276. *Reactions of Nucleic Acids and Their Components. Part I. The Reaction of Acid Imino- and Thiol Groups with Formaldehyde.*

By S. Lewin.

The characterisation of (acid) imino- and thiol groups by formaldehyde titration is described. Addition of formaldehyde to such groups results in elevation of pH in contradistinction to the depression encountered when (basic) amino-groups are used. Compounds examined include succinimide, hydantoin, uracil, mercaptoethanol, mercaptoacetic acid, xanthine, and adenine.

The characterisation of basic amino-groups by Sörensen titration has been applied extensively to amino-acids,[2] but comparatively little to nucleic acid components. Taylor [3] titrated cytosine with formaldehyde in the acid region; Gulland, Jordan, and Taylor [4] titrated the barium salt of thymic acid with acid and found that addition of formaldehyde caused a significant pH depression in the region below pH 5·5. Lewin, in preliminary communications,[5] suggested that the (acid) amino-groups and the thiol group might be characterised by adding formaldehyde during the acid titration; this would cause a pH elevation in contradistinction to the pH depression noted in the Sörensen titration of amino-groups. Experimental evidence is now presented in support of this. The approach will be used for discriminating between (acid) imino- and (basic) amino-groups for pK allocations to the respective groups, and will be used to elucidate the existence of imino-groups which may exist in certain compounds, *viz.*, the amide system, $\cdot CO{-}NH\cdot \rightleftharpoons \cdot C(OH){:}N{-}$.

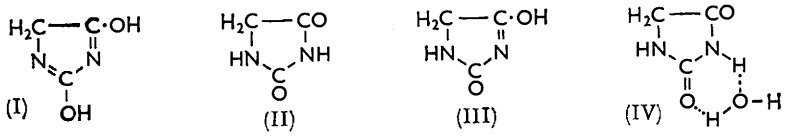
Characterisation of a basic amino-group by means of the Sörensen titration depends on the pH depression which accompanies displacement of the equilibrium $\cdot NH_2 + H^+ \rightleftharpoons \cdot NH_3^+$ to the left on superimposition of the reaction of the free amino-group with formaldehyde: $\cdot NH_2 + CH_2O \rightleftharpoons \cdot NH\cdot CH_2\cdot OH$. The proposed characterisation of (acid) imino- and thiol groups is based on displacement of the equilibria $>NH \rightleftharpoons >N^- + H+$ and $\cdot SH \rightleftharpoons \cdot S^- + H^+$ to the left on superimposition of the corresponding reversible interactions $>NH + CH_2O \rightleftharpoons \cdot N\cdot CH_2\cdot OH$ and $\cdot SH + CH_2O \rightleftharpoons \cdot S\cdot CH_2\cdot OH$.

Imino-groups.—Succinimide. The ionisation of succinimide is usually represented as

[1] Sörensen, *Biochem. J.*, 1908, **7**, 45.
[2] Cf. French and Edsall, *Adv. Protein Chem.*, 1945, **2**, 277.
[3] Taylor, Thesis, London, 1946.
[4] Gulland, Jordan, and Taylor, *J.*, 1947, 1131.
[5] Lewin, *Biochem. J.*, 1956, **64**, 30P, 31P.

removal of a proton from imino group. Consequently, interaction of the un-ionised succinimide fraction with formaldehyde, in a pH region where the ionisation equilibrium exists, should result in elevation of pH in accordance with the above scheme. Fig. 1 shows that this is the case. As succinimide tends to be hydrolysed in aqueous solution, it is essential to carry out the titrations as rapidly as possible.

Hydantoin. Hydantoin is represented in the literature as form (I) or (II) but presumably may exist also as (III). According to Albert,[6] the formulæ are without



appropriate physicochemical evidence. Fig. 2 shows that addition of formaldehyde in the titration of hydantoin results in elevation of the pH. Thus, at least some of the hydantoin must ionize as an acidic imino-form. It must be emphasised that this interpretation of the pH elevation does not exclude tautomerism between the above three forms, nor does it exclude the presence of forms in which a water molecule is hydrogen-bonded to the

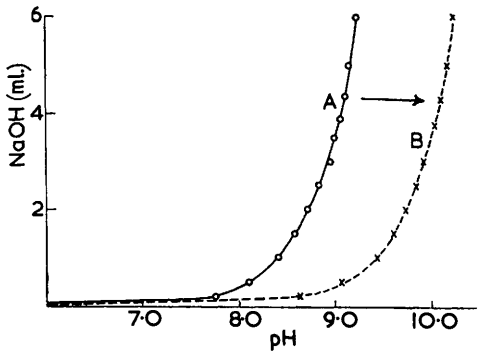


FIG. 1. Titration of 0·05M-succinimide (100 ml.) in presence and absence of aqueous formaldehyde at 19·5° with 0·183N-sodium hydroxide.

(A) Plus 1 ml. of water. (B) Plus 1 ml. of 38% formaldehyde.

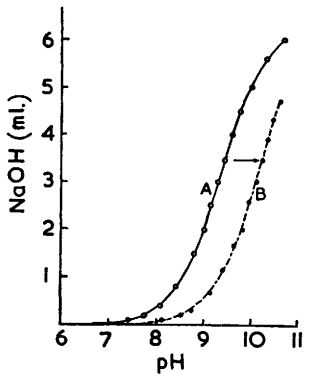


FIG. 2. Titration of 0·02M-hydantoin (50 ml.) in absence and presence of formaldehyde at 20° with 0·183N-sodium hydroxide.

(A) Plus 2 ml. of water. (B) Plus 2 ml. of 38% formaldehyde.

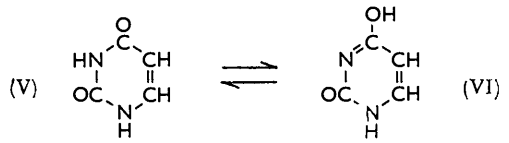
reacting group, *e.g.*, (IV), as the superimposition of the formaldehyde reaction on the overall equilibrium should result in elevation of the pH.

Uracil. Uracil is usually represented as existing in tautomeric equilibrium (V) ⇌ (VI). Titration of uracil (Fig. 3) shows that addition of formaldehyde results in a pH elevation. As in the above case, therefore, a form (or forms) exists possessing an acidic imino-group, but this does not exclude the presence of other tautomeric or hydrated groups. Titration of xanthine also showed a pH elevation in the region of pH 6·3—8.

Basically, the interpretation that the pH elevation is due to the interaction of the formaldehyde with an imino-group, and not with the alternative tautomer involving the hydroxy-group, depends on the conclusive evidence that compounds containing acid hydroxy-groups, such as phenol, do not show pH variations or changes in other physicochemical properties, on addition of formaldehyde (Birch and Harris [7]) whereas those possessing amino- or imino-groups do. The results have been confirmed in this laboratory.

[6] Albert, " Heterocyclic Chemistry," The Athlone Press, University of London, 1959, p. 153.
[7] Birch and Harris, *Biochem. J.*, 1930, **241**, 1086.

Thiol Groups.—Mercaptoethanol. The pH elevation encountered on addition of formaldehyde to mercaptoethanol is illustrated in Fig. 4.



Mercaptoacetic acid. Calcium mercaptoacetate was titrated with hydrochloric acid in absence and presence of formaldehyde. Fig. 5 shows that over the pH range of pH 8—11

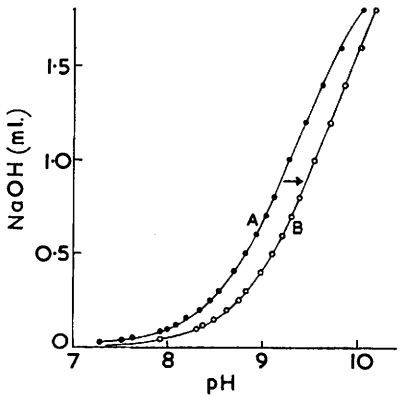


FIG. 3. Titration of 0·02M-uracil (100 ml.) in presence and absence of formaldehyde at 30° with 1·00N-sodium hydroxide.

(A) Plus 4 ml. of water. (B) Plus 4 ml. of 13M-formaldehyde.

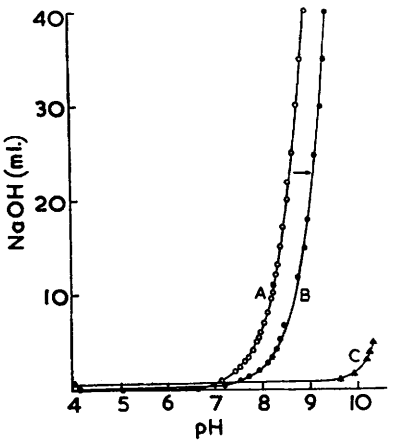


FIG. 4. Titration of mercaptoethanol (10 ml.) in absence and presence of formaldehyde with 0·183N-sodium hydroxide.

(A) Plus 100·5 ml. of water. (B) Plus 100 ml. of water and 0·5 ml. of 38% formaldehyde. (C) Plus 100 ml. of water and 10 ml. of 38% formaldehyde.

the presence of formaldehyde resulted in pH elevation. Over the titration range of the carboxyl group the curves are practically coincident, the formaldehyde curve being slightly displaced to the left, by *ca.* 0·02 pH unit. This shows that substitution of the ·S·CH_2·OH for the ·SH group hardly affects the pK value of the carboxyl group. This will be referred to below.

General Note.—As the titration of acidic imino- and thiol groups takes place over the alkaline region, often at pH >10, the following should be taken into consideration: (1) Formaldehyde has a pK value of *ca.* 12·3. Its neutralisation of hydroxyl ion can result in lowering of the pH value even in a pH region as low as 8, depending on the formaldehyde concentration.

By using a high large molar ratio of imine or thiol to formaldehyde the equilibrium concentration of formaldehyde is reduced effectively, and the negative effect of its ionisation on the pH elevation becomes insignificant when the equilibrium constant of the complex formation is very much greater than the ionisation constant of formaldehyde. (2) At pH ⩽~10 water behaves as an acid and its effect on the titration curve increases progressively with rise in pH value. This effect can be reduced by using high concentrations of the substance to be titrated but a correct ion must be made in accurate measurements.[8]

[8] Jordan and Taylor, *J.*, 1946, 994.

Assignment of p*K* *Values for Adenine.*—Titrations of adenine with hydrochloric acid and with sodium hydroxide, with and without formaldehyde, were carried out at 20°, 30°, and 40°, using both constant and non-constant volume procedures. In all the cases examined the presence of formaldehyde resulted in a pH depression in the acid range and pH elevation in the alkaline range. An example is illustrated in Fig. 6. On this basis the acid p*K* value is assigned to the basic group, and the higher, alkaline, p*K* is assigned to the imino-group, in agreement with the allocation by Taylor [3,9] which was based on comparisons of p*K*'s of different compounds.

As pointed out in the previous cases, this interpretation does not exclude the presence of other forms such as those involving intramolecular hydrogen bonding, *e.g.*, between the amido-hydrogen and the 1-nitrogen atom, or intermolecular hydrogen bonding between two adenine molecules. It is indeed likely that adenine in aqueous solution is associated

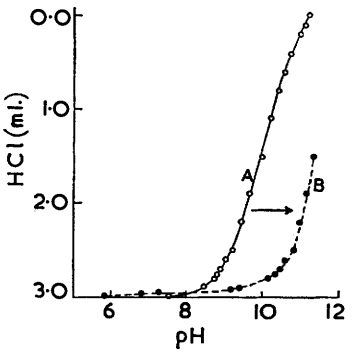


FIG. 5. Titration of calcium mercaptoacetate (0·55 g. in 100 ml.) in absence and presence of formaldehyde at 25° with 1·0N-hydrochloric acid.

(A) Plus 0·1 ml. of water. (B) 38% Plus 0·1 ml. of formaldehyde.

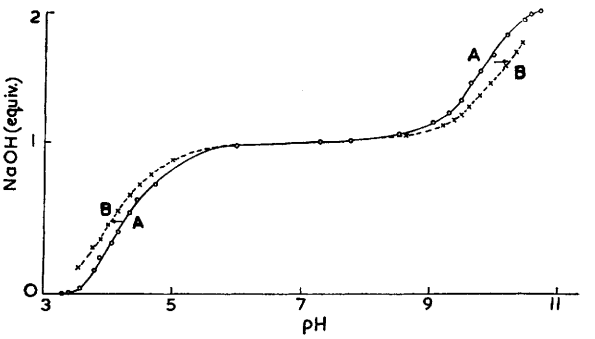
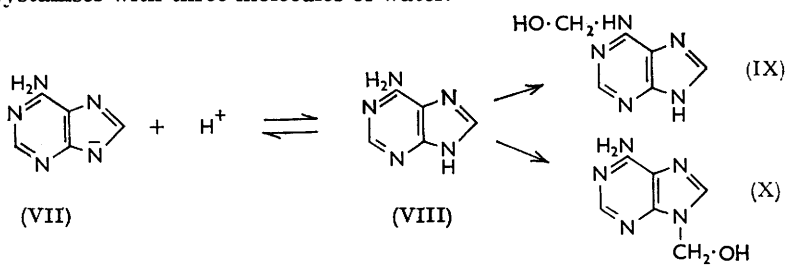


FIG. 6. Titration of 0·00227M-adenine hydrochloride solution (220 ml.) with and without added formaldehyde at 19° by aqueous sodium hydroxide.

(A) Plus 2 ml. of water. (B) Plus 2 ml. of 38% formaldehyde.

with water molecules by means of hydrogen bonds, since it was shown by Broomhead [10] that adenine hydrochloride semihydrate is hydrogen bonded in the solid state. Also adenine crystallises with three molecules of water.[11]



The presence of a second group capable of reacting with formaldehyde could be misconstrued as invalidating the p*K* allocation. Thus in the case of adenine it could be argued that the pH elevation obtained in the alkaline region could be due to displacement of the equilibrium associated with the interaction with the amino-group and not necessarily with the imino-group (cf. VII ⟶ IX + X).

[9] Taylor, *Nature*, 1949, **164**, 750.
[10] Broomhead, *Acta Cryst.*, 1948, **1**, 324.
[11] "Handbook of Physics and Chemistry," Chemical Rubber Publ. Co., 37th ed., 1955—6, pp. 718—719.

That argument is erroneous in that it tacitly assumes that the pK value of the imino-group in (IX) is significantly different from that in (VIII), for which there is no evidence. Rather the reverse is likely by analogy with the mercaptoacetic acid–formaldehyde reaction. The pK value of a group covers the various species concerned with the group; replacement of one species by another which does not possess significantly different acid properties is unlikely to affect the equilibrium. Further consideration will be advanced in later papers to show that the formaldehyde complex-formation does not, in simple cases, affect the pK values of acid groups present in a particular molecule.

Experimental.—The chemicals used were obtained from the following sources: succinimide (British Drug Houses), hydantoin (British Drug Houses), uracil (British Drug Houses and Nutritional Biochemicals Co.), mercaptoethanol (Light and Co. Colnbrook, Bucks.), calcium mercaptoacetate (Light and Co., Colnbrook, Bucks.), adenine (California Foundation for Biochemical Research and British Drug Houses), xanthine (Na) (Nutritional Biochemicals Co.).

Sodium hydroxide solutions were carbonate-free. Titrations were carried out under anaerobic carbon dioxide-free conditions with nitrogen free from oxygen and carbon dioxide. Constant temperature was maintained in the thermojacket pH vessels used.[12] pH measurements were made with Doran pH meters and Radiometer pH meters, lithium glass electrodes, and internal bridge calomel electrodes.[13] Glass-electrode calibrations were by potassium hydrogen phthalate and sodium borate.[14] Glass containers were cleaned and steamed.[15]

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PHYSICAL BIOCHEMISTRY LABORATORY,
SOUTH-WEST ESSEX TECHNICAL COLLEGE, LONDON, E.17. [*Received, July 19th,* 1961.]

[12] Lewin, *Chem. and Ind.*, 1953, 1193; *Lab. Practice*, 1958, 209.
[13] Lewin, *J. Sci. Instr.*, 1954, **31**, 302.
[14] Nat. Bur. Standards, U.S., Letter Circular LC993, August 19th, 1950; Lewin. *Lab. Practice*. 1955, 41.
[15] Lewin, *Lab. Practice*, 1957, 573.