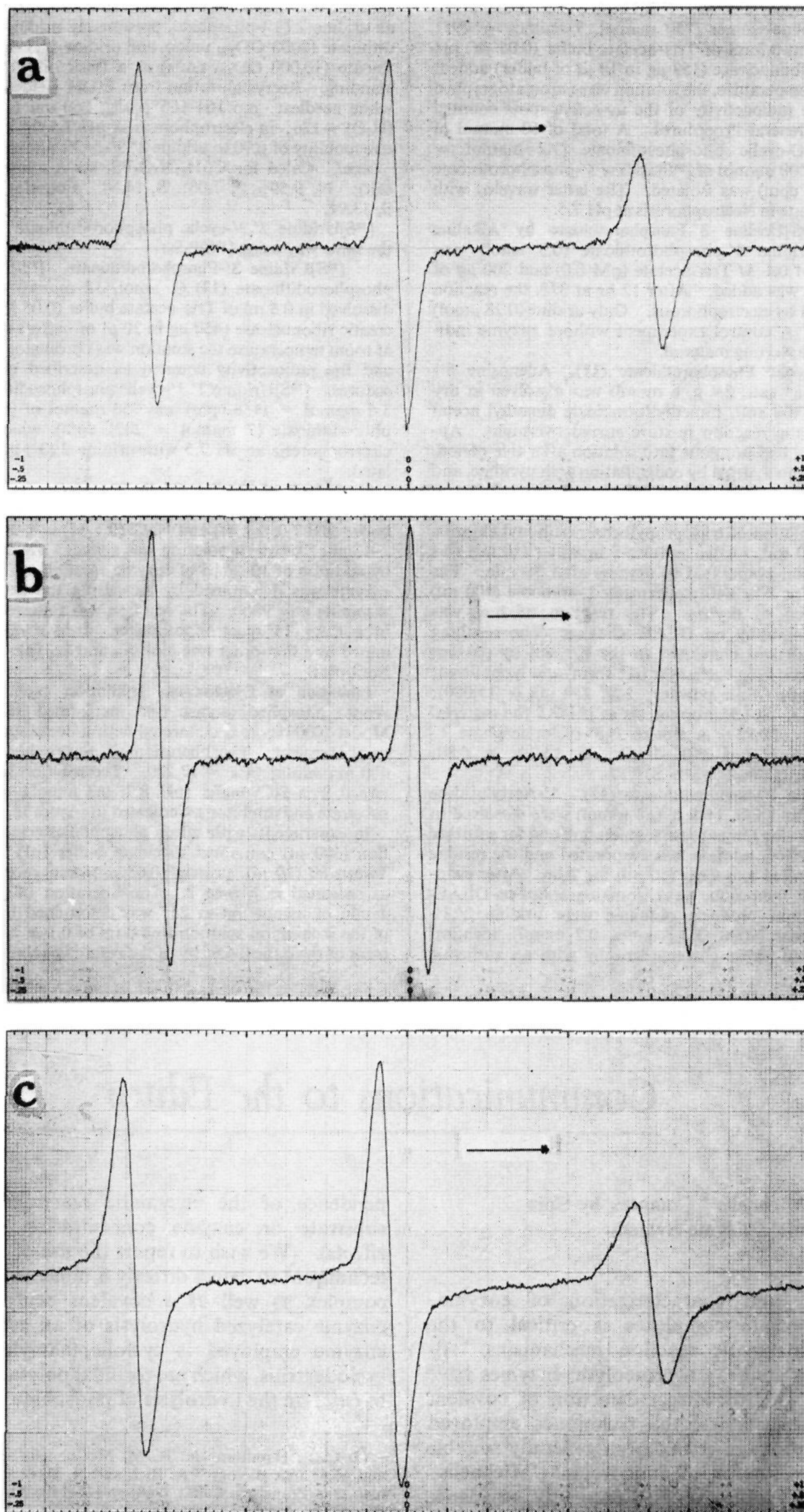
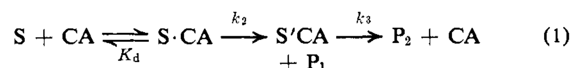


Figure 1. (a) Esr spectrum of I (10^{-4} *M*) in the presence of cycloheptaamylose (4×10^{-3} *M*) at pH 5.75 and 25.0°. (b) Esr spectrum of uncomplexed ester I at pH 5.75 and 25.0°. (c) Esr spectrum of acyl cycloheptaamylose III at pH 5.75 and 25.0°. The arrows in the figure cover 5 G.

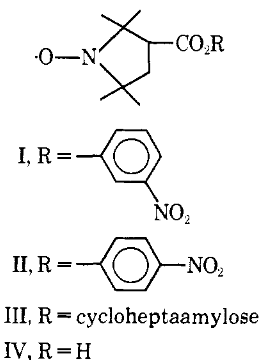
esters *via* the intermediate formation of inclusion complexes.

Rate measurements on the hydrolyses of phenyl esters have provided evidence[4] that catalysis of these reactions by the cycloamyloses (cyclodextrins) occurs by the kinetic scheme illustrated by eq 1 which is similar to that demonstrated to hold for catalysis by α-chymotrypsin.[1,5]



$$S + CA \underset{K_d}{\rightleftharpoons} S \cdot CA \xrightarrow{k_2} S'CA \xrightarrow{k_3} P_2 + CA \quad (1)$$
$$+ P_1$$

The substrate employed in the present work was the ester I. A similar compound II reacts with serine-195 at the active site of α-chymotrypsin to give a spin-labeled acyl enzyme species.[6]



From visible absorption measurements on the rate of production of the *m*-nitrophenolate ion from I at pH 9.7 (carbonate buffer) and 25.0° in the presence of varying amounts of excess cycloheptaamylose we calculated a value of $K_d = 7.5 \pm 0.6 \times 10^{-4}$ *M* and $k_2 = 6.9 \times 10^{-3}$ sec^{-1}.

From the work of Bender, *et al.*,[4] the value of K_d would not be expected to be significantly different in acidic solution. When a solution of I (10^{-4} *M*) and cycloheptaamylose (4×10^{-3} *M*) in phosphate buffer at pH 5.75 was examined using a Varian E-3 spectrometer the esr signal illustrated in Figure 1a was observed. For comparison the esr spectrum of I in the absence of the cycloamylose is shown in Figure 1b. The rotational correlation time τ for I was calculated[7] to be 0.35×10^{-10} sec. The spectrum of Figure 1a is attributed to substrate–cycloheptaamylose "Michaelis" complex (S·CA) with $\tau = 3.34 \times 10^{-10}$ sec. *m*-Nitrophenolate ion did not form in an appreciable quantity during the course of the esr measurement. By varying the concentration of excess cycloheptaamylose the dissociation constant K_d was estimated as $6 \pm 2 \times 10^{-4}$ *M* at pH 5.75, in good agreement with the value obtained from the kinetic studies at pH 9.7.

The acyl cycloheptaamylose III was prepared, using the procedure described by Bender,[4] from cycloheptaamylose and I at pH 9.6. Gel filtration chromatography (G-10 Sephadex, pH 5.75 phosphate buffer) was employed to separate the required intermediate from the unreacted ester and the products of hydrolysis.

The value of τ calculated from the esr spectrum observed for a solution of the acyl cycloheptaamylose III (Figure 1c) at pH 5.75 was 5.04×10^{-10} sec. A comparison of this rotational correlation time with the one measured for the "Michaelis" complex indicates that the spin label is somewhat more immobilized in III than in the noncovalent complex.

On raising the pH to 9.6 the rate of deacylation of III was studied by following the increase in height of the high-field hyperfine component of the spectrum, as the more rapidly tumbling acid IV is formed. The rate constant for the deacylation step was calculated as $k_3 = 3.2 \times 10^{-5}$ sec^{-1}.

In summary, using the ester substrate I, we have observed directly the "Michaelis" complex S·CA and the acyl cycloheptaamylose complex S'CA by esr spectroscopy. The rotational correlation time τ for the "Michaelis" complex was found to be intermediate between those found for the uncomplexed substrate I and the acyl cycloheptaamylose III, although it was closer to the one for the latter species. Specificity in the action of proteolytic enzymes has been interpreted by Bender, *et al.*,[8] in terms of an interaction of the R′ group of the acyl function (R′C=O) with the enzyme surface, rigidifying the whole set of bonds involved in the reaction so that the acyl group occupies the correct position for reaction even in the ground state. In agreement with this picture it is quite clear that the R′ group of the acyl function of the ester I, the part of the substrate containing the nitroxide moiety, is significantly immobilized in the "Michaelis" complex with the model enzyme cycloheptaamylose. The results of esr measurements on Michaelis complexes of substrates with the cycloamyloses and with proteolytic enzymes will provide a critical test for the proposed theory of enzyme specificity.

Acknowledgment. The support of the Petroleum Research Fund of the American Chemical Society is gratefully acknowledged. We also wish to thank Professor F. J. Kezdy for a helpful discussion.

(5) In eq 1, S represents the ester, CA the cycloamylose, S·CA the inclusion or "Michaelis" complex, S'CA the acylcycloamylose, P_1 the product alcohol, and P_2 the product acid.

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Methoxy Groups As Probes for Delocalized Cations. Substituent Effects on 2-Norbornyl Solvolysis Rates

Sir:

The effect on solvolysis rates by substituents remote from the reaction site is a standard way to detect the presence or absence of charge delocalization.[1] As the following examples (I–IV) suggest, methoxy groups may be more sensitive[2] for this purpose than the commonly employed methyl groups.[1,3-8]

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