155. *Reactions of Nucleic Acids and Their Components. Part III.* The Interaction of Adenine and Uracil with Formaldehyde.*

By S. Lewin.

The interactions of formaldehyde with adenine and uracil have been examined by using the pH-variation method and spectrophotometry in the temperature range 20—40°. The results with adenine are interpretable in terms of a major interaction of the un-ionized ($N_{(10)}$) amino-group and a minor interaction with the corresponding cation, each with one molecule of formaldehyde. Evidence for reaction of formaldehyde with the $N_{(9)}$–H group of purines has been obtained spectrophotometrically. The acid imino-group of uracil also reacts with one molecule of formaldehyde. The reactions of the neutral molecules are exothermic.

New structural configurations, involving hydrogen-bonded hydrations of the nitrogenous groups of adenine and of uracil, are advocated.

The Adenine–Formaldehyde Reaction

Introduction.—The reactions of formaldehyde with the free amino-groups of amino-acids have been extensively investigated (for references see French and Edsall [1]) by potentiometric and other methods. In the reactions of amino-acids with formaldehyde [2] and with aldoses (Lewin *et al.*[3,4]) it was established that the amino-group reacts with both one and two molecules of formaldehyde or aldose and that the equimolar reactions are exothermic, while those with two molecules can be athermic.[4] Other free amino-groups might be expected to behave similarly. However, spectrophotometric kinetic investigations [5] of the reaction of formaldehyde with the " amino "-groups of several nucleotides showed that the reactions are of first order with respect to formaldehyde; also Feldman, [6] by gravimetric methods, concluded that two adenine molecules combine with one formaldehyde molecule.

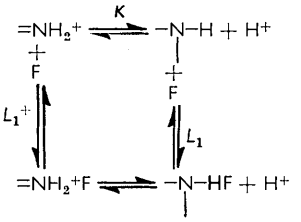
* Part II, *J.*, 1962, 1466.

[1] French and Edsall, *Adv. Protein Chem.*, 1945, **2**, 277.
[2] Levy and Silverman, *J. Biol. Chem.*, 1937, **118**, 723; Balson and Lawson, *Biochem. J.*, 1936, **30**, 1257.
[3] Lewin, *Biochem. J.*, 1956, **63**, 14; Lewin and McCall, *ibid.*, 1957, **65**, 30P.
[4] Lewin and Kosinski, *Trans. Faraday Soc.*, 1958, **54**, 222.
[5] Grossman, Levine, and Allison, *J. Mol. Biol.*, 1961, **3**, 47; Hoard, *Biochem. Biophys. Acta*, 1960, **40**, 62; Haselkorn and Doty, *J. Biol. Chem.*, 1961, **236**, 2738.
[6] Feldman, *Biokhimyia*, 1960, **25**, 563.

In Part I we showed that addition of formaldehyde to adenine results in depression or elevation of pH depending on whether the titration region of the first or of the second dissociating group of adenine ($pK_1 \cong 4{\cdot}1$, $pK_2 \cong 9{\cdot}8$) is involved. These variations were attributed to respective interactions with a basic amino-group (pK_1) and acid imino-group (pK_2). In this investigation we are concerned with the reaction in the titration region corresponding to the basic pK_1. The " amino "-group of unsubstituted adenine—free from the possibility of hydrogen bonding with the 2-hydroxy-group of ribose and the complicating influence of the phosphoric acid residue present in nucleotides—could be expected to react with one and two molecules by analogy with other free amino-groups. We propose to show that adenine—whether neutral or positively charged—reacts reversibly with only one molecule of formaldehyde, to allocate the site of acid dissociation and reaction in adenine, to advance feasible explanations for these conclusions, and also to evaluate the thermodynamic constants of the reaction.

We have established experimentally relations between the pH depressions (ΔpH) which accompany the addition of formaldehyde to adenine, and the formaldehyde concentration ([F]); we have also derived equations which account for these findings by postulating that (a) the reaction is reversible (see Scheme), (b) hydrogen-ion dissociation, and reaction with formaldehyde, take place at the same nitrogen atom (see p. 800), and (c) both neutral and cationic adenine react each with only one molecule of formaldehyde.

The reaction scheme utilized is:



Using this scheme (see Appendix 1,A) we derived the equation

$$\text{antilog}\Delta\text{pH} = (1 + L_1[\text{F}])/(1 + L_1^{+}[\text{F}]),$$

where L_1 and L_1^{+} represent the respective equilibrium constants for reaction of formaldehyde with neutral and with cationic adenine.

Therefore, a plot of $\{(\text{antilog}\Delta\text{pH}) - 1\}/[\text{F}]$ against $\{\text{antilog}\Delta\text{pH}\}$ should yield a straight line with a slope of L_1^{+} and an intercept of L_1; alternatively a plot of $\{(\text{antilog}\Delta\text{pH}) - 1\}/\{[\text{F}](\text{antilog}\Delta\text{pH})\}$ against $1/\text{antilog}\Delta\text{pH}$ should be linear with a slope of L_1 and an intercept of L_1^{+}.

We have also followed the formaldehyde–adenine interaction spectrophotometrically in buffered solutions. The reaction is accompanied by a considerable increase in optical density and by bathochromic shifts. We have related experimentally the optical-density changes, at specified wavelengths, with the formaldehyde concentration. Using the above scheme we have derived equations for the reaction of one molecule of formaldehyde with one molecule of cationic adenine or with one molecule of neutral adenine.

For very acid solutions, where only the cationic form of adenine need be considered, we derived the equation

$$\Delta A_{ac}/[\text{F}] = \varepsilon_2 L_1^{+} C - L_1^{+} A_{mix}$$

where A_{mix}, ΔA_{ac}, and ε_2 represent, respectively, the absorbance of the formaldehyde–adenine solution, the *increase* in absorbance following reaction, and the molar absorption coefficient of cationic adenine, and C is the total adenine concentration. Hence, a plot of $\{\Delta A_{ac}/[\text{F}]\}$ against A_{mix} should be linear with a slope of $-L_1^{+}$ and an intercept of $\varepsilon_2 L_1^{+} C$.

We have established that this equation accounts satisfactorily for the experimental

data and have also shown that the value of L_1^+ determined spectrophotometrically agrees well with that determined by the pH-depression method.

For neutral values of pH, where only the neutral form of adenine (molecular absorption coefficient ε_3) need be considered, we have derived the equation

$$\Delta A_{neut}/[F] = \varepsilon_3 L_1 C - L_1 A_{mix}$$

Hence, a plot of $\{\Delta A_{neut}/[F]\}$ against A_{mix} should be linear with a slope of $-L_1$ and an intercept of $(\varepsilon_3 L_1 C)$.

In this region the relation was obeyed only partially at low concentrations of formaldehyde; and the lack of agreement can be attributed to the presence of the unsubstituted acid imino-group $N_{(9)}$–H which interacts with formaldehyde and affects the optical-density values.

Results.—(*a*) *The pH depression method.* The pH of a stock solution of adenine (0·005M) was adjusted with hydrochloric acid to a desired initial value in the range pH 4·9—5·2. A series of solutions was prepared of constant adenine concentration and total volume, but the formaldehyde concentration was varied. The solutions were thermostatically

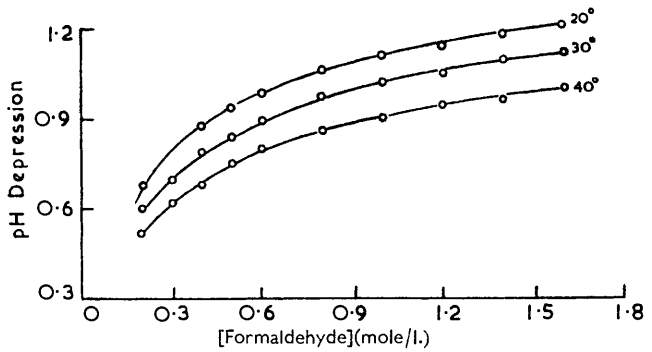


FIG. 1. Variation of the pH-depression value with formaldehyde concentration in the formaldehyde–adenine reaction at various temperatures.

controlled at 20°, 30°, and 40° (±0·03) for 4 days. The pH of the solutions dropped continuously to equilibrium. The rate of drop was lowest at low formaldehyde concentrations (<1M), when equilibrium took 40—48 hr. The equilibrium pH-depression values increased with increase in formaldehyde concentration; they also decreased with temperature rise thus showing the reaction to be exothermic, in accord with previous studies [7] (see Fig. 1).

The experimental results have been recalculated and plotted as a function of {(antilog ΔpH) − 1}/[F] against antilogΔpH (see Fig. 2) and as function of {(antilog ΔpH) − 1}/[F] antilogΔpH against 1/antilogΔpH (see Fig. 2a).

To ensure a more accurate limitation of the errors in computing L_1 and L_1^+ from the respective slopes and intercepts, the results of both plots were pooled and averaged. The computed values are given in Table 1.

TABLE 1.

Thermodynamic constants for adenine–formaldehyde interactions ($\Delta G°$ in kcal. mole^{-1}).

T °c	L_1	L_1^+	$-\Delta G°$ (neutral)	$-\Delta G°$ (acidic)
20°	22·15	0·85	1·80	−0·095
30	18·0	0·83	1·74	−0·112
40	13·2	0·87	1·60	−0·087

Error limits: L_1 ±0·7; L_1^+ ± 0·07; $-\Delta G°$ (neutral) ± 0·03; $-\Delta G°$ (acidic) ± 0·007.

Hence, the experimental data are in accord with the formulation of reversible interactions of one molecule of formaldehyde with one molecule of neutral (" amino ") adenine,

[7] Frieden, Ph.D. Thesis, 1942, Univ. of California, Los Angeles; Lewin, unpublished observations.

and of one molecule of formaldehyde with one molecule of the positively charged " ammonium " form of adenine.

The value of the heat of reaction of the neutral adenine with formaldehyde was computed, from the straight-line plot of the logarithm of L_1 against the reciprocal of absolute temperature, to be $4{\cdot}8 \pm 0{\cdot}8$ kcal. mole^{-1}. The corresponding entropy change computed by the equation $-\Delta S^\circ = \partial \Delta G^\circ / \partial T$ is 6 ± 6 cal. deg.$^{-1}$ mole^{-1} at 25°, and 14 ± 6 cal. deg.$^{-1}$ mole^{-1} at 35°. These values cover also those which are obtained by using the equation $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T$.

(*b*) *Spectrophotometry.* The interaction of formaldehyde with adenine was followed spectrophotometrically at 30° and 40° by using DK-2 Beckman recording and manual

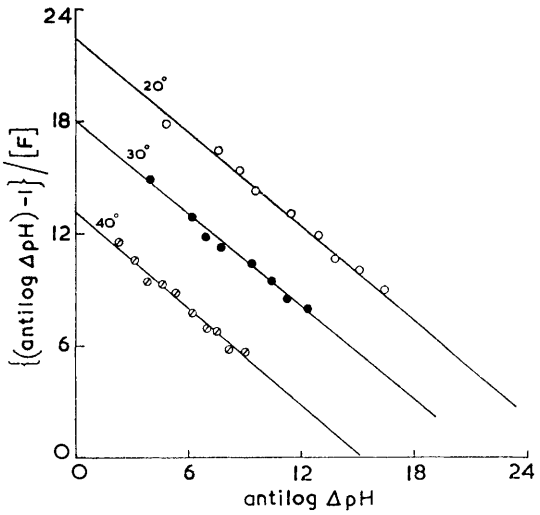


FIG. 2. Variation of the expression {(antilog ΔpH) − 1}/[F] with the antilog ΔpH at 20°, 30°, and 40° in the reaction of the amino-group of adenine with formaldehyde. Initial pH values are 4·8—5·1.

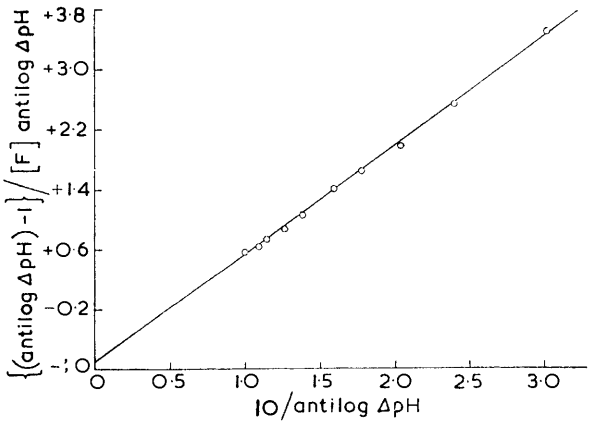


FIG. 2a. Variation of the expression {(antilog ΔpH) − 1}/[F] (antilog ΔpH) with the reciprocal of antilog ΔpH, at 40°.

Unicam S.P. 500 spectrophotometers. Buffers of various ionic strengths were used. The concentration of adenine varied between 5×10^{-5}M with 1-cm. path length, and 2×10^{-4}M with 0·5-cm. path length in silica cells.

In Fig. 3 the optical densities of the adenine and adenine–formaldehyde solutions, in acetate-sodium chloride–hydrochloric acid buffers ($I = 0{\cdot}1$), having pH values of 1·26—1·40 at 30°, are plotted against the wavelength. The optical density values at 280 mμ were compared with those determined by using the S.P. 500 Unicam spectrophotometer. Agreement was within ±1%.

The data were recalculated and plotted as a function of $\Delta A_{ac}/[F]$ against A_{mix} (see Fig. 4). The computed value of L_1^+ was 0·77 ± 0·04, which agrees fairly well with the value of 0·83 ± 0·05 deduced from the ΔpH measurements, considering the difference in the ionic strengths employed in the two techniques. The mean value of ε_3 determined when either $A_{mix} = 0$ or $\{\Delta A_{ac}/[F]\} = 0$, was found to be (1·46 ± 0·03) × 10^4 at 280 mμ, pH 1·26—1·40, and I = 0·1 at 30°. The corresponding value for ε_2 (ammonium) under identical conditions, determined in this investigation, is (5·0 ± 0·05) × 10^3.

The absorption spectra of the adenine–formaldehyde interaction in phosphate buffers, in the pH range 6·9—7·4 were also determined (see Fig. 5). In these solutions the plot of

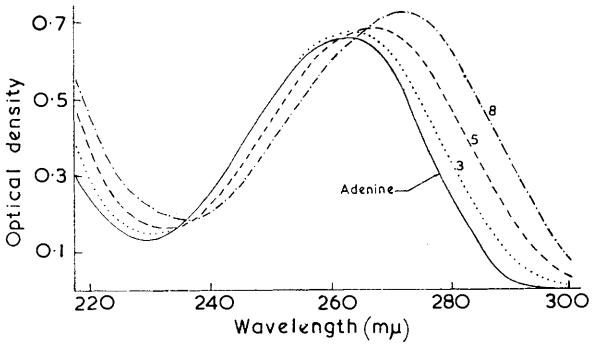


FIG. 3. Absorption spectra of adenine–formaldehyde solutions in the pH range 1·4—1·6 at 30°. [Adenine] = 5 × 10^{-5}M; HCl–acetic acid–NaCl buffers, ionic strength = 0·1; 1-cm. silica cells. Absorption spectra were measured after incubation for 48 hr. at 30°.

Full curve, adenine only; curve 3, +0·2M-formaldehyde; curve 5, +1·0M-formaldehyde; and curve 8, +4·0M-formaldehyde.

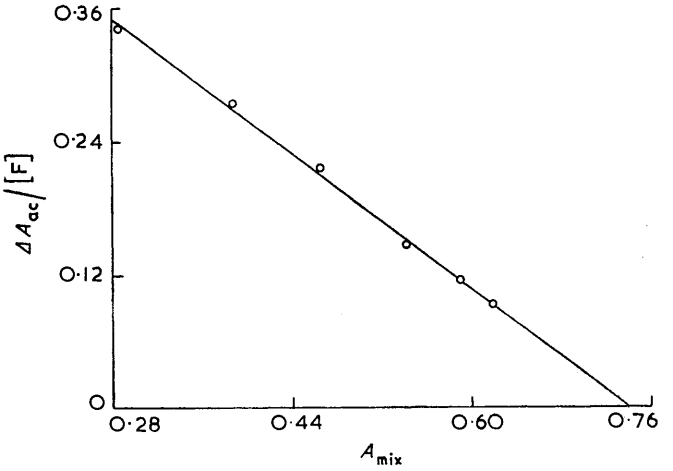


FIG. 4. Variation of the expression $\Delta A_{ac}/[F]$ with A_{mix} at about pH 1·4 at 30°.

$\Delta A_{neut}/[F]$ against A_{mix}, or the corresponding plot of $[F]/\Delta A_{neut}$ against [F], was linear only up to *ca.* 0·3M formaldehyde and the value of L_1 evaluated at 275 mμ was in fair agreement with that determined by the ΔpH method. However, despite careful measurements taken after incubation for 2 or 3 days to ensure equilibrium, the results were not reproducible, particularly at wavelengths other than 270 mμ. It is of interest that the spectrophotometric results obtained in acetic acid–acetate buffers at pH 4·7 gave comparatively straight lines (see Fig. 6). By using certain simplifying assumptions, it is possible to derive a relationship to give an approximate value of L_1. However, because of the uncertainty involved, more reliance is to be placed on the values of L_1 determined by the ΔpH method.

The difficulty encountered in evaluating spectrophotometrically the value of L_1 can be attributed to the presence of the free acid ·NH group in the adenine molecule. This group contributes to the optical density in the wavelength range 245—275 mμ; its reaction with

formaldehyde is bound to affect this contribution. This interpretation is supported by the spectrophotometric results of the interaction of unsubstituted purine with formaldehyde, which is accompanied by hypochromicity (see Fig. 7).

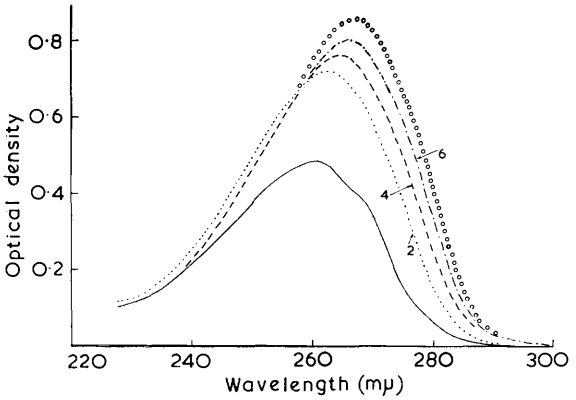


FIG. 5. Variation of the absorption spectrum of adenine–formaldehyde solutions in phosphate buffers (I = 0·1) at about pH 7·2 at 40°; [adenine] = 5 × 10^{-5}M. Solutions were incubated for about 60 hr. at 40° before measurements were made in 1-cm. silica cells.

Full curve, adenine only; curve 2, +0·1M-formaldehyde; curve 4, +0·5M-formaldehyde; curve 6, +2M-formaldehyde; and final curve +4M-formaldehyde.

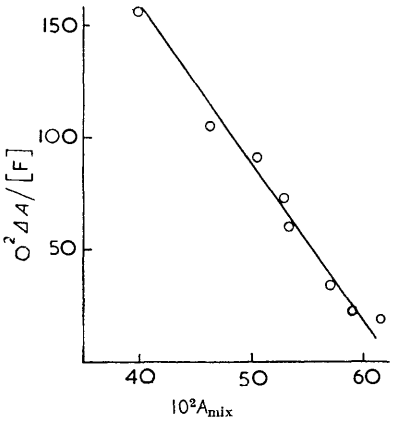


FIG. 6. Adenine–formaldehyde reaction; variation of the expression $\Delta A/[F]$ with A_{mix} in acetic acid–acetate buffers at pH 4·7, I = 0·04, 30°, 275 mμ.

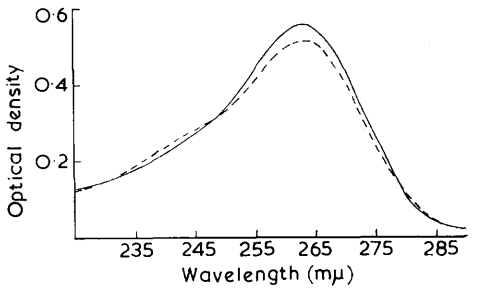


FIG. 7. Variation of the absorption spectrum of purine-formaldehyde solutions in acetate buffer at pH 4·38, 30°, 1-cm. silica cells.

—— purine only; - - - - purine–formaldehyde.

Elimination of the reactive hydrogen atom of this group by substitution of ribose (cf. adenosine,[8] cytosine, and cytidine [9]) results in very good agreement between the L_1 constants determined by the ΔpH method and by spectroscopy.

THE URACIL–FORMALDEHYDE REACTION

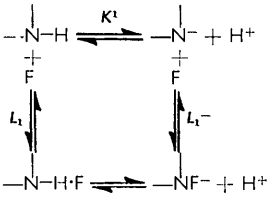
Introduction.—Fraenkel-Conratt [10] investigated the effect of formaldehyde on a number of nucleic acid components spectrophotometrically, but recorded negative results in the case of uracil. However (see Part 1), the addition of formaldehyde to uracil results in pH elevation in the dissociation region (p$K \cong 9{\cdot}5$). This was attributed to the reversible reaction of the uracil acid-imino-group with formaldehyde.

We shall now produce further evidence to show that formaldehyde can react reversibly with uracil, using both the pH elevation method and spectrophotometry.

[8] Lewin and Ford, unpublished observations.
[9] Lewin and Humphreys, unpublished observations.
[10] Fraenkel-Conratt, *Biochim. Biophys. Acta*, 1954, **15**, 307.

(*a*) *pH elevation method.* We have related experimentally the pH elevations accompanying the formaldehyde–uracil reaction, with formaldehyde concentration; we have also derived equations which account for these findings, postulating that: (i) the uracil–formaldehyde reaction is reversible (see later), (ii) acid dissociation and reaction with formaldehyde take place at the same nitrogen atom (see Discussion section); and (iii) the neutral form of uracil reacts with only one molecule of formaldehyde.

The reaction scheme utilized is



By using this scheme it will be shown (see Appendix II) that, when the formaldehyde reaction with the negatively charged form of uracil is very small compared with that of un-ionized uracil, then

$$\text{antilog}\Delta\text{pH} = 1 + L_1[\text{F}],$$

where L_1 represents the equilibrium constant of the formaldehyde reaction with un-ionized uracil.

Therefore, a plot of antilogΔpH against [F] should yield a straight line with an intercept of unity and a slope equal to L_1.

(*b*) *Spectrophotometry.* Experimental evidence will be presented to show that addition of formaldehyde results in well-defined spectral changes.

Results.—(*a*) *Thermodynamic constants of the ionization of uracil.* Levene, Bass, and Simmons [11] computed the pK value of uracil as 9·45 at 25°. This value was confirmed by Taylor.[12] Shugar and Fox [13] give 9·5. No data appear available for other temperatures. The information is relevant to this investigation and so the constants were evaluated.

The uracil solutions were kept under pure nitrogen as described previously (Lewin and Tann [14]). Sodium chloride in various proportions was added to keep the ionic strength constant at $I = 0{\cdot}01$. The results are given in Table 2. The plot of pK_c against $1/T$ was a straight line, from which ΔH was computed to be $7{\cdot}2 \pm 0{\cdot}5$ kcal. mole^{-1}. The Debye-Huckel equation gives $pK_a = pK_c + 0{\cdot}5\sqrt{I}$, where p$K_a$ is the thermodynamic or activity pK. Hence, the slope of the plot of pK_c against $1/T$ should retain its value if pK_a were substituted for pK_c, and hence $\Delta H° = 7{\cdot}2 \pm 0{\cdot}5$ kcal. mole^{-1}. The computed values of the standard free-energy changes and standard entropy changes are given in Table 2.

TABLE 2.

Thermodynamic constants of the ionization of uracil ($\Delta G°$ and $\Delta H°$ in kcal. mole^{-1}; $\Delta S°$ in cal. deg.$^{-1}$ mole^{-1}).

Temp.	20°	25°	30°	35°	40°	45°	50°
pK_c	9·47		9·28		9·15		8·98
$\Delta G°$	12·79		12·96		13·20		13·38
$\Delta S°$		−17		−24		−18	

p$K_c \pm 0{\cdot}01$ at 20—40°, $\pm 0{\cdot}02$ at 50°; $\Delta G° \pm 0{\cdot}03$; $\Delta S° \pm 5$.

(*b*) *Measurements of pH elevation.* The experimental technique used was identical with that used in the adenine–formaldehyde reaction except that the pH range was 8—9,

[11] Levene, Bass, and Simmons, *J. Biol. Chem.*, 1926, **70**, 229.
[12] Taylor, Thesis, 1946, London University.
[13] Shugar and Fox, *Biochim. Biophys. Acta*, 1952, **9**, 199.
[14] Lewin and Tann, *J.*, 1962, 1466.

and much higher uracil concentrations (0·02M) were used to reduce errors arising from the formaldehyde ionization (p$K \cong 12{\cdot}3$) to negligible proportions.

The attainment of equilibrium in the interaction of formaldehyde with uracil is strikingly different from that involving the basic " amino "-group of adenine. The former takes place normally in less than half an hour and the pH elevation remains constant for 10—15 hours, but drops later—particularly at higher temperatures—to lower pH values, thus showing that secondary reactions are subsequently encountered. (The effect cannot be due to absorption of carbon dioxide, as the solutions were kept under pure nitrogen.) The interaction with the " amino "-group of adenine takes about 2 days to reach equilibrium and, once equilibrium has been reached, the pH-depression values remain constant for several days.

The linear relation of the plots of antilog(pH elevation) against formaldehyde concentration at 20° and 40° in Fig. 8 shows that one molecule of formaldehyde reacts with one molecule of neutral uracil.

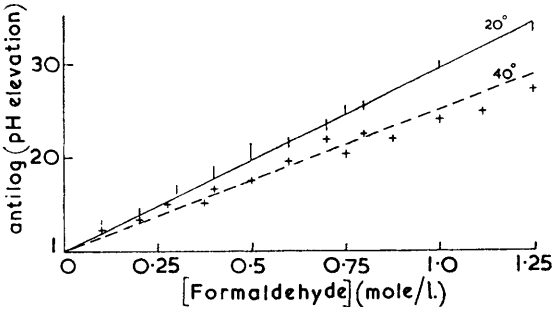


FIG. 8. Variation of the antilog (pH elevation) with [formaldehyde] in the uracil–formaldehyde reaction at 20° and 40°. [Uracil] from 0·02M to 0·015M; initial pH values are 8—8·4.

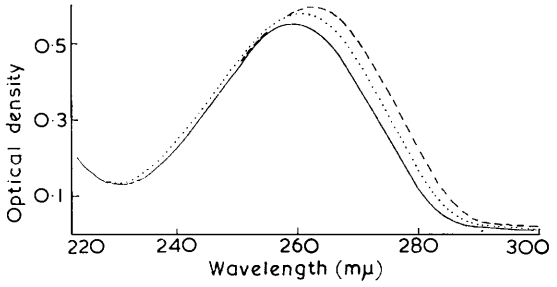


FIG. 9. Variation of the absorption spectrum of uracil–formaldehyde solution in acetic acid–acetate buffers ($I = 0{\cdot}1$) at pH 4·7 at 30°; [uracil] = $6{\cdot}67 \times 10^{-5}$M. The solutions were incubated at 30° for 60 hr. before measurements were made in 1-cm. silica cells. —— uracil only; · · · +0·267M-formaldehyde; — — — +2M-formaldehyde.

The L_1 values computed from the plots are $1{\cdot}9 \pm 0{\cdot}15$ and $1{\cdot}5 \pm 0{\cdot}2$ kcal mole^{-1} at 20° and 40°. The heat of the reaction was computed to be $2{\cdot}3 \pm 2$ kcal. mole^{-1}.

(*c*) *Spectrophotometry.* The interaction of formaldehyde with uracil was examined spectrophotometrically, the same procedures being used as for the adenine–formaldehyde interaction. The results show conclusively that changes (see Fig. 9) in optical density and bathochromic shifts take place. These are similar to those noted for the adenine–formaldehyde reaction, but are considerably smaller. This result is to be expected from comparison of L_1 values of the two reactions determined by the pH-variation method. The smallness of the changes in optical density resulted in accurate spectrophotometric evaluation of L_1 values being impracticable.

DISCUSSION

FORMULATION OF THE ADENINE–FORMALDEHYDE REACTION

Elucidation of the site of formaldehyde attachment to the adenine molecule is assisted by the following considerations:

(a) *Each formaldehyde molecule requires one hydrogen atom to be present on the reacting*

nitrogenous group. This is shown by the general observation that basic free amino-groups can react with both one and two formaldehyde molecules (for references see French and Edsall [1]). Acid imino- and mercapto-groups react with only one molecule of formaldehyde (Lewin [15]). For this and other reasons the formulation of the reversible interaction in terms of a Schiff's base, *e.g.*, (A) is not nowadays adopted, instead the reactions are formulated as (B)—(D).[1]

$$-NH_2 + H{\cdot}CHO \rightleftharpoons -N{=}CH_2 \quad (A)$$
$$>NH + H{\cdot}CHO \rightleftharpoons >N{\cdot}CH_2{\cdot}OH \quad (B)$$
$$-NH_2 + H{\cdot}CHO \rightleftharpoons -NH{\cdot}CH_2{\cdot}OH \quad (C)$$
$$-NH_2 + 2H{\cdot}CHO \rightleftharpoons -N(CH_2{\cdot}OH)_2 \quad (D)$$

(*b*) *The variation of* pH *with formaldehyde takes place only in the ionization range of the group with which reaction takes place.* The pH variations result from superimposition of the formaldehyde reaction on the original acid–base equilibrium and consequent displacement. Theoretically, they can be formulated as

pH depressions: $-NH_3^+ \rightleftharpoons H^+ + -NH_2$; $-NH_2 + H{\cdot}CHO \rightleftharpoons >N{\cdot}CH_2{\cdot}OH$
pH elevations: $H^+ + >N^- \rightleftharpoons >NH$; $NH + H{\cdot}CHO \rightleftharpoons >N{\cdot}CH_2{\cdot}OH$

This interpretation is shown by reference to the reaction of formaldehyde with mercaptoacetic acid and with purine.

Mercaptoacetic acid. Addition of formaldehyde to mercaptoacetic acid at pH 3—11 does not cause elevation of pH in the dissociation range of the carboxyl group ($pK_1 \cong 4{\cdot}7$) but it does cause considerable elevation of pH in the dissociation range of the thiol group ($pK_2 \cong 10{\cdot}2$). Spectrophotometrically, the reaction with formaldehyde can be demonstrated over the entire pH range examined.[16]

Purine. Addition of formaldehyde to purine at pH 3—11 causes no variation of pH in the first-ionization range ($pK_1 \cong 2{\cdot}4$), which is due to the association of a ternary nitrogen with H^+, and a clear elevation of pH in the ionization range of the acid-imino-group ($pK_2 \cong 8{\cdot}9$). Again, the reaction of formaldehyde with purine can be demonstrated spectrophotometrically over the entire pH range [16] (see also Fig. 7).

(*c*) *The pH-variation method is concerned only with formaldehyde attachment and protonation on the same site.* This follows from the above considerations and also from the fact that elimination of a particular ionizable group capable of interaction with formaldehyde results simultaneously in the disappearance of the particular pK value and the associated pH variation. This can be illustrated by the following.

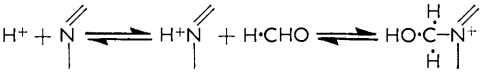
Glycine and acetic acid. Elimination of the amino-group, by substitution of H for amino, results in disappearance of the second dissociation constant of glycine ($pK_2 \cong 9{\cdot}8$) and the associated formaldehyde pH depression.

Adenine, purine, and adenosine. Titration of adenine shows that addition of formaldehyde results in depression of pH in the region of the first dissociation constant ($pK_1 \cong 4{\cdot}1$) and an elevation of pH in the region of the second dissociation constant ($pK_2 \cong 9{\cdot}8$).[15,17] Elimination of the amino-group of purine, by substitution of H for amino, results in disappearance of the original pK_1 of the basic " amino "-group and its associated pH depression. Elimination of the H-9, by ribose substitution, results in disappearance of the corresponding pK_2 and the associated pH elevation.

(*d*) *For formaldehyde depression to take place, a covalently bound hydrogen must be attached to the site of reaction.* (i) We have seen in (*a*) that one hydrogen atom must be present at the site of reaction for formaldehyde attachment to be possible. We can now add a further limitation, namely, that pH depressions cannot result from reaction of

[15] Lewin, *Biochem. J.*, 1956, **64**, 31P.
[16] Lewin, unpublished observations.
[17] Part I, *J.*, 1962, 1462.

formaldehyde with a tertiary nitrogen to which a proton is attached (E), because the reaction results in removal of H^+ ions and therefore in pH elevation.



(E)

(ii) Further, the observation that addition of formaldehyde in the first dissociation range ($pK_1 \cong 2{\cdot}4$) of purine does not result in a variation of pH shows that the above reaction is insignificant.

Allocation of Site of Acid Dissociation and Reaction.—Two related approaches, based on the above criteria, will be used to show that the formaldehyde pH variation method selects N-10 as the site of reaction:

(1) *Selection of* N-10 *as the site of first acid dissociation* ($pK_1 \cong 4{\cdot}1$). The choice can be appreciated on correlation of data from the solid state with those from solution.

(*i*) *Solid state.* The structure of solid adenine hydrochloride has been examined by *X*-ray methods by Cochran [18] and by Broomhead.[19] Cochran concluded that there are two hydrogen atoms situated on N-10, one " hydrogen atom covalently bound to N-1 " and one on N-9. The positive charge on the molecule was considered as distributed over N-10, N-9, and N-1.

We would stress that while the distribution of electron deficiency (or its counterpart of positive-charge distribution) can be readily pictured as taking place over the whole molecule, the potential hydrogen ion—present before ionization as a covalent hydrogen atom or as a proton bound to an electron pair associated with another atom—must originate on a definite site or be bonded between two definite sites; and it is this position which is to be selected.

Cochran's [18] results appear to show that N-1 carries rather more electron density than any of the other three, N-9 having the lowest density and therefore being the most positively charged.[20] It is logical to accept that the higher the electron density the less likely is the hydrogen atom to ionize as an H^+. Hence, if we assume that the results from the solid state can be extended to aqueous solution, H-1 is the least likely to ionize. Of the two remaining positions, H-9 is more positively charged than either of the two H-10. However, the overall electron deficiency on the 10-NH_2 group is made up of the contributions of the two electron-deficient H-10 atoms. Hence, the electron deficiency on the 9-NH group may well be below that of the N-10 group.

(*ii*) *Solution.* The titration curve of adenine shows two acid–base dissociations ($pK_1 \cong 4{\cdot}1$; $pK_2 \cong 9{\cdot}8$); pK_2 is generally allocated to the acid dissociation of 9-NH (based on comparisons of p*K*'s of different compounds by Taylor,[21] and on an associated formaldehyde pH elevation [17]). This leaves pK_1 as representing the dissociation of the 10-NH_2^+ group. [Hence, by criterion (*c*), formaldehyde should react at N-10.]

(2) *Selection of* N-10 *as site of reaction by elimination of all other nitrogen sites.* There are five nitrogen sites in the adenine molecule, namely, 10, 1, 3, 7, 9.

(i) *Exclusion of positions* 3 *and* 7. These nitrogen atoms do not possess a covalent hydrogen and therefore cannot give rise to pH depression, nor are they likely to react [criterion (*d*)].

(ii) *Exclusion of position* 1. Suppose the NH group at position 1 does not ionize in the pH region examined. Then if it reacts with formaldehyde; whether as a part of cationic adenine or of neutral adenine, by criterion (*b*) no pH variation should be obtained.

Suppose that N-1, despite the above considerations is the site of acid–base dissociation, thus $N + H^+ \rightleftharpoons NH^+$ (in disagreement with Cochran's conclusions that the group is NH), then by criterion (*d*) reaction with formaldehyde should result in pH elevation and not depression.

[18] Cochran, *Acta Cryst.*, 1951, **4**, 81.
[19] Broomhead, *Acta Cryst.*, 1948, **1**, 324; 1951, **4**, 92.
[20] Cochran, personal communication.
[21] Taylor, Thesis, 1946, London University; *Nature*, 1949, **164**, 1750.

Experimentally, no formaldehyde pH variation is demonstrable in the range of pK_1 ($\cong$ 2·4) of purine that is concerned with protonation of a ternary nitrogen atom.

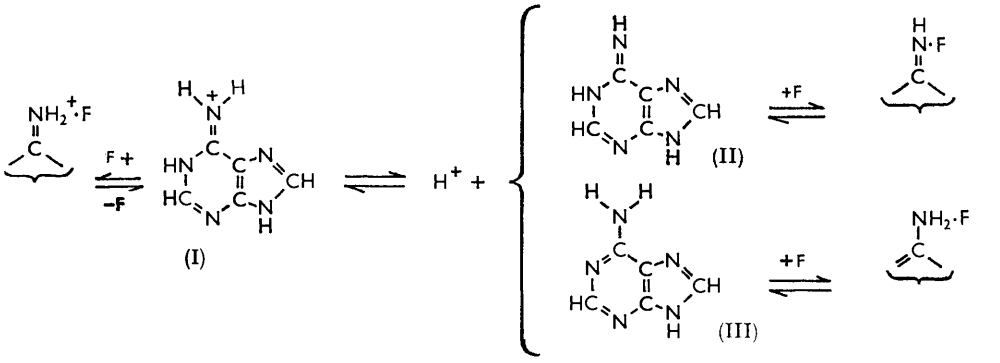
(iii) *Exclusion of position* 9. Formaldehyde addition in the pK range ($\cong$ 9·8) of the NH group at position 9 results in pH elevation and not depression.

Hence, only position 10 remains as the site of reaction with formaldehyde. The minor reaction, namely that of formaldehyde with cationic adenine, is associated with a potential pH elevation which lowers the potential pH depression of the reaction with neutral adenine to that experimentally observed. This intrinsic pH elevation also depends on acid dissociation and reaction taking place on *one* nitrogen atom; and it might be argued that this nitrogen need not be N-10. However, this hypothesis can be discounted for the following reasons: requirement of two pK values in the acid titration region, for which there is no experimental evidence; the unlikely requirement that the NH_2^+ group at position 10, which can be regarded as possessing one covalent hydrogen, should not react with formaldehyde, while a ternary NH^+ should; and no pH variation with formaldehyde could be demonstrated in the first acid dissociation range of purine and in those of other ternary nitrogen compounds examined.[16]

The conclusion is then that whether neutral adenine or cationic adenine is involved, the method of pH variation with formaldehyde allocates protonation and reaction to the N-10 site.

Consider now the experimental conclusion that only one molecule of formaldehyde reacts with one molecule of adenine whether it is cationic or neutral. This requirement can be satisfied readily in the cationic reaction by the explanation that only covalent hydrogen is capable of significant reaction with formaldehyde. If neutral adenine were to exist solely in the imino-form (II)—which results directly from ionization of the cation—only one hydrogen would be available for reaction and the requirement of reaction with only one mole of formaldehyde would be satisfied. However, it is generally recognized that neutral adenine in solution may exist in both the amino- and imino-forms. Further, the following findings favour the interpretation that the amino-form, and not the imino-form, is prevalent in solution: the qualitative resemblance between the ultraviolet spectra of aminopurines and those of the dimethylaminopurines (Mason [22]); the 6-amino-9-methylpurine spectrum possesses in the near-infrared two bands due to N–H stretching vibrations separated by *ca.* 100 cm.$^{-1}$, a characteristic of primary amines (Mason [23]); the close similarity of the spectra of sodium adenylate and those of the 6-dimethylamino-derivative of adenosine (Steiner and Beers [24]); X-ray measurements on nucleic acid result in the formulation of the adenine residue as involving the amino-form (Pauling and Corey [25]).

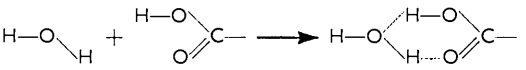
The overall preliminary picture of the formaldehyde–adenine reaction may be:



[22] Mason, *J.*, 1954, 2071.
[23] Mason, " The Chemistry and Biology of Purines," CIBA Foundation Symposium, 1957, p. 71.
[24] Steiner and Beer, " Polynucleotides," Elsevier Publ. Co., 1961, p. 29.
[25] Pauling and Carey, *Arch. Biochem. Biophys.*, 1956, **65**, 164.

If the predominance of the amino-form (III) is accepted, the requirement of reaction with one mole of formaldehyde can be met by assuming that only the imino-form (II) reacts, or that only one of the 10-H atoms of (III) is available for reaction, the other being bonded elsewhere so strongly that it is incapable of reaction. There does not appear to be any reason why the amino-form should be barred from reaction. Consequently, it is necessary to explain how one of the 10-H atoms can be so strongly bonded as to make it unavailable for reaction with formaldehyde. We propose to advance formulations which involve strong bonding *via* associated water molecules to another part in the adenine molecule.

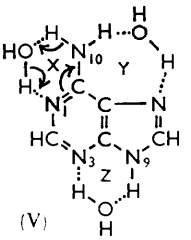
It was shown by Broomhead [18] that in adenine hydrochloride semihydrate, the water is hydrogen bonded. Also Cochran's [19] results suggest that 1-H is bonded on to the oxygen of a water molecule as the distance (1·94 Å) being significantly less than the sum of the Van der Waal's radii of oxygen and hydrogen, which is (1·2 + 1·4) = 2·6 Å. It is also known that adenine crystallizes with three water molecules,[26] and it is therefore more than likely that it is hydrated in solution. Hydration is known to be dependent on hydrogen bonding in many cases; for example, the accepted hydration of the carboxyl group can be represented by the resonance of the hydrated structure, as well as its hydrogen



(IV)

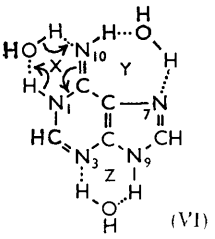
bondings, contributing to its stability. In the adenine molecule, the three associated water molecules can be represented as being hydrogen bonded in the (x), (y), and (z) configurations of the hydrated form (V) of (III) and the corresponding hydrated form (VI) of (II). From the same overall structural configuration, it will be seen that there are two alternative resonating structures each of which is capable of contributing strongly to its particular configuration. The strength of the resonance-supported bond may well be such as to make N-10 unavailable for interaction with formaldehyde.

(*a*) *Unavailability of the hydrogen at position* 10 *bonded towards* N-1. By analogy with the resonance of the hydrated carboxyl group, the hydrogen-bonded structure in (x)



(V)

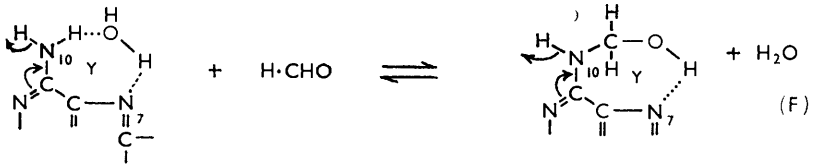
Hydrated form of (III)



(VI)

Hydrated form of (II)

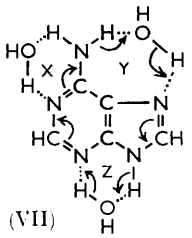
should be quite stable as compared to the non-resonating hydrogen bonded structures in (y) and (z). This representation and the unavailability of the 10-H bonded in the (x) configuration being assumed, the monomolecularity of the formaldehyde reactions can be represented as substitution of water by formaldehyde in the (y) position. Thus,

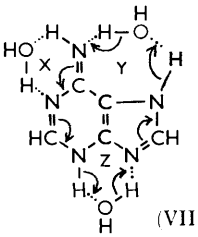


(F)

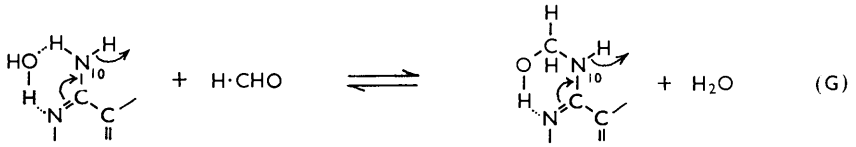
[26] " Handbook of Chemistry and Physics," The Rubber Publ. Co., 37th edn., 1955—56, p. 719.

(*b*) *Unavailability of the hydrogen at position* 10 *bonded towards* N-7. An alternative resonating structure involving both the (Y) and (Z) positions is shown below:


(VII)


(VIII)

This resonance involves the hydrogen on N-10 in the (Y) configuration and excludes the previous resonance (*a*). In this case, the 10-H bonded towards N-7 (and the 9-H bonded towards N-3) are held strongly, and could be considered unavailable for formaldehyde reaction, while the remaining 10-H bonded towards N-1 is comparatively weakly held and therefore capable of interaction with formaldehyde, thus


(G)

It should be noted that resonance (a) in the (X) position and resonance (b) in the (Y–Z) positions while mutually exclusive in the same molecule may take place simultaneously in different molecules.

The reaction of the imino-group at position 9 with formaldehyde—demonstrated by pH elevation in the alkaline pH region and by spectrophotometry of the purine–formaldehyde reaction and by the deviations in the adenine–formaldehyde reaction in the acid pH region—is compatible with resonance in (X), but does not necessarily exclude the (Y–Z) resonance. Exclusion of the latter can be expected to occur in adenosine and other ribose-substituted purines. Related influences in the formaldehyde interactions and in nucleic acid denaturation will be considered separately.

Let us now consider briefly the conclusions of other investigators concerning the site of reaction and protonation.

(a) *Site of reaction.* The conclusion that formaldehyde reacts with the N-10 site is in agreement with the conclusions of several investigators. Thus, Steiner and Beers [24] noted that inosine monophosphate, which does not possess an amino-group, does not give the formaldehyde depression; these authors also titrated polyadenylic acid in presence of formaldehyde, and related the reaction in terms of the amino-group. Hoard [27] followed the formaldehyde–cytidylic acid reaction in terms of the amino-group. Haselkorn and Doty [28] allocated the site of formaldehyde reaction with several mono- and poly-nucleotides to the amino-group.

(b) *Protonation.* Bendich,[29] Cohn,[30] and Jordan [31] allocate the position of H^+ in adenine to the amino-group at N-10. There is, however, a school of thought [32,33,24] which considers that the protonation should be allocated to the N-1 position and refer to Jardetzky and Jardetzky [34] and to Cochran [18] for major support. Thus, Jardetzky and

[27] Hoard, *Biochim. Biophys. Acta*, 1960, **40**, 62.
[28] Haselkorn and Doty, *J. Biol. Chem.*, 1961, **236**, 2738.
[29] Bendich, in " The Nucleic Acids," ed. Chargoff and Davidson, Academic Press, New York, Vol. I, 1955, p. 114.
[30] Cohn, ref. 29, p. 227.
[31] Jordan, " The Chemistry of Nucleic Acids," Butterworths, 1960.
[32] Pullman and Pullman, *Nature*, 1961, **189**, 725.
[33] Rich, Davies, Crick, and Watson, *J. Mol. Biol.*, 1961, **3**, 71.
[34] Jardetzky and Jardetzky, *J. Amer. Chem. Soc.*, 1959, **82**, 222.

Jardetzky,[34] using nuclear magnetic resonance, investigated the structures of several purines, pyrimidines, and their ribosides and concluded that their " results can be understood in terms of a definite tautomeric structure of adenosine in which the hydrogen is attached to N-1 and the positive charge is distributed among the nitrogen atoms of the adenine ring. Further evidence for such a structure is provided by the *X*-ray crystallographic data for adenine hydrochloride according to which the proton is located on N-1 rather than N-10 of the amino-group," reference being made to Cochran [18] and to Broomhead.[19] We have the following comments:

(1) The first part of the quoted statement is compatible with Cochran's conclusions. The last part ascribing to Cochran and to Broomhead the allocation of a proton to N-1 rather than N-10 can be interpreted as signifying an assignation of a proton and not a hydrogen atom; such an interpretation is erroneous. Neither Cochran nor Broomhead advocated the allocation of a positive charge to N-1 rather than N-10; indeed, the reverse interpretation is correct.

Cochran [18] states specifically that " In the pyrimidine ring...there is a hydrogen atom covalently linked to N-1." No electron deficiency is given for 1-NH, but a deficiency of 0·4 electrons has been determined for the 10-NH_2 site. Indeed, Cochran is of the opinion that " 1-H carries rather more electron density than any of the other three " namely 10-H, 10-H′, and 9-H. Since the higher the electron density, the greater is the energy required for ionization, H-1 is logically the least likely to ionize.

The confusion concerning allocation of H^+ seems to have arisen by the use—by the Jardetzkys, and by other authors in the case of polyadenylic acid [33] and of adenine [24]—of the term " proton " where they should have used " hydrogen atom." [20]

Broomhead [19] specifically allocates protonation (the positioning of the positive charge) to N-10 or N-7 but excludes N—1.

(2) Previous to the quoted statement, the Jardetzkys state that " the NH_2 of adenine and cytosine as well as the NH protons have not been obtained in either acidic or basic solution. These protons may be exchanging very rapidly with the solvent or their peaks may be covered by the broad solvent resonance line."

It must be emphasized that we do not question the applicability of nuclear magnetic resonance spectroscopy to allocation of hydrogen nuclei. However, the absence of the amino- and imino-proton peaks in the case of adenine, and the uncertainty as to the cause, limit effectively the weight to be attached to extension of the Jardetzkys' assumed conclusions as to the location of H^+ in adenosine to the case of adenine.

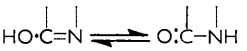
Pullman and Pullman,[32] while allocating the formaldehyde reaction to the amino-group of adenine, also allocated protonation and alkylation to position 1. They base their allocation on the work of the Jardetzkys [34] and also refer to the work of Brookes and Lawley.[35] We have already commented on the limitation of the weight to be attached to the interpretation of " proton " allocation in the Jardetzkys' statement, particularly where unsubstituted adenine is involved. Brookes and Lawley's [35] work involved the irreversible methylation of adenosine at N-1 and N-3. This allocation is not disputed, but the correctness of its extension to allocating a positively-charged hydrogen is more than questionable, particularly so because Brookes and Lawley were dealing with irreversible reactions while the formaldehyde reaction recorded in this investigation was concerned with adenine and was reversible.

The Uracil–Formaldehyde Reaction

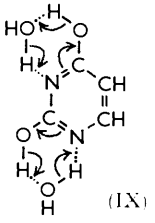
In agreement with criteria (*a*), (*b*), and (*c*) of the pH variation method and with the established observations [16,36] that hydroxyl groups do not show any pH variation on formaldehyde addition, the site of formaldehyde attachment can be assigned to the –CO·NH group responsible for ionization ($pK \simeq 9{\cdot}5$).

[35] Brookes and Lawley, *J.*, 1960, 539.
[36] Birch and Harris, *Biochem. J.*, 1930, **241**, 1086.

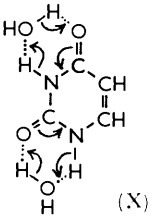
The comparatively small value of the equilibrium constant for the uracil–formaldehyde 1 : 1 reaction cannot be related to those involved in the amino–adenine reaction with formaldehyde because of the opposite acidic and basic properties which result, respectively, in pH depression and pH elevation. However, by analogy with hydrated adenine and other hydrogen-bonded hydrations, we would advocate that the structural-hydrated uracil analogues of the tautomerism



be formulated as



(IX)



(X)

As interaction with formaldehyde takes place only on a nitrogen atom where a covalently bound H is found, reaction can be restricted to form (X). The interaction of only one formaldehyde molecule can be demonstrated by the pH-elevation results because an interaction with the second, non-dissociating acid imido-group, present in the uracil molecule, cannot express itself in a pH variation.

An hypothesis that the site of reaction could be C-5, in accord with the results obtained by Cline, Fink, and Fink [37] for the uracil–formaldehyde reaction, must be rejected because the experimental conditions of the present investigation and the nature of the present reaction are fundamentally different from those of Cline, Fink, and Fink. The investigation recorded in this paper was carried out in the slightly alkaline region of pH 8—8·8 at 20—40°; it was reversible and equilbrium was quickly obtained. Cline, Fink, and Fink [37] used extreme conditions, *e.g.*, 0·42N-potassium hydroxide—the pH of which must be >13—and kept the mixture for 73 hours at 50°. Alternatively, they used 0·5N-hydrochloric acid—the pH of which must be <1—and refluxed for 25 hours. The product of their reaction was identifiable by paper chromatography, thus showing the reaction to be irreversible. Paper chromatography of the adenine–formaldehyde and uracil–formaldehyde solutions in this investigation failed to show the presence of any spots additional to those of the reactants. This is to be expected because the reactions are reversible; and as soon as the conditions of the reaction were reversed in the process of paper chromatography, the product was bound to break down.

The significance of these findings and structural formulations in the application to the interaction of formaldehyde with nucleic acids will be considered elswhere.

Experimental

Adenine and uracil (California Foundation for Biochemical Research, and Nutritional Biochemical Company) were found to be chromatographically pure. Formaldehyde (British Drug Houses, and Hopkin & Williams Ltd.) was purified by ion exchange, assayed by the bisulphite method, and checked by titration. Sodium hydroxide solutions were carbonate-free. All solutions were incubated anærobically under nitrogen (free from carbon dioxide and oxygen), and all measurements were carried out under the same conditions. Solutions used for pH measurements contained toluene for bacteriostatic purposes. pH measurements were made with Doran and Radiometer pH meters, lithium-glass electrodes, internal-bridge calomel electrodes,[38] and thermojacket pH vessels.[39] Glass electrodes were calibrated by use of potassium hydrogen phthalate and sodium borate.[40] Glass containers (Pyrex) were cleaned

[37] Cline, Fink, and Fink, *J. Amer. Chem. Soc.*, 1959, **81**, 2521.
[38] Lewin, *J. Sci. Instruments*, 1954, **31**, 303.
[39] Lewin, *Chem. and Ind.*, 1953, 1193.
[40] Nat. Bur. Standards, U.S. Letters Circular, August 19th, 1950; Lewin, *Lab. Practice*, 1955, 441.

and steamed.[41] Spectrophotometric measurements were made on manual Unicam S.P. 500 and the DK-2 Beckman recording spectrophotometers. Constant temperatures (±0·03°) were employed throughout.

I thank Mr. M. Freeman and Mr. E. R. Davis for technical assistance in the re-checking of some of the experiments. Thanks are due to the D.S.I.R. for assistance.

Appendix I

Mathematical Formulation of the Reaction of Formaldehyde with Neutral Adenine and its Cation.

(A) *The* pH-*Depression Approach.*—The following have been taken into consideration for the scheme given on p. 793:

(a) *Law of mass action requirements.*

$$K = [\rangle NH][H^+]/[=NH_2^+] \quad (1)$$

$$L_1^+ = [=NH_2{}^+F]/[=NH_2^+][F] \quad (2)$$

$$L_1 = [\rangle NH{\cdot}F]/[\rangle NH][F] \quad (3)$$

(b) *Electrical neutrality requirements.* In the initial state (denoted by i), *i.e.*, in the absence of formaldehyde,

$$[=NH_2^+]_i + [H^+]_i = [Cl^-]_i + [OH^-]_i, \quad (4)$$

where $[Cl^-]$ represents the concentration of the anion arising from acid addition. In the approximate pH range 4—5 $[OH^-]$ does not exceed 10^{-9}; the adenine concentration is to be not below 10^{-3}, while the acid concentration is not to be below *ca.* 10^{-4}. Hence, $[OH^-]$ can be neglected in comparison with $[Cl^-]$, and the last equation reduces to

$$[=NH_2^+]_i + [H^+]_i = [Cl^-]_i. \quad (5)$$

In the presence of formaldehyde, when equilibrium (denoted by e) has been obtained,

$$[=NH_2{}^+F]_e + [=NH_2^+]_e + [H^+]_e = [Cl^-]_e + [OH^-]_e \quad (6)$$

As before, this can be reduced to

$$[=NH_2{}^+F]_e + [=NH_2^+]_e + [H^+]_e = [Cl^-]_e. \quad (7)$$

If we compare only those cases where

$$[Cl^-]_e = [Cl^-]_i \quad (8)$$

and combine equations (5), (7), and (8), we obtain

$$[=NH_2^+]_i + [H^+]_i = [=NH_2{}^+F]_e + [=NH_2^+]_e + [H^+]_e \quad (9)$$

(c) *Total requirements.* The total of the concentrations of the various forms of adenine before and after combination with formaldehyde are equal. Thus,

$$[=NH_2^+]_i + [\rangle NH]_i = [=NH_2^+]_e + [=NH_2{}^+F]_e + [\rangle NHF]_e + [\rangle NH]_e. \quad (10)$$

From equations (9) and (10) we obtain

$$[\rangle NH]_i - [H^+]_i = [\rangle NHF]_e + [\rangle NH]_e - [H^+]_e, \quad (11)$$

and, using equations (1) and (3), we have

$$\frac{[=NH_2^+]_iK - [H^+]_i}{[H^+]_i} = \frac{[=NH_2^+]_e\,K}{[H^+]_e} + \frac{L_1[=NH_2^+]_eK\,[F]_e}{[H^+]_e} - [H^+]_e. \quad (12)$$

Putting $\Delta pH = (pH_i - pH_e)$ and neglecting $[H^+]_i^2$, we have

$$\text{antilog}\Delta pH = \frac{[=NH_2^+]_e\{1 + L_1[F]_e\} - \{[H^+]^2_e/K\}.}{[=NH_2{}^+F]_e + [=NH_2^+]_e + [H^+]_e - [H^+]_i} \quad (13)$$

[41] Lewin, *Lab. Practice*, 1957, 213.

Further appropriate substitution and rearrangement give

$$\text{antilog}\Delta\text{pH} = \frac{1 + L_1[\text{F}]_e - \{[\text{H}^+]_e^2/K[{=}\text{NH}_2^+]_e\}}{1 + L_1^+[\text{F}]_e + \{[\text{H}^+]_e - [\text{H}^+]_i\}/[{=}\text{NH}_2^+]_e} \quad (14)$$

For the expression $\{[\text{H}^+]_e^2/K[{=}\text{NH}_2^+]_e\}$ to be negligible compared with the term $\{1 + L_1[\text{F}]_e\}$, the values of equilibrium pH, K, adenine concentration, L_1, and $[\text{F}]_e$ should be as high as possible.

With adenine, attainment of these conditions is rather limited. Its low solubility—in practice the highest concentration utilizable is 4×10^{-3}M—and associated low buffering capacity mitigate against steady pH measurements at pH value much higher than *ca.* 5·3. At pH 5 the value of the expression $\{[\text{H}^+]_e^2/K[{=}\text{NH}_2^+]_e\}$ is *ca.* 10^{-3}, while at pH 4 it is 0·1.

It will have been noted in the Results section that the lowest value of L_1 (at 40°) is over 13. Hence, if we use formaldehyde concentrations greater than 0·25, the minimum value of the expression $\{1 + L_1[\text{F}]_e\}$ is *ca.* 4·25, and, if the equilibrium pH is restricted to values higher than pH 4, the *highest* error in neglecting $\{[\text{H}^+]_e/K[{=}\text{NH}_2^+]_e\}$ is reduced to less than *ca.* 1%, and the error decreases progressively with increase in formaldehyde concentration. It can also be computed that when these limitations are adhered to, and a value of L_1^+ of *ca.* 0·9 is assumed, neglect of the expression $\{[\text{H}^+]_e - [\text{H}^+]_i\}/[{=}\text{NH}_2^+]_e$ in the denominator results in an error of a similar magnitude.

Hence, provided that the above restrictions are adhered to, equation (14) can be reduced to the form

$$\text{antilog}\Delta\text{pH} = (1 + L_1[\text{F}]_e)/(1 + L_1^+[\text{F}]_e). \quad (15)$$

Since the formaldehyde concentration initially present is at least a thousand times greater than the adenine concentration, we may substitute $[\text{F}]_i$ for $[\text{F}]_e$.

(B) *Spectrophotometry.*—The following have been taken into consideration when using the scheme given on p. 793:

(i) The optical absorptions of neutral adenine and its cation and the products of their reaction with formaldehyde. Any contribution of the un-ionized acid 9-imino group and its formaldehyde derivative to optical absorption has been neglected.

The optical density of the adenine–formaldehyde mixture, A_{mix}, at a specified temperature, pH, and wavelength is equal to the sum of the contributions of the individual components. Thus,

$$A_{\text{mix}} = \text{A}_1 + \text{A}_2 + \text{A}_3 + \text{A}_4, \quad (16)$$

Hence, where 1 = $\rangle$NH, 2 = $=\text{NH}_2^+$, 3 = $\rangle$NHF, and 4 = $=\text{NH}_2^+\text{F}$.

$$A_{\text{mix}} = \varepsilon_1 C_1 + \varepsilon_2 C_2 + \varepsilon_3 C_3 + \varepsilon_4 C_4 \quad (17)$$

where ε is the molar absorption coefficient.

(ii) *Law of mass action.* Utilizing equations (1), (2), and (3) for substitution into the equation

$$C_1\text{H} = [\rangle\text{NH}]/\{[\rangle\text{NH}] + [{=}\text{NH}_2^+] + [\rangle\text{NHF}] + [{=}\text{NH}_2^+\text{F}]\} \quad (18)$$

(where C without suffix represents the overall concentration) and parallel equations, followed by substitution into equation (17) and rearrangement, we obtain

$$A_{\text{mix}} = \frac{\varepsilon_1 C + \varepsilon_3 L_1[\text{F}]C + [\text{H}^+]\{\varepsilon_2 + \varepsilon_4 L_1^+[\text{F}]\}C/K}{1 + L_1[\text{F}] + [\text{H}^+]\{1 + L_1^+[\text{F}]\}/K} \quad (19)$$

In very *acid* conditions, say pH 1·5, $[\text{H}^+]/K > 500$; then the last term in the numerator exceeds considerably the combined values of the first two terms. The same applies to the denominator, and the last equation after cancellation, rearrangement, and substitution of

$$\Delta A_{\text{ac}} = A_{\text{mix}} - (\varepsilon_2 C), \quad (20)$$

reduces to

$$\Delta A_{\text{ac}}/[\text{F}] = \varepsilon_2 L_1^+ C - L_1^+ A_{\text{mix}} \quad (21)$$

This equation indicates that a plot of $\Delta A_{\text{ac}}/[\text{F}]$ against A_{mix} will be straight, with slope of $-L_1^+$ and intercept of $\varepsilon_2 L_1^+ C$. Alternatively $[\text{F}]/\Delta\text{A}_{\text{ac}}$ plot against $[\text{F}]$ yields an intercept of the reciprocal of L_1^+.

In the pH region greatly *above* the pK value, say pH 7·1, $[H^+]/K \cong 10^{-3}$. Then the last term in the numerator of equation (19) becomes negligible compared with the first two terms. The same consideration applies to the denominator. Finally, with aid of

$$\Delta A_{neut} = A_{mix} - \varepsilon_1 C \quad (22)$$

we have

$$\Delta A_{neut}/[F] = \varepsilon_3 L_1 C - A_{mix} L_1. \quad (23)$$

A plot of $\Delta A_{neut}/[F]$ against A_{mix} will therefore be straight, of slope $-L_1$ and intercept $\varepsilon_3 L_1 C$.

Appendix II

Mathematical Formulation of the Reaction of Formaldehyde with Uracil in Terms of the pH Elevation.

The following were taken into consideration for the scheme on p. 798:

(*i*) *Law of mass action.*

$$K' = [N^-][H^+]/[\rangle NH] \quad (24)$$

$$L_1 = [\rangle NHF]/[\rangle NH][F] \quad (25)$$

$$L_1^- = [\rangle NF^-]/[N^-][F] \quad (26)$$

(*ii*) *Requirements of electrical neutrality.* As in Appendix I, in the absence of formaldehyde,

$$[N^-]_i + [OH^-]_i = [M^+]_i + [H^+]_i \quad (27)$$

where M^+ represents metal-ion concentration (alkali added to adjust the pH). In the approximate pH range 5—9 $[H^+]$ and $[OH^-]$ do not exceed *ca.* 10^{-5}, and if the imino-acid and metal-ion concentrations are much greater, the last equation reduces to

$$[N^-]_i = [M^+]_i. \quad (27a)$$

In the presence of formaldehyde, when equilibrium has been attained, and on comparison of only those cases in which the solutions of the acid imino-compound and of the acid imino-formaldehyde contain the same quantity of added alkali, the last equation reduces to

$$[N^-]_i = [N^-]_e + [NF^-]_e. \quad (28)$$

(3) *Total requirements.*—The sum of the concentrations of the various forms of the acid imino-compound, before and after combination with formaldehyde, are equal; thus

$$[\rangle NH]_i + [\rangle N^-]_i = [\rangle NHF]_e + [\rangle NF^-]_e + [\rangle NH]_e + [\rangle N^-]_e. \quad (29)$$

Equation (29) then reduces to

$$[H^+]_i\{1 + L_1^-[F]_e\} = [H^+]_e\{1 + L_1[F]_e\}. \quad (30)$$

By use of the expression

$$\Delta pH = pH_e - pH_i \quad (31)$$

equation (30) may be rearranged to

$$[H^+]_i/[H^+]_e = \text{antilog}\Delta pH = (1 + L_1[F]_e)/(1 + L_1^-[F]_e). \quad (32)$$

If, however, the amount of interaction of formaldehyde with the negatively charged N^- form is very small compared with that of the neutral $\rangle NH$ group, then $L_1^-[F] \ll 1$ (and when the formaldehyde concentration is very much greater than that of the acid imino-compound we have $[F]_e$ practically equal to $[F]_i$), the last equation reduces to

$$\text{antilog}\Delta pH = 1 + L_1[F]_i \quad (33)$$

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