eration of zero-point energy differences for a C–H stretching vibration of 2900 cm.$^{-1}$ is 7.8, and the largest deuterium isotope effect reported in electrophilic aromatic substitution is 6.67.[7] Thus, the isotope effect weakening produced by the symmetrical stretching vibration in the transition state of a three-center reaction must be near its minimum value in this case.

The O–H stretching vibration in the solvated proton occurs at 2900 cm.$^{-1}$,[8] and the value predicted for the other isotope effect, $k_1^{H_2O}/k_1^{D_2O}$, is 7.8 as well. This is considerably greater than the observed value of 2.93. But the transition states of the two steps in the exchange reaction are the same, and, if the observed value of k_2^H/k_2^D is near its maximum value, the observed value of $k_1^{H_2O}/k_1^{D_2O}$ must be near its maximum value also. This discrepancy can be understood in terms of a predicted secondary effect of the water molecules solvating the proton.[9] When a proton is transferred from its solvent shell, the solvating water molecules revert to ordinary water. Since the O–H stretching vibration in liquid water is 3400 cm.$^{-1}$, this process is accompanied by considerable bond-tightening. It has been estimated that the deuterium isotope effect on this change is 0.7 per O–H bond for an equilibrium process,[9a] and the prediction has been made that this will reduce the kinetic isotope effect on proton transfer to a maximum value of about 3.6.[9b] The observed effect of 2.93 is in good agreement with this prediction.

TABLE I

RATES OF AROMATIC HYDROGEN EXCHANGE BETWEEN 1,3,5-TRIMETHOXYBENZENE AND 0.050 *M* $HClO_4$ AT 25°

Substrate	Solvent	$10^2 k_2$ (M^{-1} min.$^{-1}$)	No. of runs
TMB-t	H_2O	3.722 ± 0.030[a]	9
TMB-d	H_2O	7.98 ± .10[a]	7
TMB-t	D_2O	6.286 ± .012[a]	5

[a] Error estimates are standard deviations of the mean values.

The difference between this approximately maximum isotope effect on proton transfer to 1,3,5-trimethoxybenzene and the other smaller isotope effects on slow proton transfer[2] is understandable in terms of the relative basicities of the various proton donors and acceptors.[9b] Isotope effects in three-center reactions will be less than their maximum value whenever the transition state is not truly symmetrical, that is, whenever the two force constants governing the symmetrical stretching vibration in the transition state are not equal.[10] In the slow proton transfer reactions on which isotope effects have heretofore been reported, the proton acceptor has usually been a strongly basic anion. In these transition states, therefore, the proton is not bound with equal strength to the acceptor molecule and the solvating water which it is leaving. Trimethoxybenzene, on the other hand, is a weaker base than these anions, and in this case the two forces holding the proton in the transition state should be more nearly equal. Thus, the isotope effect with trimethoxybenzene should be stronger than those reported before.

This isotope effect on proton transfer to trimethoxybenzene is twice the value which was used to estimate that H_2O is five times as strong an acid as D_2O.[2b] Similar reasoning with the data for trimethoxybenzene gives a ten-fold difference in acid strength between H_2O and D_2O. This unreasonably high isotope effect emphasizes the danger inherent in basing conclusions on the assumption that isotope effects will have essentially constant values.

DEPARTMENT OF CHEMISTRY
ILLINOIS INSTITUTE OF TECHNOLOGY
CHICAGO 16, ILLINOIS A. J. KRESGE
UNION CARBIDE RESEARCH INSTITUTE
TARRYTOWN, NEW YORK Y. CHIANG

RECEIVED JUNE 18, 1962

CELLULOSE COLUMNS CONTAINING POLYRIBONUCLEOTIDES AND RIBONUCLEIC ACIDS[1]

Sir:

Recent studies[2,3] have shown that polydeoxyribonucleotides can be covalently linked to cellulose and then be employed as chromatographic adsorbents which selectively bind polynucleotides complementary to them in base sequence. The methods of synthesis utilized the glucosidic hydroxyl groups of T4 DNA[2] and the terminal phosphate groups of thymidine oligonucleotides.[3] This suggested to us that the free hydroxyl groups on ribose C'_2 in polyribonucleotides might be available for similar reactions. We have found that phosphocellulose can indeed be linked to synthetic polyribonucleotides and made into columns capable of binding and desorbing polynucleotides. The specificity of adsorption with respect to the nature of the bases, salt concentration and temperature, is very similar to that for the formation of helical complexes in solution. Columns also have been prepared from natural ribonucleic acids. Use of these columns may constitute a chromatographic method for isolating cellular components complementary in base sequence to the RNAs.

We followed a procedure similar to Bautz and Hall's[2] adaptation of Khorana's carbodiimide reaction for forming phosphate-ester bonds between acetylated phosphocellulose (Serva, 0.78 meq. P/g.) and each of the listed polyribonucleotides: poly-A, poly-C, poly-I, poly-U, bacteriophage virus RNA, *E. coli* transfer and ribosomal RNAs. Nucleotide polymers and cellulose were dissolved in pyridine and reacted with dicyclohexyl carbodiimide at 115° for one hour. After the reaction product was isolated, it was chopped in a Waring blender at 4°, ground in a mortar, and washed extensively with neutral buffer at 80° to liberate pyridine and starting materials. After removal of

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(1) Abbreviations used: poly-A or simply A, polyriboadenylic acid; poly-C or C, polycytidylic acid; poly-I or I, polyinosinic acid; poly-U or U, polyuridylic acid; tris, tris-(hydroxymethyl)-aminomethane.

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(3) P. T. Gilham, *J. Am. Chem. Soc.*, **84**, 1311 (1962).

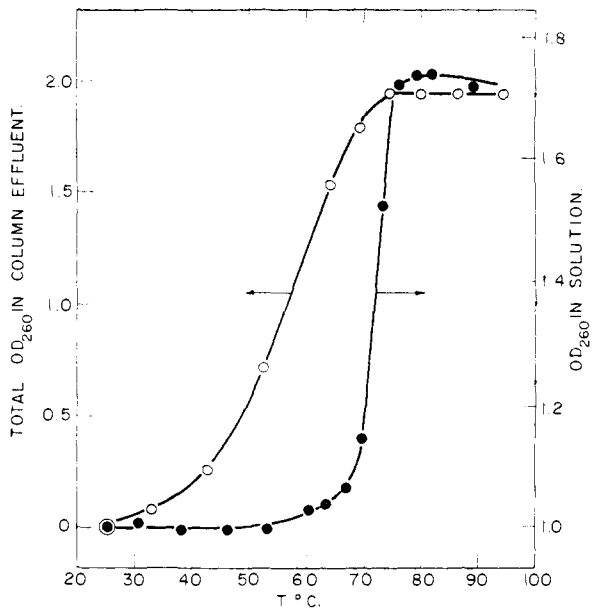

Fig. 1.—Melting curves for poly-A + poly-U complex in solution and on column; the same polymer samples were used for both experiments; all samples were in 0.01 *M* tris buffer, pH 7.4, and 0.5 *M* NaCl: ●, optical density in solution for 1:1 mixture of poly-A + poly-U; ○, elution of poly-U from poly-A column.

fine cellulose particles, the preparations were packed into chromatographic columns.

By using C^{14}-labeled poly-A and transfer-RNA, we estimate that 10 to 30% of each polymer reacted with phosphocellulose; of this, 50 to 90% was discarded with the fine particles. For example, upon hydrolysis with ribonuclease, the poly-C cellulose column yielded 1.3 μmoles of cytidylic acid (0.4 mg.), thus confirming that little more than 1% of the original 30 mg. of polynucleotide was fixed in the chromatography column. This poly-C column specifically adsorbed 0.34 μmole of poly-I, presumably by forming a 1:1 hydrogen-bonded complex, indicating that the poly-C on the column was about 25% efficient in binding complementary polymers. The attachment of phosphocellulose to these lower molecular weight polyribonucleotides appears to proceed to a much smaller extent than its linkage to T4 DNA.[2] This difference may reflect a greater accessibility of sidechain glucosidic hydroxyls than of the backbone ribose C'_2 hydroxyl groups. We are investigating the possibility that there may be a component of mechanical entrapment in the cellulose–pyridine gel of the reaction mixture. This suggestion appears unlikely, however, since the small transfer-RNA molecules were retained to the same extent as the much larger poly-A strands.

Column specificity was determined by loading the column with solutions of various polynucleotides under complexing conditions until it was saturated. Elution was carried out under conditions designed to destroy nucleotide complexes (low salt or high temperature). Recovery of the test polymer was always quantitative upon elution with 0.001 *M* tris. Conditions for column loading and elution are listed in Table I, together with the quantities of

TABLE I

BINDING CAPACITY OF VARIOUS POLYRIBONUCLEOTIDE-CELLULOSE COLUMNS[a]

Cellulose column	Amount of solution bound in mμmoles[b] Poly-A	Poly-C	Poly-I	Poly-U
Poly-A	157	<3	277	180
Poly-C	57	18	344	30
Poly-I	52	62	135[c]	<4
Poly-U	27	<3	<2	15[d]
Control, no polynucleotide added	<2	<3	<5	<2

[a] Unless otherwise noted, all runs were performed at 23 ± 2°. Polymers were introduced onto the columns in 0.5 *M* NaCl, 0.01 *M* tris (buffer), *p*H 7.4, and eluted with 0.001 *M* tris, *p*H 7.4. [b] The polynucleotide content of the different columns varied. Accordingly, the adsorption figures for different columns cannot be compared directly. [c] Adsorbing buffer contained 1 *M* NaCl. Only 18 mμmoles of poly-I was adsorbed when 0.5 *M* NaCl was used, and 11 mμmoles with 0.1 *M* NaCl. [d] 18 mμmoles of poly-U was bound at 4° in a solution of 0.01 *M* $MgCl_2$, 0.001 *M* tris.

polyribonucleotides which were adsorbed by the columns at high salt concentration and released at low concentration. The reactivity displayed by the columns closely parallels the specificity of helical complex formation shown by these polymers in solution, where it has been shown that these homopolymers react: A + U,[4] A + I,[5] C + I,[6] A + A,[7] I + I,[8] and U + U.[9] This agreement is evidence that the chromatographic columns are retaining polyribonucleotides by means of hydrogen bonding. Many of the features of the solution reactions are retained on the columns. For example, the complex I + I + I forms only in high salt. These results also suggest that the A + A reaction occurs at neutral pH, accounting for the hypochromicity of poly-A in solution at pH 7. The poly-C column shows some nonspecific adsorption, which may be caused by an impurity in the polymer.

An experiment was performed in which gradually decreasing concentrations of NaCl (at pH 7.4 and 24°) were employed to elute poly-U from the poly-A column. The results showed that the A + U complex dissociates at the same salt concentration on the column as in the solution.

The hydrogen bonds between polynucleotide strands can be broken also by heating. Figure 1 compares melting curves for the A + U complex in solution (as determined by hypochromism) and on the poly-A column (measured by elution of poly-U at increasing temperatures). The transition on the column is less sharp, and is lower by 14°. This difference could be explained if the poly-A on the column is firmly attached to the cellulose at several places, leaving shorter segments of poly-A free to combine helically with poly-U. We are investigating the use of cellulose with a lower density of phosphate groups to overcome this problem.

The ability to make polyribonucleotide columns specific in their chromatographic properties may

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(8) A. Rich, *Biochim. Biophys. Acta*, **29**, 502 (1958).
(9) M. N. Lipsett, *Proc. Natl. Acad. Sci. U. S.*, **46**, 445 (1960).

prove useful for the study of natural ribonucleic acids.[10]

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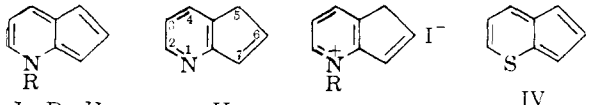
DEPARTMENT OF BIOLOGY ALICE J. ADLER
MASSACHUSETTS INSTITUTE OF TECHNOLOGY
CAMBRIDGE, MASSACHUSETTS ALEXANDER RICH

RECEIVED AUGUST 8, 1962

THE TAUTOMERISM OF 5H-1-PYRINDINE

Sir:

Recently much interest[1] has centered on heterocyclic analogs of azulene, which were first described forty years ago by Armit and Robinson.[2] Such compounds are iso-π-electronic with azulene with the heteroatom (N, O, S) providing an electron pair instead of a (—CH=CH—) group; they are expected to resemble azulene in their ultraviolet and visible absorption spectra and general aromatic properties. We wish to report evidence for the existence of the parent pseudo-azulene[3] of the pyrindine group discussed by Armit and Robinson,[2] namely, 1H-1-pyrindine (Ia).



Ia, R = H
b, R = Me

II

IIIa, R = H
b, R = Me

IV

Robison[4] prepared 5H-1-pyrindine (II) and noted that the freshly distilled liquid had an orange color which was discharged on dilution with organic solvents, and that a 10^{-4} *M* solution in cyclohexane was completely transparent above 305 mμ. This color was also discharged slowly by freezing the material. Anderson, *et al.*,[5] suggested that the color was due to the presence of the tautomeric pseudoazulene (Ia).

We have confirmed Robison's observations and have obtained spectral evidence which suggests that the orange color of (II) is indeed due to the presence of (Ia). Pyrindine (II) was left overnight at room temperature with a large excess of methyl iodide. The residue, obtained by evaporation of the excess methyl iodide, was crystallized from methanol to give cream colored crystals (50%) of pyrindine methiodide (IIIb), m.p. 248–250° (dec.). *Anal.* Calcd. for $C_9H_{10}IN$: C, 41.69; H, 3.86; N, 5.41. Found: C, 41.30; H, 3.60; N, 5.27. A dark orange oil of 1-methyl-1-pyrindine (Ib) was obtained by treating (IIIb) with alkali; this material could not be isolated as it was converted rapidly into an infusible solid, insoluble in organic solvents. In this way it resembled its sulfur analog[6] (IV) and differed from the more stable pseudoazulenes prepared by Anderson, *et al.*[5] However, its ultraviolet and visible spectrum was obtained by treating pyrindine methochloride (obtained by passing an aqueous solution of (IIIb) through a Dowex 1 chloride column) with alkali: $\lambda_{max.}^{0.1\,N\,NaOH}$ 220, 256, 320, 456 mμ (log ϵ = 3.53, 4.06, 3.56, 2.84). The spectrum was recorded rapidly as (Ib) readily decomposed in aqueous solution, even at pH 8.

Figure 1 shows the visible absorption spectrum of (Ib) with a thousand-fold reduced ϵ scale and that of the colored impurity in *neat* pyrindine with full ϵ scale. Despite the different solvents, the two absorption bands are strikingly similar; they have $\epsilon_{max.}$ values of 685 and 0.76, respectively. If it is assumed that N-methylation of (Ia) does not alter the $\epsilon_{max.}$ of its visible band appreciably, then at 20° 5H-1-pyrindine (II) contains 1.1 parts per 1000 of the colored pseudoazulene tautomer (Ia) in equilibrium with it. This equilibrium can be shifted by dilution or freezing. It is noteworthy that N-methylation causes a hypsochromic shift of 14 mμ, but this may be due to the different solvents involved.

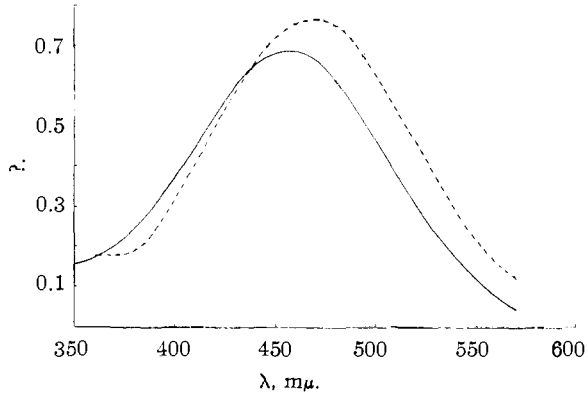


Fig. 1.—Full curve is for 1-methyl-1-pyridine (Ib) in 0.1 *N* NaOH; ordinate, 10^{-3} ϵ value. Broken curve is for neat 5H-1-pyridine at 20°; ordinate, ϵ value.

This estimate of approximately 0.1% of pseudoazulene (Ia) in tautomeric equilibrium with (II) has been confirmed by pK_a measurements. The latter compound has pK_a 5.7 (determined potentiometrically) whereas (Ib) has pK_a 8.7 (determined spectroscopically[7]). If (Ia) is assumed to have the same pK_a as (Ib) (ΔpK_a for N-methylation is normally[8] about 0.2 unit), and if both tautomers (Ia) and (II) are assumed to have the same conjugate acid (IIIa), then it follows[8] that the tautomeric equilibrium constant is 10^3 in favor of (II). Tautomerism between (II) and the other possible 2,3-cyclopentenopyridine, 7H-1-pyrindine has not been investigated. Comparison of the ultraviolet spectra of (II), 2-vinyl-[9] and 3-vinylpyridine[10] was not helpful.

UNIVERSITY CHEMICAL LABORATORY
CAMBRIDGE, ENGLAND C. B. REESE

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(3) Term originated by R. Mayer, see reference (6).

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