terpretation, one of us (D.R.D.) examined the tissues without prior knowledge of which was the experimental side.

Four hemibladders were bathed on both surfaces by isotonic Na^+ Ringer solution. Vasopressin (Pitressin) was added to the serosal medium of the experimental half of each preparation to a final concentration of 40 to 200 milliunits per milliliter. An identical volume of Na^+ Ringer solution was added to the serosal medium of the control half. The short-circuit current of all the experimental sides increased after addition of hormone. The tissues were fixed 15 to 20 minutes after addition of hormone.

The control side was characterized by closely apposed lateral margins (Fig. 1A). The sample treated with vasopressin showed enlarged intercellular spaces (Fig. 1B).

Three experiments were carried out in identical fashion with isotonic choline$^+$ Ringer solution. In the absence of Na^+, the addition of vasopressin did not alter the transepithelial potential, which remained close to zero. However, electron micrographs revealed that the results were unchanged from those obtained with Na^+ Ringer solution. In every case, the intercellular spaces of the experimental side were considerably more open than those of the control.

Vasopressin may increase the intercellular spaces in the absence of net transfer of salt and water. This increase may be related to another effect of vasopressin, the relaxation of the smooth muscle of toad bladder (*8*). The distribution of the submucosal smooth muscle and collagen may be such that muscle contraction results in increased pressure against the basement membrane, directed from serosa to mucosa. Since the basement membrane is porous (*9*), intercellular fluid would then be extruded into the submucosa, and the cells would be more closely apposed.

In other experiments (*10*), hemibladders were very loosely mounted, and one side was distended by an applied hydrostatic gradient. The nondistended control half revealed markedly open intercellular spaces, while the spaces of the distended half were tightly closed.

We conclude that although dilation of the intercellular spaces may result from increased net transepithelial water flow, other experimental conditions may produce similar morphologic changes. Therefore, dilation of the intercellular spaces in itself does not indicate increased net transport of water across epithelial membranes.

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Noncovalent Binding of a Spin-Labeled Inhibitor to Ribonuclease

Abstract. *The stable free radical 2,2,6,6-tetramethyl-4-hydroxypiperidin-1-oxyl monophosphate has been synthesized; it binds to ribonuclease. The selective changes in the nuclear magnetic resonance spectrum of the enzyme produced by the free radical make it possible to define qualitatively the region of the enzyme to which it binds. The radical appears to occupy a site similar to that to which inorganic phosphate binds which is close to or within the active site of the enzyme.*

Stable free radicals, or spin labels, were first used for investigating the structure of proteins by McConnell and co-workers (*1*) and subsequently by others (*2–4*). Most of these investigators studied the electron spin resonance spectrum of a free radical covalently attached to a protein and obtained information about changes in the relative rotational freedom of the spin label and hence about changes in its environment on the protein under various conditions (*1, 2*). The unpaired electron of these free radicals also increases the relaxation rate of a nearby nucleus, and this effect can be used to give detailed information about the environment of the spin label. Sternlicht and Wheeler (*3*) showed that a marked broadening of the nuclear magnetic resonance (NMR) spectrum of lysozyme resulted in covalent attachment of a spin label to the protein, although specific effects were not observed. Mildvan and Weiner (*4*) have reported that the effects of a spin-labeled analog of nicotinamide-adenine dinucleotide (NAD) on the relaxation rates of the protons of ethanol and acetaldehyde in the presence of liver alcohol dehydrogenase (E.C.1.1.1.1) could be used to give detailed information on the mode of binding of substrates to the enzyme.

We have now studied the effects of a spin-labeled inhibitor on the NMR spectrum of bovine pancreatic ribonuclease A (E.C.2.7.7.16). The inhibitor used was 2,2,6,6-tetramethyl-4-hydroxypiperidin-1-oxyl monophosphate (III). Ribonuclease is competitively inhibited by phosphate, sulfate, and arsenate (*5*), and by many phosphate monoesters (*6*). We hoped, therefore, that compound III would similarly bind to the active site of the enzyme.

Compound III was prepared as follows: A freshly prepared 2.0 molar solution of dimorpholinophosphobromidate (*7*) in chloroform (12.8 ml, 0.0255 mole) was added at room temperature to a chloroform solution (12.8 ml) of 2,2,6,6-tetramethyl-4-hydroxypiperidin-1-oxyl (I) (*8*) (4.00 g, 0.0232 mole) and tribenzylamine (7.34 g, 0.0255 mole), which was left at room temperature in a sealed flask for 17 hours. More 2.0 molar reagent (6.4 ml) and tribenzylamine (3.67 g) were added, and the reaction mixture was set aside for another 24 hours. Chloroform was then removed at reduced pressure, and the residue was triturated with benzene (two 50-ml portions). Insoluble tribenzyl hydrobromide (10.09 g) was filtered off, the solvent was removed from the filtrate at 75°C (1 mm-Hg), and the residual dark red syrup (18.03 g) was chromatographed over silica gel (720 g) in chloroform containing increasing amounts of methanol (2 percent maximum). Tribenzylamine (5.53 g) was eluted first, and then 2,2,6,6-tetramethylpiperidin-1-oxyl-4-phosphoryldimorpholidate (II) (5.47 g) was eluted. Crystallization twice from cyclohexane containing a trace of ether

resulted in pale orange-brown prisms (2.64 g), m.p. 99.5° to 100.5°C. Analysis showed (percent) C, 52.57; H, 8.61; N, 10.95. The calculated values (percent) are, for $C_{17}H_{33}N_3O_5P$: C, 52.30; H, 8.52; N, 10.77. Analytical grade Biorad AG 50W-X8 ion-exchange resin (50 to 100 mesh) in its hydrogen form was washed once with distilled water. The wet resin (37 ml) was added to a solution of 2,2,6,6-tetramethylpiperidin-1-oxyl-4-phosphoryldimorpholidate (II) (3.70 g) in distilled water (37 ml). The mixture was magnetically stirred and heated to 65°C under nitrogen for 2½ hours and then filtered hot. More water (40 ml) at 65°C was added, and the mixture was stirred at this temperature for 30 minutes and filtered while hot. This process was repeated four more times. The combined aqueous filtrates were evaporated at 100°C (0.5 mm-Hg) leaving a resin (1.23 g) which slowly crystallized from water (~ 2 ml), after several days at room temperature, to give off-white prisms (0.48 g), m.p. approximately 190° to 203°C, of the monohydrate (probably) of 2,2,6,6-tetramethyl-4-hydroxypiperidin-1-oxyl phosphate (III). Heating at 140°C (0.5 mm-Hg) for 2 hours resulted in compound III. Analysis showed (percent) C, 42.96; H, 7.80; N, 5.46. Calculated values (percent) for $C_9H_{19}NO_5P$ are C, 42.85; H, 7.59; N, 5.55.

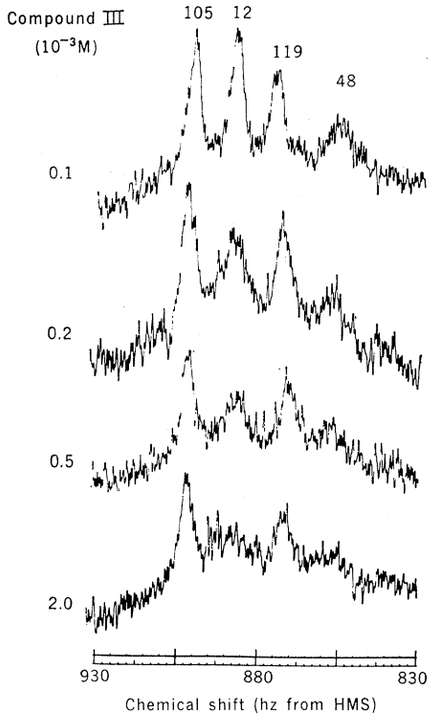
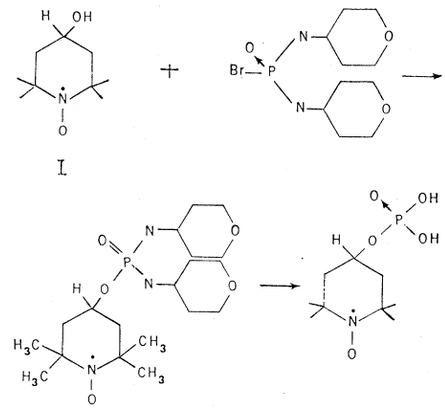


Fig. 1. Imidazole C(2) proton region of the NMR spectrum of 0.0065*M* ribonuclease [0.2*M* NaCl, *p*H (meter reading) 5.5, 99.8 percent D_2O] in the presence of increasing concentrations of compound III (2,2,6,6-tetramethyl-4-hydroxypiperidin-1-oxyl monophosphate). The spectra were obtained at 100 Mhz with a Varian HA-100 spectrometer at the normal probe temperature of 32°C. The external standard was hexamethyldisiloxane (HMS). Each spectrum was time-averaged over 40 to 50 sweeps with a C1024 computer of average transients. The C(2) protons of His^{105}, His^{12}, His^{119}, and His^{48} are identified in the figure.

Figure 1 shows the effect of increasing concentrations of compound III on the histidine C(2) proton absorptions in the NMR spectrum of ribonuclease. The assignment of the peaks to particular histidine residues in the enzyme is taken from Meadows *et al.* (*9*). The spectrum in the presence of 0.1 m*M* compound III is identical to that in its absence, but as the concentration is increased to 2 m*M*, the C(2) proton peak of histidine-12 (His^{12}), and to a lesser extent that of His^{119}, is broadened, while that of His^{105} is unaffected throughout. The His^{48} C(2) proton peak does not appear to be appreciably broadened, though it is not possible to be certain of this; the peak is very broad even in the absence of compound III, and the overlap of the peaks as they become broader makes an accurate estimate of linewidths impossible at higher concentrations of compound III. Apart from the disappearance of a small shoulder which can be assigned to the C(4) proton peak of His^{12} (*10*), addition of compound III at concentrations up to 5 m*M* had no effect on the main aromatic region of the NMR spectrum of ribonuclease.

In view of the difficulty of determining linewidths with sufficient accuracy under these conditions, quantitative conclusions about the distance between the unpaired electron and the different histidine C(2) hydrogens cannot be drawn at present. It is clear, however, that the unpaired electron is relatively close to His^{12}, somewhat farther from His^{119}, and distant from His^{105}, His^{48}, and the phenylalanine and tyrosine residues. This is consistent with the x-ray structure of the enzyme (*11*) which shows that His^{12} and His^{119} are close together in a cleft in the molecule, while the other two histidines are much farther away. The identification of the region of the molecule containing His^{12} and His^{119} residues as the



active site was originally based on chemical modification studies (*12*), and it has since been amply confirmed by the results of x-ray diffraction (*11, 13*) and NMR (*14, 15*). Addition of cytidine or cytidine nucleotides to ribonuclease causes an upfield shift of a peak in the aromatic region of the NMR spectrum of the enzyme (*14, 15*). This shift has been assigned (*15*) to phenylalanine-120, which has been shown by x-ray diffraction (*13*) to be in the pyrimidine binding site of the enzyme. Since compound III has no effect on the phenylalanine and tyrosine region of the NMR spectrum of ribonuclease, it appears that the free radical portion of this inhibitor does not extend into the cytidine binding site. This study indicates that spin-labeled inhibitors used in conjunction with NMR provide a means for determining the amino acid residues in the immediate vicinity of an inhibitor binding site, which will be particularly valuable when quantitative measurements of the distance from the unpaired electron to groups on the enzyme become possible.

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Anaphylatoxin Release from the Third Component of Human Complement by Hydroxylamine

Abstract. *Treatment of highly purified preparations of the third component of complement (C3) with 0.5*M *hydroxylamine at 20°C for 15 to 30 minutes, followed by acidification, resulted in dissociation of a peptide from the C3 molecule. The isolated fragment (molecular weight, 7600) resembled enzymatically liberated anaphylatoxin (C3a) with respect to size, charge, amino acid composition, and biological activity. Its capacity to contract smooth muscle was inhibitable by antihistamines; it also produced tachyphylaxis and desensitization of the guinea pig ileum to C3a. Thus native C3 probably contains an esterlike bond and hydroxylamine-liberated anaphylatoxin may represent one of the polypeptide chains of the C3 molecule.*

Recently, anaphylatoxin could be identified with two enzymatically produced split products derived from the components of complement C3 and C5. Both fragments, designated C3a and C5a, respectively (*1*), exhibit smooth muscle contracting activity and the capacity to change capillary permeability. They are not only chemically distinct but also biologically in that C3a anaphylatoxin can cause contraction of guinea pig ileum desensitized to the action of C5a anaphylatoxin and vice versa. This field of research has been reviewed (*2*).

The C3a anaphylatoxin has been shown to be liberated from the native C3 molecule by the following enzymes: C3 convertase (*3–5*), C3 inactivator complex (*4*, *6*), trypsin, and plasmin (*5*). The ease with which C3a may be cleaved off its precursor, particularly by trypsin (see below), indicated the presence of a site in the native C3 molecule which is highly susceptible to enzymatic attack. The following experiments were carried out to determine whether a biologically active, C3a-like piece can be liberated from C3 by chemical dissociation.

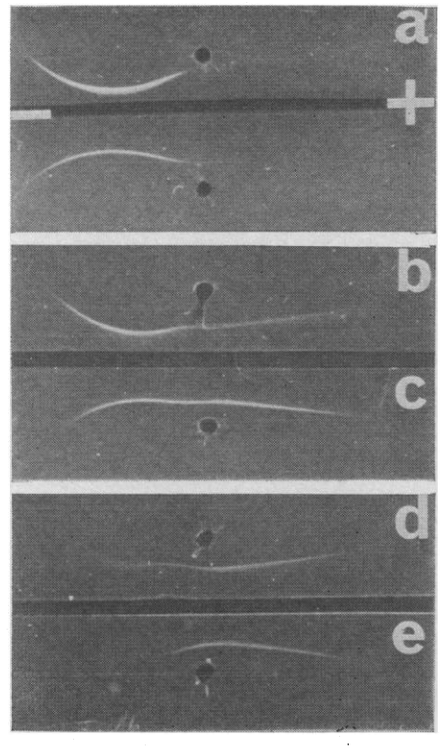

Fig. 1. Change of immunoelectrophoretic appearance of isolated C3 caused by treatment with hydroxylamine for 5 to 60 minutes. Concentration of C3 was 3 mg/ml, that of NH_2OH was 0.5*M*; 20°C, *p*H 7.0. Electrophoresis was carried out for 2 hours at *p*H 8.6 with a potential gradient of 2 volt/cm; patterns were developed with a rabbit antiserum to human C3. The cathode is to the left. (a) Control; (b) 5 minutes; (c) 15 minutes; (d) 30 minutes; (e) 60 minutes.

Samples (30 mg) of highly purified human C3 (*7*) in 10 ml of phosphate buffer, *p*H 7.0, ionic strength 0.1, were treated with 0.5*M* NH_2OH for 15 to 30 minutes at 20°C. The NH_2OH solution was prepared as described by Lipmann and Tuttle (*8*). The reaction between NH_2OH and C3 was stopped by transferring the reaction mixture to an ice bath and by increasing the hydrogen ion concentration with 1*N* HCl to *p*H 3.8; the reaction mixture was then dialyzed at 4°C (overnight) against acetate buffer, *p*H 4.0, ionic strength 0.1. Separation of the C3 products was accomplished at *p*H 4.5 either by filtration on a Sephadex G-100 column or by preparative electrophoresis on a polyacrylamide gel column according to the procedures described for the isolation of C3a (*5*). Enzymatically liberated C3a anaphylatoxin was prepared by treatment of 30-mg batches of C3 in phosphate buffer, *p*H 7.5, with either trypsin [1 percent (by weight), 30 seconds, 20°C, followed by addition of soybean trypsin inhibitor (2 percent by weight)] or C3 convertase (1 to 2 percent by weight), 20 minutes, 37°C (*5*). The maximum yield of C3a in both instances was approximately 1 mg. Anaphylatoxin activity was assayed on segments of guinea pig ileum in a Schultz-Dale bath (*4*).

Figure 1 shows the effect of treatment with 0.5*M* NH_2OH on the immunoelectrophoretic appearance of isolated C3. Within minutes, an electrophoretically faster migrating component appeared which resembled the hemolytically inactive form of C3 (*9*) or its major fragment, C3b (*5*). As judged by the immunoelectrophoretic patterns, the reaction with NH_2OH was complete after 30 to 60 minutes at 20°C, and there was no evidence for a second reaction product which would have indicated cleavage of the C3 molecule. However, analytical disc electrophoresis at *p*H 4.5 (Fig. 2) revealed in NH_2OH-treated C3 a minor component which migrated toward the cathode with the same mobility as C3a produced either by trypsin or C3 convertase (*5*). This fragment could not be liberated by exposure of C3 to acid *p*H alone,