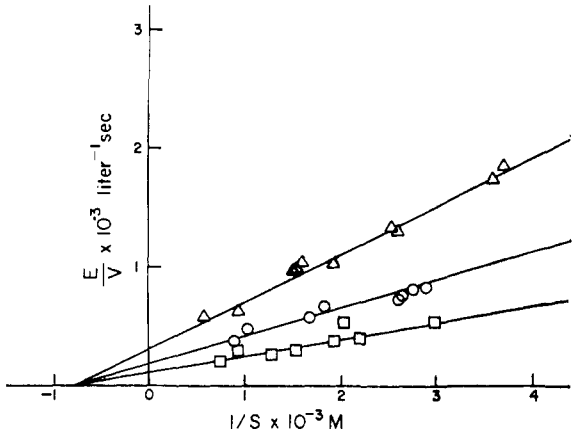

Figure 2. Plot of total enzyme divided by the initial velocity *vs.* the reciprocal of the substrate concentration for the reaction of bovine ribonuclease-A with *o*-nitrophenyl hydrogen oxalate anion at various concentrations of collidine perchlorate buffer (Δ, 0.2 *M*; ○, 0.1 *M*; □, 0.05 *M*) at 30° and pH 5.9.

exhibit no initial burst reaction with *o*-NPO⁻ though the reaction representing the second phase (Figure 1) is still present. His_{12}-carboxymethylated RNase (as a 2.15×10^{-5} *M* solution in 0.1 *M* phosphate buffer containing 0.05% phenol) was indistinguishable from RNase at the same concentration (for 18 kinetic runs for each).[8] Because we could not obtain His_{12}-carboxymethylated RNase as other than a dilute solution, we can say no more. His_{119}-carboxymethylated RNase exhibits an initial burst reaction smaller than that of S-protein.

The catalysis of the hydrolysis of cytidine 2′,3′-cyclic phosphate by RNase is known to be greatly dependent upon the integrity of His_{12} and His_{119}, as well as upon the tertiary structure of the enzyme which is assured by the intactness of all four disulfide bonds. Of these features the intactness of the four disulfide bonds of the S-protein is essential for *o*-NPO⁻ activity. The lack of activity of the cyanoethylated RNase suggests the involvement of an amino group in binding of *o*-NPO⁻ or in the catalytic process. The inability of oxidized RNase and reduced and carboxymethylated RNase to exhibit the initial burst and the almost complete lack of reactivity of bovine serum albumin, lysozyme, pepsin, and trypsinogen with *o*-NPO⁻ provide evidence that we are not dealing with a completely nonspecific protein reagent.

The initial burst is not subject to product inhibition at substrate levels nor inhibition by phenol (to 0.17 *M*), cytidine (at 3×10^{-4} *M*), and phosphate or chloride ion (at 1.22×10^{-3} *M*). RNase activity is known to be only weakly inhibited by cytidine,[9] and binding of phosphate and chloride ions[10,11] is also weak. Cytidylic acid, ATP ($K_i = 3.8 \times 10^{-3}$ *M*), and CTP are strong competitive inhibitors of the cyclic phosphate,[9] whereas toward *o*-NPO⁻ they are strong noncompetitive inhibitors ($K_i \cong 1.5 \times 10^{-4}$ *M*, 1×10^{-4} *M*, and 8×10^{-4} *M* for cytidylic acid,[12] ATP, and CTP, respectively). The cytidylic inhibition may be employed to determine what derivatives of RNase not possessing RNase activity still bind inhibitor. Thus, the reaction of S-protein with *o*-NPO⁻ is inhibited by cytidylic acid ($K_i = 6.6 \times 10^{-3}$ *M*). Cytidylic acid also inhibited the reaction of His_{119}-carboxymethylated RNase. From this result binding of cytidylic acid is not dependent on the intact nature of His_{12} or His_{119}. It has been reported that neither of the carboxymethylhistidine derivatives of RNase complexes with 2′-cytidylate.[13] Our data are not in accord with these results and tend to support the data of Ross, Mathias, and Rabin that cytidylic acid is bound to carboxymethylribonuclease.[14] Iodoacetate (at 1.8×10^{-3} *M*) and collidine or lithium perchlorate (for both, $K_i = 0.03$ *M*; Figure 2) are noncompetitive inhibitors of *o*-NPO⁻, whereas anions are known to be competitive inhibitors of the cyclic phosphate.[9,15,16] Thus, anionic species which are known to be competitive inhibitors of cytidine 2′,3′-cyclic phosphate are noncompetitive inhibitors of *o*-NPO⁻. It is known that anions bind to more than one site on RNase,[17-19] and the reaction of *o*-NPO⁻ likely occurs near one of these sites. The noncompetitive inhibition is probably due to the binding of cytidylic acid or other inhibitor at an anion binding site other than the active site toward *o*-NPO⁻. One might conclude that the positive binding site afforded by Arg_{10} and the group His_{12} is not essential to *o*-NPO⁻ activity.

All data reported herein have been collected at the single pH of 5.9 and at a temperature of 30° and, unless otherwise indicated, in 0.1 *M* collidine perchlorate buffer.[20,21]

(8) His_{119}-carboxymethylated RNase was found to have approximately 1% activity toward cyclic phosphate compared to the native enzyme while the His_{12} derivative had less than 1% activity.

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(12) A mixture of 2′- and 3′-cytidylic acid (Sigma) was employed. Both are competitive inhibitors of RNase.

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(21) We gratefully acknowledge the kindness of F. M. Richards, C. H. W. Hirs, A. R. Crestfield, and J. P. Riehm in providing us with ribonucleases, and the National Institutes of Health for financial support. In addition we wish to thank Drs. A. R. Crestfield, G. G. Hammes, S. Moore, F. M. Richards, and W. H. Stein for helpful suggestions.

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The Direct Observation of a Cationic Intermediate in a Carbonyl-Assisted Solvolysis

Sir:

The participation of the carbonyl oxygen in the solvolysis of γ-halobutyrophenones (Ia,b) has been suggested by Oae[1] and by Pasto and Serve.[2] To explain the observed rate enhancement relative to the corre-

(1) S. Oae, *J. Am. Chem. Soc.*, 78, 4030 (1956).

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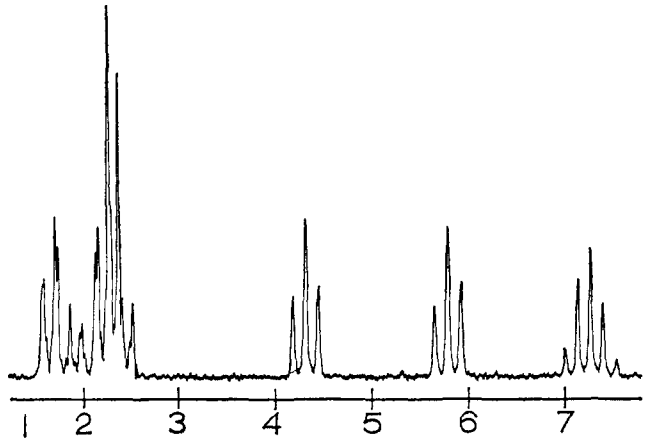
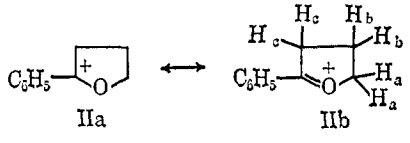


Figure 1. The nmr spectrum obtained by dissolving 4-*p*-bromobenzenesulfonoxybutyrophenone in trifluoroacetic acid at ~40°.

sponding *n*-butyl halides, they postulated that a cation of structure II might be an intermediate in the reaction.

$$\underset{1}{C_6H_5}\overset{\overset{O}{\|}}{\underset{2}{C}}\underset{3}{CH_2}\underset{4}{CH_2}CH_2X$$

Ia, X = Cl
b, X = Br
c, X = $OSO_2C_6H_4Br$ (OBs)
d, X = O_2CCF_3
e, X = O_2CH



In the course of the investigation of the formolysis of 4-phenyl-3-butynyl 1-brosylate, we have found that the acid-catalyzed addition of solvent to the triple bond to give enolformates, III (*cis* and *trans*), is a facile reaction.[3] The enolformates, III, were observed to

$$C_6H_5\overset{\overset{O-CHO}{|}}{C}=CHCH_2CH_2OBs$$
III

react further to give a species of unknown structure (IV), characterized by its nmr spectrum: τ 4.29 (triplet, two protons), 5.78 (triplet, two protons), 7.25 (pentuplet, two protons), and 1.5–2.5 (multiplet, nine protons). Since the conversion of enol esters to their corresponding ketones is an established reaction,[4] we have prepared the brosylate (Ic) and examined its solvolysis in formic and trifluoroacetic acids.

We find that when Ic is dissolved in either acid at room temperature, a spectrum identical with that of IV is obtained. The spectrum in trifluoroacetic acid is shown in Figure 1. If formic acid-d_2 is substituted for formic acid the triplet at τ 5.78 disappears (and so can be assigned to the two protons on the carbon α to the carbonyl) and the pentuplet at τ 7.25 becomes a slightly broadened triplet. Thus the protons at τ 4.3 must be those on carbon 4 in Ic. This absorption is at much lower field than would be expected for those protons in the brosylate itself, and is, in fact, in the region of

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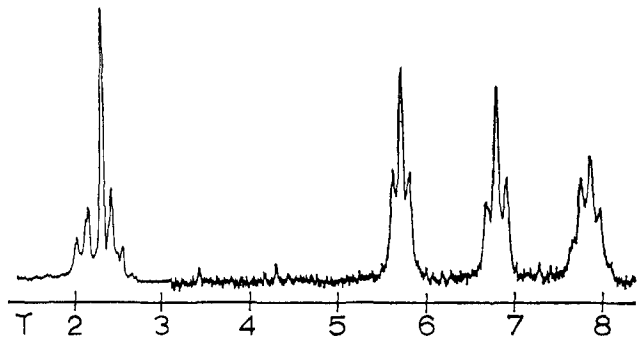

Figure 2. The nmr spectrum of 4-*p*-bromobenzenesulfonoxybutyrophenone in trifluoroacetic acid at −3°.

absorption for protons on carbon next to a positive charge.[5]

We suggest that the observed spectrum (Figure 1) is actually that of the cation II, formed by a very rapid ionization of Ic. Support for this suggestion is provided by the observation that a solution of 2-phenyl-4,5-dihydrofuran (V) with an equivalent amount of perchloric acid in trifluoroacetic acid gives a spectrum which is predominantly the same as that shown in Figure 1.

The fact that the absorption assigned to H_a in II should be a sharp triplet is not obviously predicted from the usual considerations of the variations of coupling constants with dihedral angle. However, the protons on the carbon next to the ether oxygen in V, a suitable model compound, give a clean triplet. The unusual vicinal coupling constants and the effect of electronegative substituents on these constants in systems similar to II have been reported[6] and are consistent with the observed spectrum.

The chemical shifts observed in the spectrum shown in Figure 1, relative to those of Ic, give some information on the charge distribution in II. The biggest shift, 1.6 ppm, is observed with the H_a protons, followed by shifts of 1.0 ppm for H_c and 0.6 ppm for H_b, clearly suggesting that IIb is a more suitable representation for the ion than IIa.

When Ic is added to precooled trifluoroacetic acid and the spectrum is taken at −3°, it is possible to observe the absorption expected for Ic, with only a small conversion to II[7] (Figure 2). Using data obtained from repeated integral scans, an approximate rate constant, $k = 1.2 \times 10^{-4}$ sec^{-1}, can be obtained for the conversion of Ic to II.

The spectrum of II in trifluoroacetic acid remains unchanged at room temperature for at least 30 days. When excess sodium trifluoroacetate is added and the solution is heated, the spectrum of Id can be observed. In formic acid, II formed from Ic is less stable and reacts at room temperature to form Ie as the major product. With added benzenesulfonic acid, which should act to reduce the concentration of formate anion, II reacts to form Ie less rapidly, and other reactions are observed.

When 4-*p*-bromobenzenesulfonoxy-2-pentanone is dissolved in trifluoroacetic acid at room temperature,

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(7) The observed line broadening is apparently a result of increased solvent viscosity at the lower temperature. In carbon tetrachloride solution, Ic has a well-resolved spectrum with similar chemical shifts.

it is possible to observe the reaction of the brosylate to form a species, VI, whose spectrum is very similar to that shown in Figure 1: τ 2.1–2.5 (quartet, four protons), 4.29 (triplet, two protons), 6.10 (triplet, two protons), 6.94 (singlet, three protons), and 7.48 (pentuplet, two protons). We conclude that an oxonium ion is formed in this system as well, and make the spectral assignments by analogy to those made for IIb. It is important to notice the presence of a phenyl substituent is not necessary for ion formation.

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A Hypochromic Effect from Pairing of Purine and Pyrimidine Bases

Sir:

Hypochromism in nucleic acids was first theoretically considered by Tinoco[1,2] and Rhodes[3] and was attributed to a dispersion interaction involving the transition moment for the lowest energy $\pi-\pi^*$ transition with those for the transitions of higher energy. It has consequently been generally supposed that hypochromism is a consequence of "vertical," or stacking, interactions between the chromophores, and indeed the spectra of polynucleotides in the single-strand stacked conformation[4,5] bears out the validity of this concept. At the same time it is known that intensity changes can come about from environmental effects of various kinds; we have attempted to ascertain whether such extraneous factors can lead to hypochromism contributions in consequence merely of base pairing.

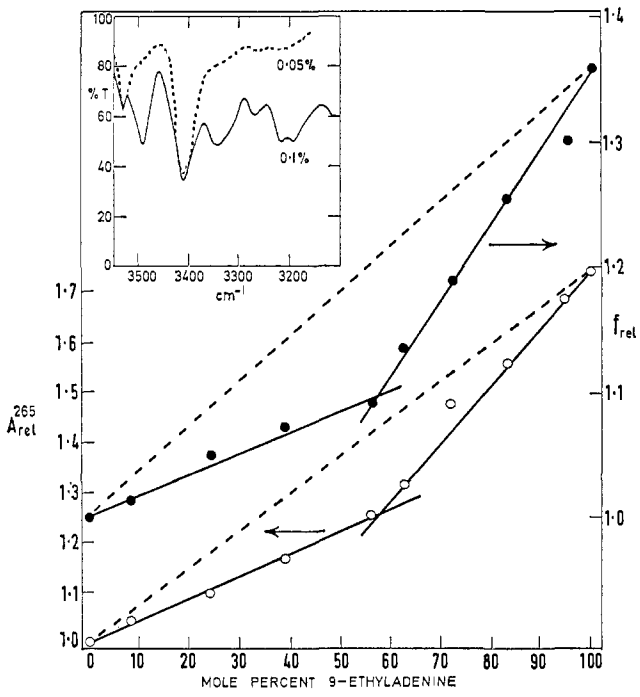

Figure 1. Absorbances and oscillator strengths, relative to 1-cyclohexyluracil, of 9-ethyladenine and 1-cyclohexyluracil mixtures. Solutions of the two bases *ca.* 0.03 *M*; solvent, chloroform; path, 0.033 mm. Inset: infrared spectra of a 1:1 mixture at similar concentration (1-mm path) showing association, and at low concentration (1-cm path) showing negligible association. Qualitatively similar results were obtained with 1-*n*-octadecyluracil and 9-*n*-octadecyladenine.

1-Cyclohexyluracil and 9-ethyladenine were obtained from Cyclo Chemical Corp. 9-*n*-Octadecyladenine was synthesized according to Davoll and Lowy's synthesis of adenosine,[6] using *n*-octadecyl bromide in place of their bromoribose derivative. For 1-*n*-octadecyluracil, the synthesis of 1-methyluracil by Hilbert and Johnson[7] was followed, using *n*-octadecyl bromide in place of methyl iodide. Chloroform was treated with sulfuric acid, followed by alkali and water, and distilled from P_2O_5. Short-path cells (0.033 mm) were used in a Beckman DK-2A spectrophotometer, and solutions of comparable concentration were examined in the infrared (Perkin-Elmer 237 grating instrument) in 1-mm and 1-cm paths, taking advantage of a good window in solvent and silica cells in the range 3500–3150 cm^{-1}. It has been shown[8-10] that 1:1 A···U pairing occurs at high concentration through hydrogen bonding. From our infrared spectra of the A and U derivatives and their mixtures we can, since we are able to observe the nonbonded form, derive the composition of an A + U solution, in terms of the two monomeric and three dimeric species. A···U pairs are favored relative to the homodimers.

Figure 1 shows relative absorbances of A + U mixtures in chloroform solution at the highest workable concentration, as well as relative oscillator strengths (taken to 4.3×10^4 cm^{-1}). It is consistently found that a hypochromic effect is present, falling in magnitude with diminishing concentration, but still measurable at 0.02 *M* in A. The point of maximum hypochromicity is displaced from A:U equivalence in the sense predicted by the presence of A_2 dimers, but this effect is close to the limits of error. From the infrared study, we find that in a 1:1 mixture of A + U, *ca.* 0.03 *M* in each, some 40% of the A is present as A···U pairs, and about one-third of the remainder as A_2. Thus for complete pairing a hypochromic decrease in oscillator strength of around 20% is predicted. It must be stated that these results are regarded as only semiquantitative since the solutions in the spectrophotometers were not thermostated. In the Beckman instrument, the temperature in the sample compartment is about 32°.

We next consider the effect of hydrogen bonding on the A and U spectra *per se*. It is remarkable that the U spectrum shows no significant perturbation by hy-

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(5) G. D. Fasman, C. Lindblow, and L. Grossman, *Biochemistry*, **3**, 1015 (1964).
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