(4) A. L. Lehninger, *Proc. Natl. Acad. Sci. U.S.*, **60**, 1069 (1968).
(5) The structures are abbreviated as follows, where NeuNAc = N-acetyl-neuraminic acid (also known as sialic acid), Gal = galactose, GalNAc = N-acetylgalactosamine, Glc = glucose, and Cer = N-(fatty acyl)sphingosine; G_{D1a} = NeuNAc-($\alpha2\rightarrow3$)-Gal-($\beta1\rightarrow3$)-GalNAc-($\beta1\rightarrow4$)-[NeuNAc($\alpha2\rightarrow3$)]-Gal-($\beta1\rightarrow4$)-Glc-($\beta1\rightarrow1$)-Cer, G_{M1} = Gal-($\beta1\rightarrow3$)-GalNAc-($\beta1\rightarrow4$)-[NeuNAc($\alpha2\rightarrow3$)]-Gal-($\beta1\rightarrow4$)-Glc-($\beta1\rightarrow1$)-Cer, G_{M3} = NeuNAc-($\alpha2\rightarrow3$)-Gal-($\beta1\rightarrow4$)-Glc-($\beta1\rightarrow1$)-Cer.
(6) (a) M. F. Czarniecki and E. R. Thornton, *J. Am. Chem. Soc.*, **98**, 1023 (1976); (b) *ibid.*, **99**, 8273 (1977).
(7) M. F. Czarniecki and E. R. Thornton, *Biochem. Biophys. Res. Commun.*, **74**, 553 (1977).
(8) M. F. Czarniecki and E. R. Thornton, *J. Am. Chem. Soc.*, **99**, 8279 (1977).
(9) (a) J.-P. Behr and J.-M. Lehn, *FEBS Lett.*, **31**, 297 (1973); (b) A. K. Bhattacharjee, H. J. Jennings, C. P. Kenney, A. Martin, and I. C. P. Smith, *J. Biol. Chem.*, **250**, 1926 (1975); (c) V. Eschenfelder, R. Brossmer, and H. Friebolin, *Tetrahedron Lett.*, 3069 (1975); (d) H. J. Jennings and A. K. Bhattacharjee, *Carbohyd. Res.*, **55**, 105 (1977); (e) E. B. Brown, W. S. Brey, Jr., and W. Weltner, Jr., *Biochim. Biophys. Acta*, **399**, 124 (1975); (f) L. W. Jaques, E. B. Brown, J. M. Barrett, W. S. Brey, Jr., and W. Weltner, Jr., *J. Biol. Chem.*, **252**, 4533 (1977).
(10) D. Chapman, V. B. Kamat, J. deGier, and S. A. Penkett, *J. Mol. Biol.*, **31**, 101 (1968).
(11) (a) W. Curatolo, D. M. Small, and G. G. Shipley, *Biochim. Biophys. Acta*, **468**, 11 (1977); (b) H. C. Yohe, D. E. Roark, and A. Rosenberg, *J. Biol. Chem.*, **251**, 7083 (1976); (c) E. Heuser, K. Lipp, and H. Wiegandt, *Anal. Biochem.*, **60**, 382 (1974); (d) R. E. Howard and R. M. Burton, *Biochim. Biophys. Acta*, **84**, 435 (1964); (e) D. B. Gammack, *Biochem. J.*, **88**, 373 (1963); (f) E. G. Trams and C. J. Lauter, *Biochim. Biophys. Acta*, **60**, 350 (1962).
(12) M. W. Hill and H. R. Lester, *Biochim. Biophys. Acta*, **282**, 18 (1972).
(13) W. Stoffel, B. D. Tunggal, O. Zierenberg, E. Schreiber, and E. Binczek, *Hoppe-Seyler's Z. Physiol. Chem.*, **355**, 1391 (1975).
(14) (a) J. R. Lyerla, Jr., and G. C. Levy, *Top. Carbon-13 NMR Spectrosc.*, **1**, 79 (1974); (b) E. Breitmaier, K.-H. Spohn, and S. Berger, *Angew. Chem., Int. Ed. Engl.*, **14**, 144 (1975).
(15) F. J. Sharom and C. W. M. Grant, *Biochem. Biophys. Res. Commun.*, **74**, 1039 (1977).
(16) M. B. Feinstein, S. M. Fernandez, and R. I. Sha'afi, *Biochim. Biophys. Acta*, **413**, 354 (1975).
(17) S. N. Rosenthal and J. H. Fendler, *Adv. Phys. Org. Chem.*, **13**, 279 (1976); (b) H. Wennerström and G. Lindblom, *Q. Rev. Biophys.*, **10**, 67 (1977).
(18) See also recent studies of myelin lipids: E. C. Williams and E. H. Cordes, *Biochemistry*, **15**, 5792 (1976).
(19) N. O. Peterson and S. I. Chan, *Biochemistry*, **16**, 2657 (1977).
(20) R. E. London and J. Avitabile, *J. Am. Chem. Soc.*, **99**, 7765 (1977).

(21) T. L. James, "Nuclear Magnetic Resonance in Biochemistry", Academic Press, New York, N.Y., 1975, pp 299–347, 15–64.
(22) D. F. H. Wallach and R. J. Winzler, "Evolving Strategies and Tactics in Membrane Research", Springer-Verlag, New York, N.Y., 1974, pp 190–230.
(23) G. M. Gray in S. Estrada-O. and C. Gitler, Ed., "Perspectives in Membrane Biology", Academic Press, New York, N.Y., 1974, pp 85–106.
(24) C.-L. Schengrund and O. W. Garrigan, *Lipids*, **4**, 488 (1969).
(25) M. Marsh and R. T. Holzbach, *Clin. Chim. Acta*, **43**, 87 (1973).
(26) T. P. Carter and J. Kanfer, *Lipids*, **8**, 537 (1973).
(27) G. Rouser, G. Kritchevsky, C. Galli, and D. Heller, *J. Am. Oil Chem. Soc.*, **42**, 215 (1965).
(28) K. Eberlein and G. Gercken, *J. Chromatogr.*, **106**, 425 (1975).
(29) L. Svennerholm, *Biochim. Biophys. Acta*, **24**, 604 (1957).
(30) (a) C. C. Winterbourn, *J. Neurochem.*, **18**, 1153 (1971); (b) T. Momoi, S. Ando, and Y. Magai, *Biochim. Biophys. Acta*, **441**, 488 (1976).
(31) G. Rouser, G. Kritchevsky, A. Yakamoto, G. Simon, C. Galli, and A. J. Bauman, *Methods Enzymol.*, **14**, 272 (1969).
(32) J. F. Tallman, E. H. Kolodny, and R. O. Brady, *Methods Enzymol.*, **35**, 541 (1975).
(33) C. Huang, *Biochemistry*, **8**, 344 (1969).
(34) M. P. Sheetz and S. I. Chan, *Biochemistry*, **11**, 4573 (1972).
(35) I. M. Armitage, H. Huber, H. Pearson, and J. D. Roberts, *Proc. Natl. Acad. Sci. U.S.*, **71**, 2096 (1974).
(36) D. Canet, G. Levy, and I. Peat, *J. Magn. Reson.*, **18**, 199 (1975).
(37) R. Freeman, H. D. W. Hill, and R. Kaptein, *J. Magn. Reson.*, **7**, 327 (1972).
(38) (a) S. J. Opella, D. J. Nelson, and O. Jardetzky, *J. Chem. Phys.*, **64**, 2533 (1976); (b) D. Canet, *J. Magn. Reson.*, **23**, 361 (1976); (c) R. K. Harris and R. H. Newman, *ibid.*, **24**, 449 (1976).
(39) M. P. N. Gent and J. H. Prestegard, *Biochemistry*, **13**, 4027 (1974).
(40) C. H. A. Seiter and S. I. Chan, *J. Am. Chem. Soc.*, **95**, 7541 (1973).
(41) C. F. Schmidt, Y. Barenholz, and T. E. Thompson, *Biochemistry*, **16**, 2649 (1977).
(42) M. P. N. Gent and J. H. Prestegard, *J. Magn. Reson.*, **25**, 243 (1977).
(43) D. Doddrell, V. Glushko, and A. Allerhand, *J. Chem. Phys.*, **56**, 3683 (1972).
(44) R. E. London and J. Avitabile, *J. Chem. Phys.*, **65**, 2443 (1976).
(45) J. Schaefer, *Top. Carbon-13 NMR Spectrosc.*, **1**, 149 (1974).
(46) Y. K. Levine, P. Partington, G. C. K. Roberts, N. J. M. Birdsall, A. G. Lee, and J. C. Metcalfe, *FEBS Lett.*, **23**, 203 (1972).
(47) B. Sears, *J. Membr. Biol.*, **20**, 59 (1975).
(48) (a) L. M. Jackman and S. Sternhell, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry", Pergamon, New York, N.Y., 1969, pp 88–92; (b) J. A. Pople, *J. Chem. Phys.*, **37**, 3009 (1962); (c) G. E. Maciel, *Top. Carbon-13 NMR Spectrosc.*, **1**, 53 (1974).
(49) M. F. Czarniecki, unpublished work, University of Pennsylvania.

Nuclear Magnetic Resonance Studies of Catecholamines. Temperature and Solvent Effects on the Association with Adenosine Triphosphate and Its Divalent Metal Ion Chelates

Joseph Granot

Contribution from the Department of Structural Chemistry, The Weizmann Institute of Science, Rehovot, Israel. Received March 9, 1978

Abstract: The effects of temperature and organic solvents on the association between catecholamines and ATP or divalent metal ion–ATP chelates have been investigated by means of ${}^1H$ NMR chemical-shift and line-width measurements. The average thermodynamic parameters for the association of catecholamines with ATP, either in the absence or in the presence of divalent metal ions, are found to be -5.0 ± 0.5 kcal/mol, -11.1 ± 1.5 eu, and -1.7 kcal/mol for $\Delta H°$, $\Delta S°$, and $\Delta G°$ (300 K), respectively. These data, coupled with the observations that organic solvents which are less polar than water or possess large electric-dipole moments disrupt the catecholamine–ATP association, are interpreted in terms of possible mechanisms for the complexation between catecholamines and ATP. It is suggested that van der Waals–London interactions provide the major stabilizing force for the ring stacking involved in the complex formation. Hydrophobic and charge-transfer interactions, as well as hydrogen bond formation and an electrostatic interaction, are also considered to contribute to the stability of the association. An upper limit of 3×10^{-6} s is derived for the mean residence time in the complexed state of catecholamines.

Recent NMR studies[1-4] have established that at the region of neutral pH catecholamines bind to ATP, either in binary complexes or in ternary complexes with divalent metal ions, mainly through stacking between the catechol and the purine rings. This interaction was found to be augmented by hydrogen bond formation involving the catecholamine hydroxyls and by an electrostatic interaction between the protonated ammonium group and the negatively charged phosphate moiety. The specific interactions between aromatic moieties are of great importance in biological systems. Numerous experiments have provided evidence that parallel base stacking is the major stabilizing force for the self-association of purine and pyrimidine nucleosides and nucleotides, the folded structure of oligo- and dinucleotides, and the secondary

Table I. Formation Constants (K_1) and Bound State Shifts (δ_1) for the 1:1 Complexes of Catecholamines with ATP or MATP at 0 and 52 °C

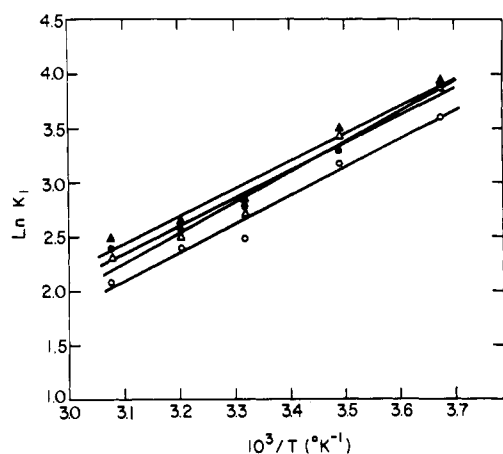| complex	pD	temp, °C	K_1, M^{-1}	δ_1, Hz				
				H_6	H_5	H_2	H_β	H_α
DA–ATP	6.9	0	49 ± 3	46	43	42	24	16
		52	11 ± 2	31	26	24	15	8
DA–MgATP	6.5	0	35 ± 3	42	39	37	25	17
		52	8 ± 2	27	24	22	16	7
DA–CoATP	6.5	0	37 ± 3	425	320	348	637	502
		52	8 ± 2	225	154	158	293	264
NE–ATP	6.9	0	50 ± 3	33	31	22	a	9
		52	12 ± 2	20	18	9	a	3
NE–MgATP	6.5	0	42 ± 3	29	27	22	a	8
		52	9 ± 2	18	16	9	a	3
NE–CoATP	6.5	0	48 ± 3	382	282	282	636	575; 922
		52	10 ± 2	198	125	125	250	250; 318

a Obscured by the HDO signal.

Figure 1. van't Hoff plots for the association constants of NE (triangles) and DA (circles) with ATP (filled symbols) and CoATP (open symbols).

structure of nucleic acids.[5-6] It is the purpose of the present study to examine the effects of temperature variation and of organic solvents on the interactions between catecholamines and ATP in aqueous solution. An attempt is made to gain some insight into the factors which govern their association. Thermodynamic data obtained from the NMR studies are compared with the results of related studies and are discussed in terms of possible mechanisms for the association.

Experimental Section

Dopamine (DA) and norepinephrine (NE) as hydrochlorides, and ATP as the sodium salt, of highest purity, were obtained from Sigma. The metal salts Co(NO$_3$)$_2$·6H$_2$O and MgCl$_2$·6H$_2$O were obtained from Merck and were dried in vacuo with slight heating before use. Deuterium oxide (99.7% D) and deuterated organic solvents were purchased from Roth, Karlsruhe, West Germany. Experimental solutions were made up by dissolving the materials in D$_2$O or in D$_2$O–solvent mixtures.[2,3]

^{1}H NMR spectra were recorded on a Bruker HFX-10 spectrometer operating at 90 MHz. The deuterium signal of the solvent was used for a field-frequency locking. A trace of dioxane in the experimental solutions served as internal standard. The error in the diamagnetic chemical shifts was estimated as 0.5–1.0 Hz. In the paramagnetic shifts, depending on the line width of the signal, the error was 3–7% of the measured shift. Temperature variations were effected by allowing dried nitrogen to flow in the annular space between the sample tube and a Dewar. The temperatures were kept constant to within ±0.5 °C by a Bruker's B-ST 100/760 control unit and were calibrated by measuring the peak separation of ethylene glycol or methanol samples. The notation of the proton resonances of the studied catecholamines is as given in Table I of ref 1.

Table II. Thermodynamic Parameters for the Formation of the 1:1 Complexes of Catecholamines with ATP or MATP

complex	$\Delta H°$, kcal/mol	$\Delta S°$, eu	$\Delta G°$ (300 K), kcal/mol
DA–ATP	−4.9 ± 0.5	−10.5 ± 1.5	−1.75
DA–MgATP	−5.0 ± 0.5	−11.4 ± 1.5	−1.58
DA–CoATP	−5.0 ± 0.5	−11.4 ± 1.5	−1.58
NE–ATP	−4.9 ± 0.5	−10.2 ± 1.5	−1.84
NE–MgATP	−4.8 ± 0.5	−10.8 ± 1.5	−1.56
NE–CoATP	−5.3 ± 0.5	−12.1 ± 1.5	−1.67

Results

Temperature Effects. The chemical shifts and line broadening induced in the proton resonances of DA and NE by varying concentrations of ATP or 1:1 M^{2+}–ATP (MATP) chelates (M = Mg^{2+},Co^{2+}) were measured in the temperature range 0–52 °C. Appropriate concentrations were used to ensure the formation of catecholamine complexes with 1:1 stoichiometry.[2,3] The data were analyzed by employing a procedure described elsewhere.[2] Calculated formation constants (K_1) and intrinsic shifts (δ_1) for the temperatures 0 and 52 °C are given in Table I. Evidently the association is significantly enhanced as the temperature is lowered, whereas at elevated temperatures the complexes are subjected to disruption.

The standard free-energy change, $\Delta G°$, given by:

$$\Delta G° = -RT \ln K_1 \qquad (1)$$

is related to the standard enthalpy change, $\Delta H°$, and to the standard entropy change, $\Delta S°$, through the equation:

$$\Delta G° = \Delta H° - T\Delta S° \qquad (2)$$

van't Hoff plots for the association constants were constructed (Figure 1) and the values of $\Delta H°$ and $\Delta S°$ were calculated from the slopes and intercepts of the straight lines through the experimental points, obtained by a least-squares analysis. It was assumed that $\Delta H°$ and $\Delta S°$ do not vary with temperature.[2,3] The thermodynamic parameters are given in Table II.

Paramagnetic line broadening induced in the resonances of ligand molecules depends in principle on two factors: the fractional concentration of the complexed molecules and the rate of exchange of the ligand between the bound and the free states.[7] An analysis[8,9] for the exchange contribution to the line broadening of catecholamines complexed with CoATP revealed that throughout the whole temperature range the exchange was too fast to produce a measurable effect on the line widths. An upper limit for the mean residence time of the catecholamines in the paramagnetic environment can be calculated with the relation $\tau_M < \Delta\nu_{2M}/\Delta\omega_M^2$, where $\Delta\nu_{2M}$ de-

Table III. Chemical Shifts ($\Delta\delta$) Induced by ATP in the Proton Resonances of DA[a] in Aqueous Solution and in Organic Solvent–D_2O Mixtures, and Electric-Dipole Moments, Dielectric Constants, and Polarity Parameters for the Solvents

| solvent system | $\Delta\delta$, Hz | | | | | solvent parameters[c] | | | |
	H_5	H_2	H_6	H_β	H_α	ϵ^d	Z value[e]	$E_T(30)^f$	μ^g
D_2O	24	22	28	16	9	77	94.6	63.1	1.85
methanol-d_4–D_2O[b]	19	17	22	12	7	33	83.6	55.5	1.70
ethanol-d_6–D_2O[b]	19	17	21	12	7	24	79.6	51.9	1.69
acetone-d_6–D_2O[b]	18	16	19	10	6	21	65.7	42.2	2.88
Me_2SO-d_6–D_2O[b]	16	14	17	9	6	47	71.3	45.0	3.96
dioxane-d_8–D_2O[b]	11	9	13	8	5	2		36.0	0

[a] [DA] = 0.13 M, [ATP] = 0.27 M. [b] Solvent–D_2O, 25%:75%, v/v. [c] The values given below are for nondeuterated solvents. [d] Dielectric constant of the solvents (from ref 11). [e] Solvent polarity parameters (from ref 12). [f] Solvent polarity parameters (from ref 13). [g] Electric-dipole moment of the solvent molecules (from ref 14).

notes the net paramagnetic contribution to the line width in the bound state, which was suitably corrected for diamagnetic and bulk-susceptibility contributions, and $\Delta\omega_M = 2\pi\delta_1$. At 300 K we get $\tau_M < 3 \times 10^{-6}$ s. Since the association of catecholamines in the ternary complexes was demonstrated to be independent of the type of the metal ion,[3] this limit is relevant to other metal–ion ternary complexes of catecholamines and actually to the binary catecholamine–ATP complexes as well.

The paramagnetic contribution to the line width in the bound state can be expressed as:[10]

$$\Delta\nu_{2M} = DR^{-6}\tau_S \qquad (3)$$

where D is a constant of the system, R is the distance between the metal ion and the proton under consideration, and τ_S is the relaxation time of the unpaired electrons of the metal ion. The temperature dependence of τ_S follows the Arrhenius expression:

$$\tau_S = \tau_S^\circ \exp(V_s/RT)$$

where V_s is the activation energy for the electronic relaxation. Typical line-broadening data are depicted in Figure 2 as a semilogarithmic plot vs. the reciprocal of the temperature. From the slopes of the lines through the experimental points, an average value of 5.1 kcal/mol was obtained for V_s.

Solvent Effect. The effects of several organic solvents such as methanol, ethanol, acetone, dioxane, and dimethyl sulfoxide on the association between DA and ATP were studied. Due to the low solubility of ATP in these solvents, mixtures of deuterium oxide–solvent had to be used. The chemical shifts of DA were measured in the absence and presence of ATP, at pD 7.0. The association shifts are summarized in Table III. In addition, several parameters which characterize the solvents, such as electric-dipole moments, dielectric constants, and polarity parameters, are also included. The latter parameters consist of the Z values derived by Kosower[12] and the $E_T(30)$ parameter proposed by Dimroth et al.,[13] both of which serve as a measure for the solvent polarity.

The association shifts of DA in the DA–ATP complex are significantly reduced in the organic solvent–D_2O mixtures relative to those in the aqueous solution. This effect may originate either due to disruption of the binary complex or due to changes in the geometry of the complex. Yet, inspection of the data in Table III reveals that within experimental error the shift ratios of the DA protons are unmodified throughout the series of solutions used in the study. Since the chemical shifts of DA are induced by ring-current effects,[1,2] this implies that the binary DA–ATP complex essentially retains its geometry. The observed effect on the chemical shifts can thus be attributed predominantly to disruption of the DA–ATP complex.

Discussion

Inspection of Table II shows that the complex formation between catecholamines and either ATP or MATP chelates
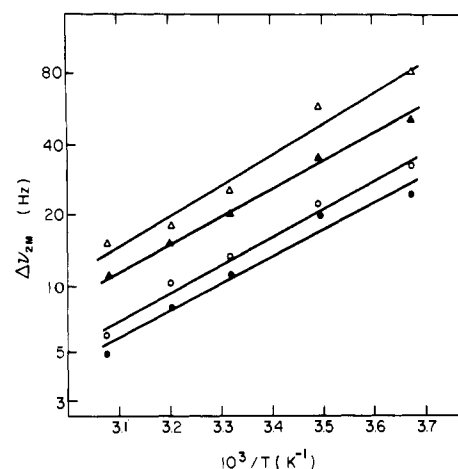

Figure 2. Semilogarithmic plot of $\Delta\nu_{2M}$ against the reciprocal temperature for the H_5 (circles) and H_β (triangles) resonances of NE (filled symbols) and DA (open symbols) bound to CoATP.

is characterized by the same changes (within experimental error) in the thermodynamic parameters. This is compatible with the former conclusion[3] that catecholamines bind in the ternary complexes only through their association with ATP, which is similar to the association in their binary complexes. The negative values obtained for both ΔH° and ΔS° indicate that the driving force for the formation of the complexes is the favorable enthalpy term which more than compensates for the entropy term. Similar magnitudes and signs for the changes of the enthalpy and entropy have been found also for the associations of epinephrine with nucleic acid bases[15] and of tryptophan with ATP,[16] AMP, and nucleic acid,[17] for self-association of purines,[18] and for intramolecular association ("folding up") of dinucleotides.[19] It appears that these associations, all of which involve ring stacking, are stabilized by similar interactions. The exact nature of the specific interactions which govern the stacking of aromatic rings is unclear. Yet certain conclusions with regard to the major stabilizing forces can be drawn.

The disruption of the DA–ATP complex upon addition of organic solvents can be interpreted by considering a hydrophobic driving force for the stacking interaction. However, the negative values for ΔS° which characterize the association of catecholamines and ATP in aqueous solution are not consistent with the classical concept of hydrophobic interactions. Due to the reduced amount of ordering of the water molecules around the complex, relative to the free molecules, the entropy is expected to increase.[20,21] Furthermore, a hydrophobic interaction by itself cannot account for the observed changes in the enthalpy of formation. It has been found that formation of hydrophobic bonds in aromatic systems is accompanied by little change in ΔH°.[22,23] A suggestion has been made that the absence of the stacking interaction in nonaqueous solvents could

be partially due to specific solvent–solute interactions, which compete with the forces that cause the stacking.[5,24] This would be true in particular in the case of stacking of polar rings such as adenine. The solvent effects on the DA–ATP association shifts follow the sequence dioxane > dimethyl sulfoxide > acetone > ethanol $\simeq$ methanol. A similar trend is observed by comparison of the dielectric constants of these solvents, with the exception of dimethyl sulfoxide. A somewhat better correlation is obtained between the trends of the experimental data and the solvent polarity parameters. The data in Table III appear to imply that the effects of two properties of the solvents, namely low polarity and large electric-dipole moment, may be responsible for disruption of the DA–ATP complex. Thus, due to its low polarity, dioxane has a large effect on the DA–ATP association. On the other hand, although dimethyl sulfoxide is much more polar, it also produces a rather large effect, apparently due to its relatively large electric-dipole moment. By means of this dipole moment the dimethyl sulfoxide molecules can interact directly with the solute molecules and consequently disturb their associations. From this discussion it follows that the disruption of the DA–ATP complex by organic solvents does not necessarily indicate involvement of hydrophobic bonds in the association. On the basis of the thermodynamic data, especially the negative value of $\Delta S°$, and the above considerations it is therefore concluded that although the presence of a hydrophobic interaction cannot be ruled out, apparently it does not play a major role in stabilization of the catecholamine–ATP association.

Theoretical studies and experimental investigations of related systems[6,15,25-29] imply the possible importance of ring interactions which consist of van der Waals–London and charge-transfer forces, in the stabilization of the catecholamine–ATP association. At a vertical separation of ca. 3.4 Å between the catechol and the adenine rings,[2] the distances between the atoms of the respective rings will be close to their van der Waals contact distances. Therefore, this interaction will be highly effective. As the van der Waals bond energy for a pair of atoms is about 1 kcal/mol, the possible formation of this bond between several pairs of ring atoms can have significant contribution to $\Delta H°$. The ring separation in the catecholamine–ATP complexes is also in the region of typical distances between molecules which form charge-transfer complexes.[30] The contribution of charge-transfer interactions to the stability of complexes depends on the ionization potential of the molecule donating the electron charge, and on the electron affinity of the acceptor molecule. It is generally accepted that both the adenine[6,26] and the catechol[31] rings are strong electron donors. The data presently at hand do not allow conclusive determination of their relative strengths as donors or acceptors. However, in view of their similar properties it seems most probable that charge-transfer interaction between catechol and adenine will not be strong. It is therefore reasonable to assume that the van der Waals–London interactions provide the major stabilizing force for the association between the rings of catecholamines and ATP.

In addition to the interactions considered above, the formation of hydrogen bonds between the hydroxyl groups of the catecholamines and suitable acceptors in the ATP molecules, as well as the electrostatic interaction between the protonated ammonium group and the negative phosphate moiety, contribute also to the enthalpy of formation of the catecholamine complexes. These interactions affect also the entropy term and can account for its observed negative value. The change in entropy upon complex formation can be described in terms of two major contributions: the solvent entropy and the configurational entropy. The first contribution, as noted above, is positive. The change in the configurational entropy originates from the decrease in the amount of degrees of freedom, principally due to restriction of the rotations about single bonds,

upon formation of the complex. Hydrogen bonds and electrostatic interactions, and to a lesser extent hydrophobic bonds, increase the effective height of the potential barrier for internal rotations. The loss of configurational entropy was estimated to vary from ca. -1 eu (per bond whose rotation is restricted) for an increase of 1–2 kcal/mol in the rotational potential barrier[32] to ca. -5 eu (per bond) for complete freezing of the rotation about single C–C bonds.[33] It should be noted that in the case of associations of small molecules, the entropy loss due to changes in the translational rotational degrees of freedom of the molecules as a whole may not be insignificant.

It is worth noting the relatively small values of the free-energy change, of ca. -1.7 kcal/mol at 300 K, associated with the formation of the catecholamine complexes. Evidently these complexes could be easily subjected to disruption by thermal energy. As it is generally accepted that association of catecholamines with ATP (either in the presence or in the absence of divalent metal ions) plays a role in the biological mechanisms of catecholamine action, this property may be of importance, particularly in view of the fact that after termination of their actions the catecholamines are taken up again in their storage sites. This process obviously requires disruption of the association between the catecholamines and their effector sites.

Finally, some remarks are to be made with regard to the effect of temperature on the chemical shifts and line widths of the complexed catecholamines. The value of 5.1 kcal/mol for the activation energy for the relaxation of the unpaired electrons of Co^{2+}, obtained under the assumption that only τ_S is temperature dependent, is larger than the values reported previously, e.g., for Co^{2+} in aqueous solutions.[34-36] This discrepancy can be interpreted by considering an additional temperature-dependent effect on the line widths. It is plausible to attribute this effect to variations in the metal ion–proton distances. Due to the sixth power dependence (eq 3), small changes in R will introduce large changes in $\Delta\nu_{2M}$. Accordingly, it then follows from the experimental results that as the temperature is lowered the metal ion and the catecholamine molecule come closer to one another. The variations of the shifts induced in the catecholamine–CoATP complexes are found to decrease with temperature faster than the expected $1/T$ dependence.[37] These pseudocontact shifts vary with R^{-3}, and thus will be also affected by changes in R. The ring current shifts induced in the binary catecholamine–ATP complexes have the same spatial dependence as the pseudocontact shifts but are intrinsically independent of the temperature.[38] The similar trends in the observed variation of both these shifts with temperature substantiate the conclusion that the intermolecular geometries, and most probably also the intramolecular conformations, vary systematically with temperature.

References and Notes

(1) J. Granot and D. Fiat, *J. Am. Chem. Soc.*, **99**, 4963 (1977).
(2) J. Granot, *J. Am. Chem. Soc.*, **100**, 1539 (1978).
(3) J. Granot, *J. Am. Chem. Soc.*, **100**, 2886 (1978).
(4) J. Granot, *FEBS Lett.*, **88**, 283 (1978).
(5) C. E. Bugg, J. M. Thomas, M. Sundaralingam, and S. T. Rao, *Biopolymers*, **10**, 175 (1971).
(6) B. Pullman, "Molecular Associations in Biology", Academic Press, New York, N.Y., 1967.
(7) T. J. Swift, in "NMR of Paramagnetic Molecules", G. N. La Mar, W. DeW. Harroks, Jr., and R. H. Holms, Ed., Academic Press, New York, 1973, pp 53–83.
(8) A. C. McLaughlin and J. S. Leigh, Jr., *J. Magn. Reson.* **9**, 296 (1973).
(9) J. Granot and D. Fiat, *J. Magn. Reson.*, **15**, 540 (1974).
(10) A. Abragam, "The Principles of Nuclear Magnetism", Oxford University Press, London, 1961, Chapter 8.
(11) E. M. Kosower, "An Introduction to Physical Organic Chemistry", Wiley, New York, N.Y., 1968, p 269.
(12) E. M. Kosower, *J. Am. Chem. Soc.*, **80**, 3253 (1958).
(13) K. Dimroth, L. Reichardt, T. Siepmann, and F. Bohlmann, *Justus Liebigs Ann. Chem.*, **661**, 1 (1963).
(14) R. C. Weast, Ed., "Handbook of Chemistry and Physics", CRC Press, Cleveland, Ohio, 1975.
(15) F. A. Al-Obeidi and H. N. Borazan, *J. Pharm. Sci.*, **65**, 982 (1976).
(16) F. Morita, *Biochim. Biophys. Acta*, **343**, 674 (1974).

(17) J. Dimicoli and C. Helene, *Biochimie*, **53**, 331 (1971).
(18) S. J. Gill, M. Downing, and G. F. Sheats, *Biochemistry*, **6**, 272 (1967).
(19) G. McDonald, B. Brown, D. Hollis, and C. Walter, *Biochemistry*, **11**, 1920–1930 (1972).
(20) W. Kauzmann, *Adv. Protein Chem.*, **14**, 1 (1959).
(21) G. Nemethy, I. Z. Steinberg, and H. A. Scheraga, *Biopolymers*, **1**, 43 (1963).
(22) H. S. Frank and M. W. Evans, *J. Chem. Phys.*, **13**, 507 (1945).
(23) D. M. Crothers and D. I. Ratner, *Biochemistry*, **7**, 1823 (1968).
(24) S. Hanlon, *Biochem. Biophys. Res. Commun.*, **23**, 682 (1966).
(25) H. DeVoe and I. Tinoco, Jr., *J. Mol. Biol.*, **4**, 500 (1962).
(26) B. Pullman, *J. Chem. Phys.*, **43**, S233 (1965).
(27) P. Claverie, B. Pullman, and J. Caillet, *J. Theor. Biol.*, **12**, 419 (1966).
(28) R. Foster and C. A. Fyfe, *Prog. Nucl. Magn. Reson. Spectrosc.*, **4**, 1 (1969).
(29) M. A. Slifkin, "Charge Transfer Interactions of Biomolecules", Academic Press, London, 1971.
(30) See ref 29, p 2.
(31) See ref 29, p 214.
(32) G. Nemethy and H. A. Scheraga, *J. Phys. Chem.*, **66**, 1773 (1962).
(33) M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **76**, 6305 (1954).
(34) R. A. Bernheim, T. H. Brown, H. S. Gutowsky, and D. E. Woessner, *J. Chem. Phys.*, **30**, 950 (1959).
(35) Z. Luz and S. Meiboom, *J. Chem. Phys.*, **40**, 1058 (1964).
(36) Z. Luz and S. Meiboom, *J. Chem. Phys.*, **40**, 2686 (1964).
(37) H. M. McConnell and R. E. Robertson, *J. Chem. Phys.*, **29**, 1361 (1958).
(38) R. A. Dwek, R. J. P. Williams, and A. V. Xavier, in "Metal Ions in Biological Systems", H. Siegel, Ed., Marcel Dekker, New York, N.Y., 1974, pp 167–169.

Communications to the Editor

Isolation and Identification of Thymine Products from DNA γ Irradiated in Oxygen-Free Aqueous Solutions

Sir:

It has been suggested[1-3] that the response of living cells to ionizing radiation involves different classes of DNA damages, in aerated and deaerated conditions.[4] In deaerated solutions the radiation-induced degradation of free pyrimidine bases has been well studied. However, the actual isolation and identification of bases damaged *in the DNA chain* in oxygen-free solutions has not been accomplished. It should be noted that the radiation chemistry of the free bases may be quite different from that of bases covalently linked to the phosphate–sugar backbone of the DNA chain.[5] We report the successful characterization of thymine fragment derivatives formed in the polymer chain by γ irradiation of oxygen-free DNA solutions.

In a first step, *Escherichia coli* DNA $^{14}CH_3$ labeled in the thymine moiety was obtained from the mutant 15 $T^-A^-U^-$.[6] The DNA solution (500 μg/mL) in 10^{-3} M phosphate buffer deaerated under vacuum by repeated cycles of freezing and pumping was irradiated in sealed vials by ^{60}Co γ rays with a dose rate of 70 Gy/min at 1000 Gy (gy = gray). After irradiation, the polymeric material was separated from the low-molecular-weight material by dialysis.

The low-molecular-weight ^{14}C material was fractionated by silica gel thin layer chromatography[6] and shown to be free thymine and its radiolysis products.[7] It may be postulated that the release of the thymine fragment[8] involves OH radical attack at C-4′ and C-1′. These compounds of low molecular weight will not be discussed further because they are of lesser biological interest than the modified DNA chain.

In a second step, the high-molecular-weight material, i.e., modified DNA chain, was treated at 90 °C with 95% formic acid for 16 h.[9] The ^{14}C labeled products liberated from the DNA chain by this acid hydrolysis have been shown to be thymine (I), 5,6-dihydrothymine (II), *cis*- (III) and *trans*-5,6-dihydroxy-5,6-dihydrothymine (IV), and 5-hydroxy-5,6-dihydrothymine (V) (Figure 1).

The structure of compound II was supported by its transformation into 2-methyl-3-ureidopropionic acid in alkaline solution.[10] The oxidation of products III and IV[11] by potassium periodate gave N^1-formyl-5-hydroxy-5-methylhydantoin,[12] which suggested the presence of a glycol group, in agreement with the expected structure. 2-Hydroxy-2-methyl-3-ureidopropionic acid, which gave a colored spot with *p*-dimethyl-
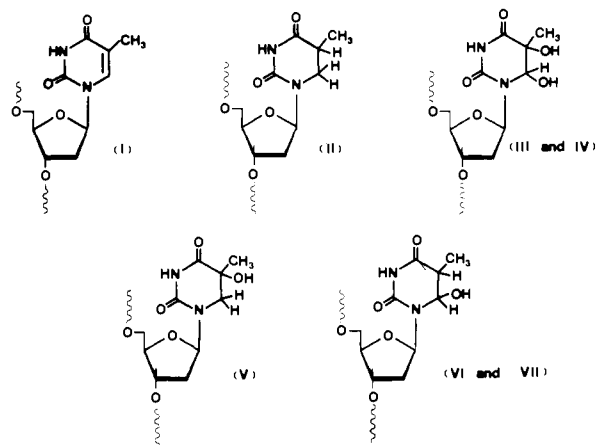

Figure 1. Unaltered thymine fragment (I) and modified thymine fragments (II–VII) in DNA chain produced by γ irradiation of DNA in deaerated aqueous solutions.

aminobenzaldehyde,[13] was obtained by alkaline solvolysis of derivative V which is consistent with the properties of 5-hydroxy-5,6-dihydrothymine.[14a] The drastic conditions of formic acid hydrolysis did not allow the isolation of 6-hydroxy "hydrate";[14b] however, isolation of VI and VII could be obtained using an enzymatic method. The polymeric material resulting from the irradiation of DNA incubated with crude enzymatic extracts of *E. coli*[15] for 2 h at 37 °C at pH 7.2 released the products I to V and in addition *cis*- (VI) and *trans*-6-hydroxy-5,6-dihydrothymine (VII).[16] The products I to VII were separated from the reaction mixture by dialysis against water and then chromatographed in the usual manner.[6,9] As expected VI and VII gave rise to thymine on warming for 10 min at 100 °C in aqueous acid solution at pH 1. Treatment of VI and VII with H_2O_2 as described previously causes their transformation to *cis*- and *trans*-6-hydroperoxy-5,6-dihydrothymine, through nucleophilic substitution of 6-OH by OOH group.[16] These peroxides were stereospecifically reduced to VI and VII. In this way VI and VII were interconverted. Finally, the structure of the products II–VII was confirmed by chemical synthesis of reference samples which were characterized by UV, IR, ^1H NMR, and mass spectrometry.[16-18]

On the basis of studies performed on model compounds,[19] a mechanism for the formation of these radiation products on the DNA chain may be postulated. When water absorbs γ rays, it decomposes to OH radicals, H atoms, and e^-_{aq} which