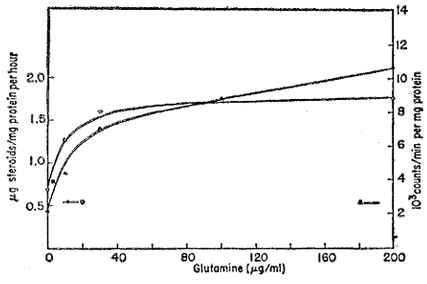

Fig. 2. Cultures were incubated in Krebs Ringer buffer containing 30 mg of bovine serum albumin per milliliter, 5.0 milliunits of ACTH per milliliter, 0.5 μc of ^{14}C-phenylalanine per milliliter, and varying concentrations of glutamine. After 2 hours steroids were extracted from the medium, and the radioactive material (insoluble in hot trichloroacetic acid) incorporated in the cells was determined.

those concentrations necessary to maintain protein synthesis adequate for the ACTH response. Transfer of freshly cultured cells does not result in this depletion of glutamine.

The dependence of steroid formation on the concentration of serum albumin is approximately linear at low concentrations and reaches a maximum at about 30 mg/ml (Fig. 3). When the serum is fractionated by the Cohn method (*7*), the albumin fraction alone is stimulatory. The effect is destroyed by heat and trypsin digestion, but it does not disappear when the albumin is extracted with organic solvents. Thus albumin appears not to act as a reservoir of bound micromolecules. Another way in which albumin could act would be by binding inhibitory substances in the incubation buffer (Table 1). Adrenal tumor cultures were incubated in growth medium containing cholesterol-^{14}C for 24 hours. The radioactive medium was removed and the cells were incubated in Krebs Ringer buffer containing 30 μg of glutamine per milliliter for 1 hour under various conditions. The medium was harvested and extracted with methylene chloride; the extract was fractionated by thin-layer silica-gel chromatography with a mixture of ether and *N,N*-dimethyl formamide (99 : 1). The radioactive material migrating with the various steroid hormones, including pregnenolone, was eluted and pooled for assay; that migrating with cholesterol was eluted and assayed separately. Adrenocorticotropic hormone stimulates the conversion of radioactive cholesterol, accumulated by the cells in the preincubation period, to steroid hormones. Pregnenolone inhibits the conversion whereas the addition of serum albumin to the incubation buffer partially reverses this inhibition.

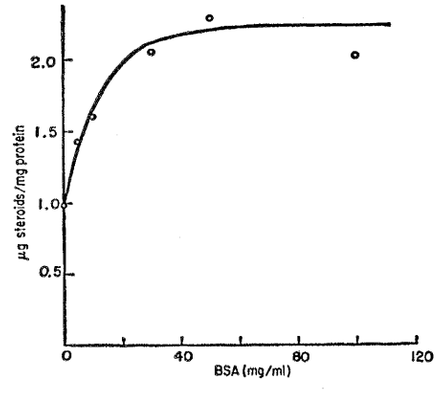

Fig. 3. Adrenal tumor cultures were incubated in Krebs Ringer bicarbonate buffer containing 5.0 milliunits of ACTH per milliliter, 100 μg/ml of glutamine, and varying amounts of bovine serum albumin (*BSA*). After 2 hours the medium was extracted, and its steroid content was determined.

Our results represent a technical improvement in the handling of adrenal tumor cultures and raise the possibility that special conditions may be required to demonstrate, in each individual instance, the potentiality of cultured cells to perform differentiated functions. As a further example, epithelium cultures of pigmented iris form melanin only when the growth rate of the cultures is decreased (*8*). Our results provide strong evidence that the loss of specialized function by adrenal tumor cells in culture is actually a change in phenotype in cells that were once functional rather than selective overgrowth by stromal elements of the tumor inoculum. The fact that our cultures lose their ability to synthesize steroids when incubated in Krebs Ringer buffer could be explained on a basis of selective overgrowth, but phenotypic alterations must be invoked to explain the restoration of the synthesizing function by glutamine and albumin.

Finally, these data represent clues in the mystery of phenotypic alterations, an important problem that is receiving more and more attention (*9*).

GORDON H. SATO
TOBY ROSSMAN
LEON EDELSTEIN
STUART HOLMES
VINCENZO BUONASSISI
Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154

References and Notes

1. V. Buonassisi, G. Sato, A. I. Cohen, *Proc. Natl. Acad. Sci. U.S.* **48**, 1184 (1962).
2. Y. Yasumura and G. Sato, in preparation.
3. G. Sato and V. Buonassisi, *Natl. Cancer Inst. Monograph 13* (1964), p. 81.
4. ———, *Wistar Institute Monograph 1* (Wistar Inst. Press, Philadelphia, 1964), p. 27.
5. R. E. Handschumacher and A. D. Welch, in *The Nucleic Acids* (Academic Press, New York, 1961), vol. 3, chap. 39.
6. J. J. Ferguson, *J. Biol. Chem.* **238**, 2754 (1963).
7. E. J. Cohn, W. L. Hughes, Jr., J. H. Weare, *J. Am. Chem. Soc.* **69**, 1753 (1947).
8. L. Doljanski, *Compt. Rend. Soc. Biol.* **105**, 343 (1930).
9. K. E. Ebner, E. C. Hageman, B. L. Larson, *Exptl. Cell Res.* **25**, 555 (1961); H. Kuramitsu and I. Harary, *Biochim. Biophys. Acta* **86**, 56 (1964); A. Fujimoto and I. Harary, *ibid.*, p. 74; H. Holtzer, J. Abbot, J. Lash, S. Holtzer, *Proc. Natl. Acad. Sci. U.S.* **46**, 1533 (1960).
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Hydrogen-Bonded Dimers of Adenine and Uracil Derivatives

Abstract. *In concentrated solutions of either 9-ethyladenine or 1-cyclohexyluracil in deuterochloroform, absorption bands in the infrared spectrum demonstrate hydrogen bonding of the adenine and uracil derivatives with themselves. In dilute solutions, there is very little hydrogen bonding. However, when dilute solutions of 9-ethyladenine and 1-cyclohexyluracil are mixed, a series of bands appear which show that these molecules are hydrogen-bonding with each other much more strongly than with themselves. A study of the stoichiometry of this association indicates formation of 1:1 hydrogen-bonded pairs in solution.*

The molecular basis for the transfer of information in biological systems is believed to be the specificity of hydrogen bonding between the constituent purines and pyrimidines of the nucleic acids. In the double-stranded form of DNA (or RNA), adenine forms hydrogen bonds with thymine (or uracil), and guanine forms such bonds with cytosine. The stability of the two-stranded molecule is governed by hydrophobic or van der Waals interactions and also by electrostatic and hydrogen-bonding interactions. Several studies have been carried out on the interaction of polynucleotides with each other in solution, but no studies have been reported which demonstrate hydrogen bonding between individual pu-

rines and pyrimidines. Studies by Ts'o and his associates (*1*) suggest that purines and pyrimidines can associate in aqueous solution, but this association is believed to be due to a vertical stacking of the unsaturated rings rather than to hydrogen bonding. More recently, however, Küchler and Tuppy (*2*) have demonstrated the retardation of complementary nucleotides, on columns, which have single nucleotides covalently linked to them. Their work suggests that it is possible for single nucleotides to interact by means of specific hydrogen bonding. In our study we have observed the infrared spectrums of adenine and uracil derivatives dissolved in deuterochloroform and obtained direct evidence concerning the hydrogen bonding of these molecules in a system in which there are no electrostatic interactions and in which hydrophobic bonding is minimum or absent. Our results indicate that 9-ethyladenine and 1-cyclohexyluracil (Fig. 1) in dilute solution form hydrogen bonds with each other to a much greater extent than with themselves.

We have studied hydrogen bonding in a nonaqueous solvent in order to decrease the effect of hydrophobic bonding and in order to provide a medium which makes possible a direct spectroscopic study of the interaction. It is difficult to obtain purine and pyrimidine derivatives which are sufficiently soluble in solvents that are suitably transparent in the infrared region. Preliminary studies carried out in carbon tetrachloride showed indications of some hydrogen bonding, but the low solubility of purine and pyrimidine derivatives made it difficult to obtain accurate measurement (*3*). However, purines and pyrimidines are available with hydrocarbon side chains attached to the glycosidic nitrogen atoms which increase the solubility considerably (Fig. 1). 9-Ethyladenine and 1-cyclohexyluracil (Cyclo Chemical Company, Los Angeles) have solubilities sufficiently high in deuterochloroform (Merck of Canada, Ltd.) so that it is possible to obtain accurate measurements of the N—H stretching vibrations with a path length of less than 1 cm.

All spectrums were observed with a Perkin-Elmer Model 421 Infrared Spectrophotometer. The wavelength calibration from the instrument was checked against ammonia vapor (*4*), and the wave numbers reported are accurate within ± 2 cm^{-1}. The limitation on the accuracy of the wave number is set by the breadth of the absorption band rather than the accuracy of the calibration. Absorption cells of fused silica were purchased (Beckman Instruments, Inc.) and the sodium chloride cells were constructed in the laboratory. Path lengths ranged from 1 mm to 10 cm, but most measurements were made with the 1-mm cells. The temperature of the solution was not measured, but the heating effect of the beam was estimated to have raised this temperature 3° to 4°C above 21°C, the temperature of the room.

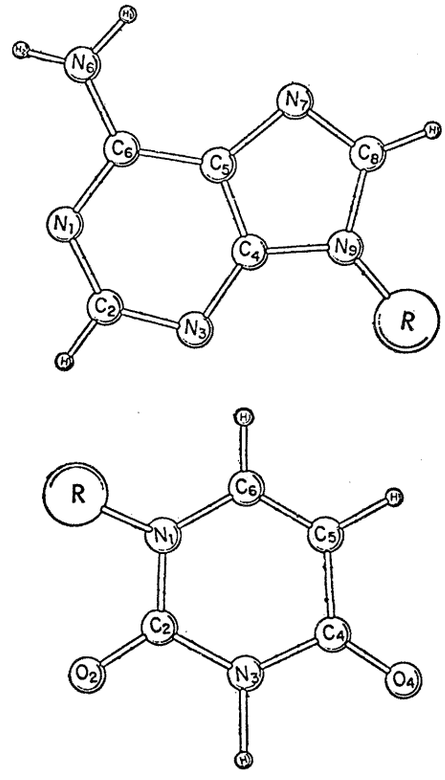
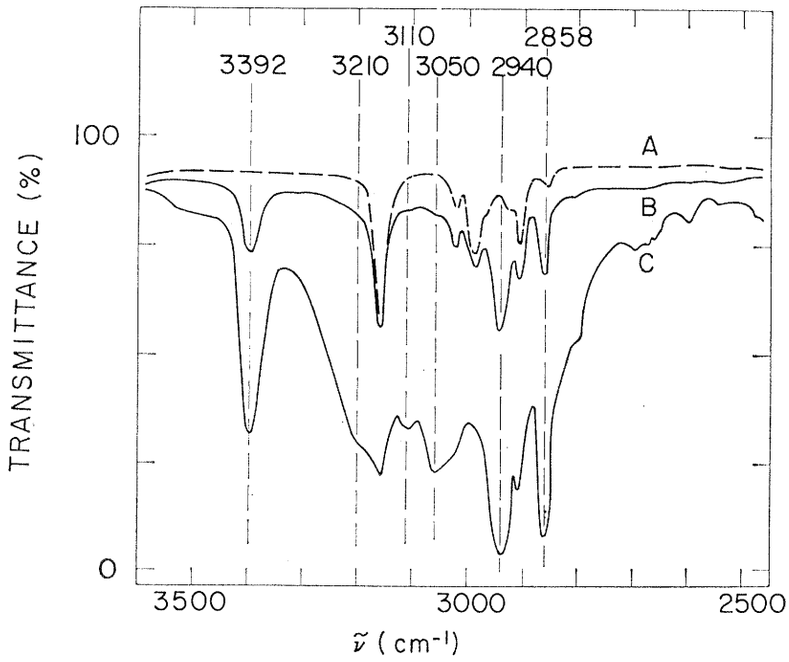


Fig. 1. Adenine and uracil derivatives used in infrared study. (Top) 9-Ethyladenine (R = ethyl). (Bottom) 1-Cyclohexyluracil (R = cyclohexyl).

In dilute solution of 1-cyclohexyluracil (Fig. 2*B*, 0.022*M*) the nonbonded N—H stretching band is prominent at 3392 cm^{-1} and the cyclohexyl C—H stretching bands are at 2940 and 2858 cm^{-1}. There is no evidence of hydrogen bonding at this concentration. However, in the saturated solution (Fig. 2*C*), bands appearing at 3210, 3110, and 3050 cm^{-1} are due to hydrogen-bonded N—H groups (*5*). This is consistent with the hydrogen bonding of uracil residues with each other. X-ray diffraction studies of crystals in the solid state (*6*) show that 1-methyluracil molecules crystallize as hydrogen-bonded pairs connected by two N—H · · · O hydrogen bonds. The infrared spectrum is consistent with this type of pairing in solution.

The infrared spectrum of 0.022*M* 9-ethyladenine (Fig. 3*B*) shows two prominent bands, the symmetric and antisymmetric NH_2-stretching vibrations at 3416 and 3527 cm^{-1}, respectively. However, there is also some indication of hydrogen bonding as shown by the weak bands at 3482 and 3312 cm^{-1}. These become much more pronounced in the saturated solution (Fig. 3*C*) in which an additional band also appears at 3255 cm^{-1} and a shoulder at about 3200 cm^{-1}. Thus, the spectrums indicate that 9-ethyladenine residues form hydrogen bonds extensively in concentrated solution and even to a slight extent in the more dilute solution. The shifts in both the symmetric and antisymmetric vibrations are consistent with the formation of N—H · · · N hydrogen bonds. In this connection it is interesting to note that adenine hydrochloride crystallizes by forming pairs connected by two hydrogen bonds between the amino group and the imidazole nitrogen-7 (in the seven-position) of adenine (*7*). There is a similar type of hydrogen bonding in the helical homopolymer of adenylic acid (*8*). However, with infrared spectrums we cannot distinguish readily between this type of pairing and hydrogen bonding where the amino group of adenine interacts with the nitrogen-1 of the purine ring.

Mixtures of ethyladenine and cyclohexyluracil solutions with various mole ratios were prepared so that the total number of solute molecules was constant. Five different solutions were made by mixing various volumes of the standard 0.022*M* solutions, and the spectrums are presented vertically for comparison (Fig. 4). The spectrum of the uracil derivative alone shows only the sharp band at 3392 cm^{-1} associated with N—H stretching. The spectrum of 9-ethyladenine shows strong symmetric and antisymmetric stretching bands of the nonbonded NH_2 group. When these solutions are mixed, a series of pronounced bands due to hydrogen bonding appear. In the mixture which is 50 percent cyclohexyluracil and 50 percent ethyladenine, there are relatively strong bands at 3490 cm^{-1} and 3330 cm^{-1}, a weaker band is visible at 3260 cm^{-1}, and a poorly defined shoulder appears



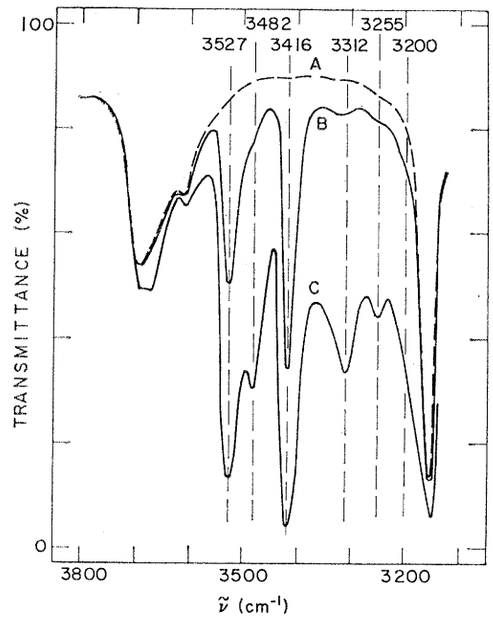
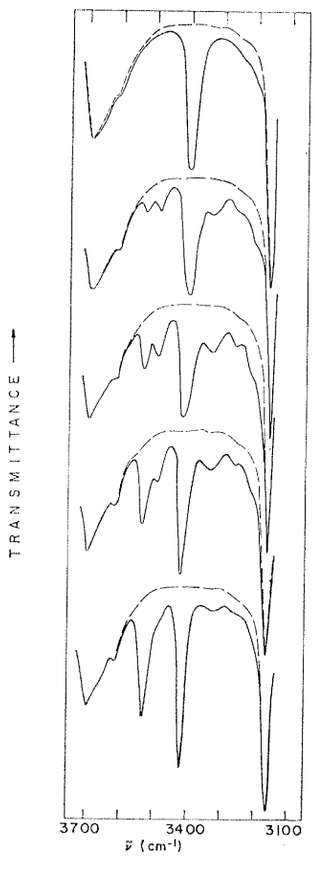


Fig. 2 (left). Infrared spectrums of 1-cyclohexyluracil dissolved in deuterochloroform from 2500 to 3500 cm^{-1}. The path length of the solutions was 0.2 mm and NaCl windows were used in the cells. The reference beam in the spectrometer was uncompensated. (*A*) The dashed line represents the solvent spectrum. (*B*) 1-Cyclohexyluracil at a concentration of 0.022*M*. (*C*) 1-Cyclohexyluracil saturated at 21°C (near 0.1*M*). Fig. 3 (right). Infrared spectrums of 9-ethyladenine in deuterochloroform from 3100 to 3800 cm^{-1}. The path length was 1 mm in these solutions and silica cells were used. (*A*) Dashed line is the solvent spectrum. (*B*) 9-Ethyladenine at a concentration of 0.022*M*. (*C*) 9-Ethyladenine saturated at 21°C (near 0.1*M*).



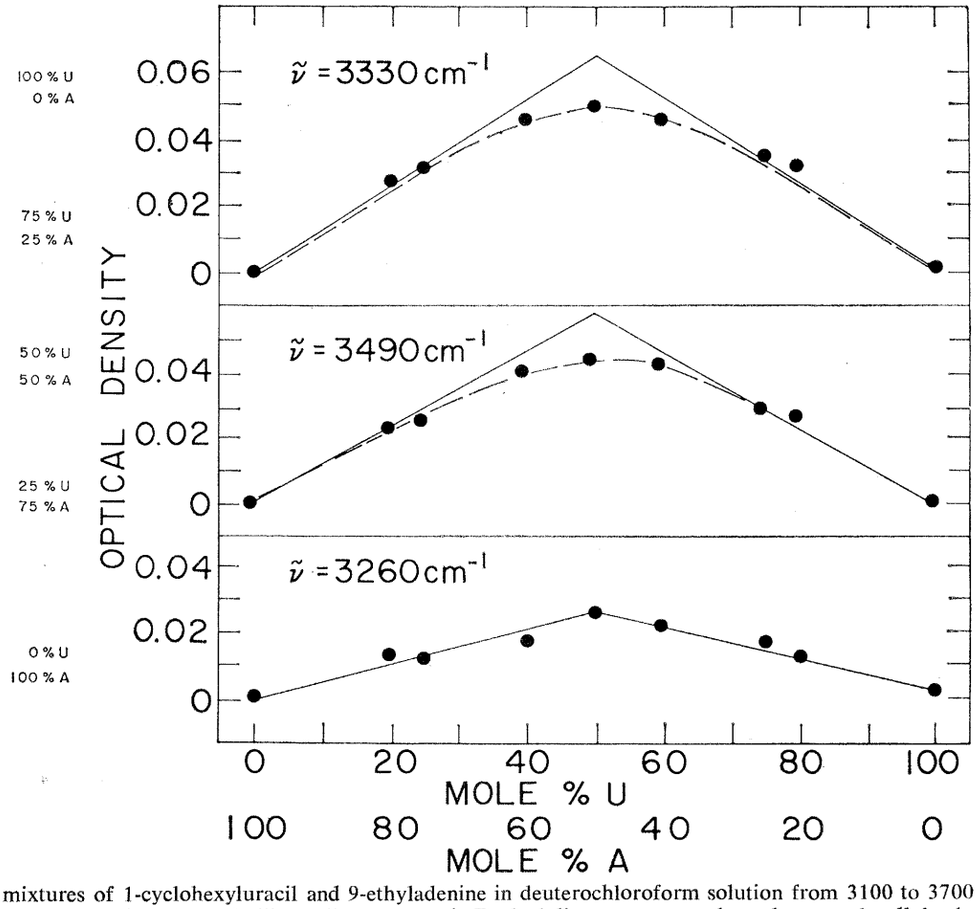


Fig. 4 (left). Infrared spectrums of mixtures of 1-cyclohexyluracil and 9-ethyladenine in deuterochloroform solution from 3100 to 3700 cm^{-1}. The path length was 1 mm in these experiments, and silica cells were used. Dashed lines represent the solvent and cell background while solid lines represent the actual spectrums. The total concentration in all cases was 0.022*M* but varying ratios (mole percent) of the uracil and adenine derivatives are shown. Fig. 5 (right). The change in the optical density of three bands due to hydrogen-bonded species obtained from solutions of ethyladenine and cyclohexyluracil in deuterochloroform. The abscissa represents the mole ratio of the uracil and adenine derivatives. The change of optical density in the lowest curve is too small to show the difference between dashed and solid lines.

at slightly over 3200 cm^{-1}. These bands are present in the mixture but absent in the spectrum of each component. This can be seen by plotting the intensity of these bands against the mole ratio (Fig. 5). The optical densities were obtained by adjusting the transmittance of the pure cyclohexyluracil and pure ethyladenine solutions to have the value zero. The solid circles represent experimental points, and the dashed lines form a smooth curve through them in the upper two graphs of Fig. 5. The solid lines are drawn approximately as tangents to the experimental curve at either end. There is a maximum rise of optical density in a 1:1 mixture of the uracil and adenine derivatives.

The stoichiometry of polynucleotide reactions has been studied in the ultraviolet absorption spectrum by analogous methods (*9*). Data of this type may be used to learn something about the equilibrium constant of the reaction. For example, if the species are interacting fully, then one obtains data which follow the solid tangential lines. The fact that the experimental points fall below the solid line is an indication that the molecules are not all hydrogen bonded into 1:1 dimers. In the lowest curve of Fig. 5, the change in optical density is too small compared to the scatter of the points to demonstrate this.

If data of the type shown in Fig. 5 are sufficiently accurate, it is possible to interpret the figure quantitatively to obtain an equilibrium constant. The data for the 3330 cm^{-1} band suggest a value of the order of 10^3 liters per mole if we assume that the interval between the solid lines and the experimental dashed line represents the unreacted species. However, more accurate data will have to be obtained at the two extremes of the mole-ratio coordinate in order to be certain that the solid lines actually represent the experimental behavior in that region.

Uracil can dimerize with adenine by two different modes of hydrogen bonding. The amino group of adenine can bond with a carbonyl oxygen atom of uracil, but the N—H group of uracil can bond with either nitrogen-1 of the adenine or the nitrogen-7 of the imidazole ring. This was first indicated when polyadenylic acid was found to combine with two moles of polyuridylic acid to form a three-stranded helical molecule (*10*). X-ray diffraction studies of single crystals containing adenine plus either thymine (*11*) or uracil (*12*) derivatives have shown pairing through two hydrogen bonds by way of the imidazole nitrogen (N-7). From our studies we cannot choose between these two types of hydrogen bonding, since both types involve formation of an N—H · · · O hydrogen bond and an N—H · · · N hydrogen bond. However, it is not surprising that in our study of the hydrogen bonding, the monomers form a dimer complex of one adenine and one uracil but not a trimer of one adenine with two uracil derivatives. Entropic effects with small molecules make it very unlikely that the stabilizing energy of the hydrogen-bonded trimer would be large enough to permit formation of detectable amounts of such a trimer.

Although adenine derivatives and uracil derivatives can form hydrogen bonds with themselves in deuterochloroform, they do so only to a limited extent compared to the fairly pronounced association of the adenine and uracil derivatives with each other to form 1:1 hydrogen-bonded pairs. This infrared study provides the first direct evidence that these monomeric molecules are hydrogen-bonding when present together in solution. In view of the nature of the solvent, it is unlikely that there is much stacking of the bases in solution, and therefore the amount of hydrophobic bonding is minimum in this solution.

Our work suggests that there are factors stabilizing the hydrogen-bonded association of adenine and uracil derivatives other than simply the number and type of hydrogen bonds. The self-dimerization of either the adenine or the uracil derivatives involves two hydrogen bonds, as is the case in the adenine-uracil dimer. Nonetheless, the latter is more stable than the former. This suggests that the purine-pyrimidine pair may be stabilized by forces such as dipole-dipole or dipole-induced dipole interactions in addition to the hydrogen bonds themselves.

ROY M. HAMLIN, JR.
R. C. LORD
ALEXANDER RICH
Departments of Chemistry and *Biology*, and *Spectroscopy Laboratory*, *Massachusetts Institute of Technology*, *Cambridge*

References and Notes

1. P. O. P. Ts'o, I. S. Melvin, A. C. Olson, *J. Amer. Chem. Soc.* **85**, 1289 (1963).
2. E. Küchler and H. Tuppy, *Biochim. Biophys. Acta* **80**, 669 (1964).
3. R. Hamlin, thesis, Massachusetts Institute of Technology, June 1963.
4. "Tables of Wavenumbers for the Calibration of Infrared Spectrometers," Commission of Molecular Structure and Spectroscopy, I.U.P.A.C., (Butterworth, Washington, D.C., 1961).
5. R. C. Lord and T. J. Porro, *Z. Elektrochem.* **64**, 672 (1960).
6. D. W. Green, F. S. Mathews, A. Rich, *J. Biol. Chem.* **237**, 3573 (1962).
7. W. Cochran, *Acta Cryst.* **4**, 81 (1951).
8. A. Rich, D. R. Davies, F. H. C. Crick, J. D. Watson, *J. Mol. Biol.* **3**, 71 (1961).
9. G. Felsenfeld and A. Rich, *Biochim. Biophys. Acta* **26**, 457 (1957).
10. G. Felsenfeld, D. R. Davies, A. Rich, *J. Amer. Chem. Soc.* **79**, 2023 (1957).
11. K. Hoogsteen, *Acta Cryst.* **12**, 822 (1959); **16**, 28 (1963).
12. F. S. Mathews and A. Rich, *J. Mol. Biol.* **8**, 89 (1964).
13. Supported by grants from NIH, NSF, and NASA.

7 April 1965

Thyroid and Parathyroid Roles in Hypercalcemia: Evidence for a Thyrocalcitonin-Releasing Factor

Abstract. *Extirpation of either the parathyroids or the thyroid in rats acutely impaired their ability to counteract hypercalcemia induced by exogenous parathormone. Thyroidectomy had the greater effect, and in rats subjected to both parathyroidectomy and thyroidectomy the calcium-lowering mechanism was not further impaired, which suggests that the parathyroids secrete a humoral factor that releases calcium-lowering thyrocalcitonin from the thyroid. This hypothesis reconciles previous conflicting reports.*

While evidence has rapidly accumulated for the presence of a fast-acting calcium-lowering hormone, investigators have arrived at contradictory conclusions as to whether the tissue of origin of such a hormone is the parathyroid (*1, 2*) or the thyroid (*3, 4*). Their conflict is apparent, not real, and all the observations reported heretofore (*1–9*) can be reconciled by hypothesizing the secretion of a humoral thyrocalcitonin-releasing factor (TCRF) by the parathyroid gland. The parathyroid may respond to ambient hypercalcemia and secrete TCRF which has no intrinsic calcium-lowering activity but which is carried by the circulation to the thyroid gland where it releases the stores of thyrocalcitonin, a polypeptide with demonstrated calcium-lowering activity (*10*). We have sought further experimental support for this hypothesis by observing the effect of the specific extirpation of either parathyroid or thyroid tissue, or both, on the ability of a young rat to counteract the prolonged hypercalcemic effect of a large exogenous dose of parathyroid hormone.