[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

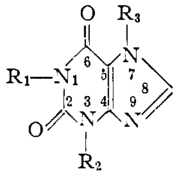
On the Nature of Xanthine and Substituted Xanthines in Solution[1]

BY LIEBE F. CAVALIERI, JACK J. FOX,[2] AUDREY STONE AND NAISHUN CHANG

RECEIVED SEPTEMBER 24, 1953

The spectra of a number of methylated xanthines have been determined in aqueous solution as a function of *p*H and have been found to fall into two distinct categories; that is, these derivatives may be placed into either of two spectral classes which are readily distinguishable regarding both the positions and the intensities of the bands. Correlation of the spectral shifts with *p*H has permitted an assignment for the sequence of ionization of the various hydrogen atoms. In the case of xanthine, for example, the first ionization is attributed to the removal of the 3-hydrogen while the second is due to the 7-hydrogen. When the 3-position is substituted, the 7-hydrogen ionizes first; the 1-hydrogen next. When the 1-position is substituted the order is identical to that of xanthine, and if the 7-position is substituted, the sequence is 3, 1. In general, the sequence of ionization of the three positions would be 3, 7, 1. The spectrally-determined apparent dissociation constants are found to be in good agreement with those obtained potentiometrically. Contrary to an earlier belief, it has been shown that xanthine, 7-methylxanthine and xanthosine each exhibit two dissociations in the *p*H range studied. The relative values of the various apparent dissociation constants are discussed in terms of structure.

The extent and mode of enolization and ionization of xanthine and substituted xanthines in aqueous solutions of varying *p*H are problems for which there are no satisfactory explicit answers. An examination of the structural formula of xanthine ($R_1 = R_2 = R_3 = H$) immediately reveals the multiplicity of possibilities relating to both questions.



Insofar as this class of compounds is of general biochemical interest, the present investigation was undertaken with the aim of clarifying these issues. We have selected as a basis for this study the ultraviolet spectral characteristics and dissociation constants of xanthine and its N-methylated derivatives. It will be shown that the sequence of ionization for the various hydrogen atoms may be determined by the spectral behavior of these compounds as a function of *p*H in a manner similar to that previously reported with pyrimidines.[3,4]

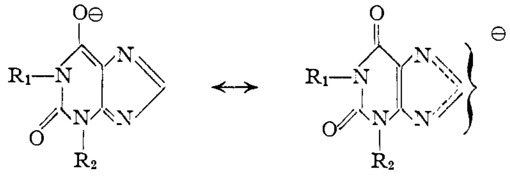
Experimental

Materials.—The sample of xanthine employed was that used in previous spectral studies[5]; 1- and 3-methylxanthine and 1,7-dimethylxanthine were kindly supplied by G. B. Elion and Dr. G. H. Hitchings, of the Wellcome Research Laboratories. A sample of 3-methylxanthine was also furnished by Dr. A. A. Christman, of the University of Michigan. The remaining samples were commercially available materials which were recrystallized to constancy with respect to spectral characteristics.

Method.—Stock aqueous solutions of the compounds were made in all cases and known quantities were delivered by means of a microburet to buffered solutions of ionic strength 0.05. Acetate, phosphate and glycinate were the buffers employed. In the high alkaline range (*p*H 12 to 14) 0.01, 0.1 and 1.0 *N* solutions of sodium hydroxide were used along with appropriate additions of sodium chloride to maintain an ionic strength of one. All spectra were run in duplicate with estimated error of ±0.003 optical density units. Concentrations were adjusted so that the important readings on the density scale of the Model DU Beckman spectrophotometer were made in the range of 0.1 to 0.7, in 1-cm. matched silica cells. Apparent dissociation constants were calculated in the usual manner.[6] Apparent molar volumes were determined by the falling drop method used previously.[7]

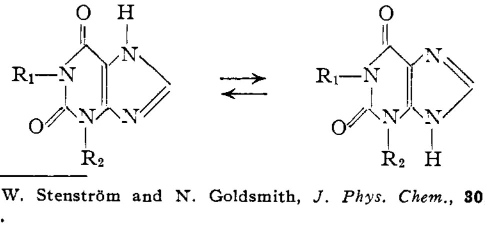
Results and Discussion

Spectral Studies.—Caffeine (1,3,7-trimethylxanthine) cannot enolize or ionize; accordingly, its spectrum remains unaltered with changes in *p*H (2–14)[8] and the characteristic selective absorption (Fig. 1) may be ascribed to a system containing carbonyl rather than hydroxyl functions. In 1,3-dimethylxanthine (theophylline, $R_1 = R_2 = CH_3$, $R_3 = H$) only the hydrogen atom of the imidazole ring can ionize. Since the free pair of electrons resulting from ionization may be transmitted readily to the 6-oxygen atom, the ionic form (Fig. 1, curve for *p*H 10.77) may be written as I.



I II

It is highly probable that I makes the major (if not the entire) contribution to the state of the ion in view of the difference in electronegativity between oxygen and nitrogen. Below *p*H 7 (*cf.* curve for *p*H 6.69, Fig. 1) the ratio $\epsilon_{\min}$ (245 mμ)/$\epsilon_{\max}$ (272 mμ) for caffeine is 0.269; $\epsilon_{\min}$ (243)/$\epsilon_{\max}$ (271) for theophylline is 0.273. The near identity of the spectrum of caffeine to that of 1,3-dimethylxanthine at ~*p*H 7 indicates that the structure of the latter in neutral solution is



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[1] This investigation was supported by grants from the National Cancer Institute, National Institutes of Health, United States Public Health Service, and from the Atomic Energy Commission, Contract AT(30-1)-910.

[2] Fellow of the Damon Runyon Memorial Fund.

[3] J. J. Fox and D. Shugar, *Bull. soc. chim. Belg.*, **61**, 44 (1952).

[4] D. Shugar and J. J. Fox, *Biochim. Biophys. Acta*, **9**, 199 (1952).

[5] L. F. Cavalieri, A. Bendich, J. Tinker and G. B. Brown, THIS JOURNAL, **70**, 3875 (1948).

[6] W. Stenström and N. Goldsmith, *J. Phys. Chem.*, **30**, 1683 (1926).

[7] L. F. Cavalieri, THIS JOURNAL, **74**, 5804 (1952); *ibid.*, **75**, 5268 (1953).

[8] In the region of *p*H 0, the spectrum is altered, indicating proton addition.

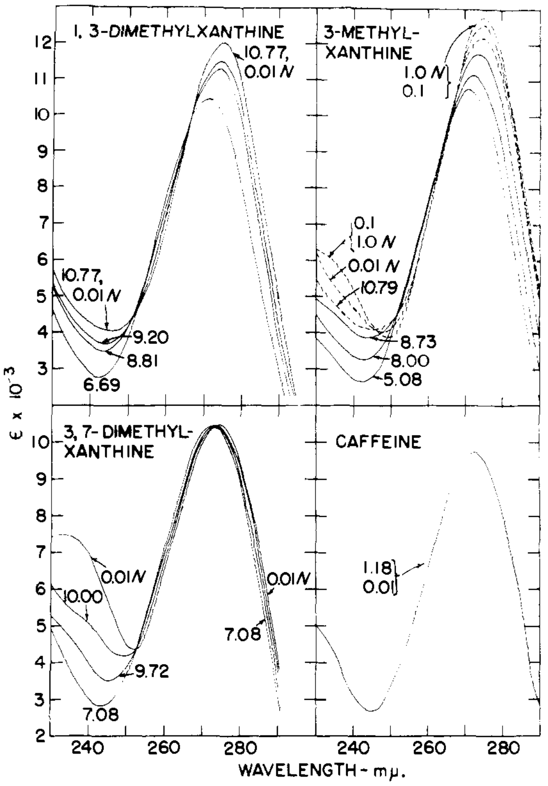
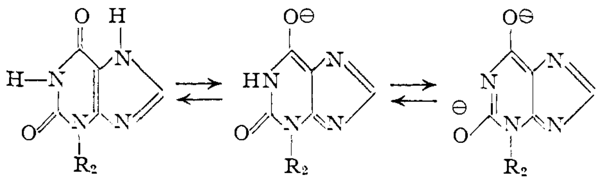


Fig. 1.—The *p*H values are those indicated. The *p*H values 12, 13 and 14 are assumed to correspond to 0.01, 0.1 and 1.0 *N* sodium hydroxide. The curves for caffeine are identical between *p*H 1.18 and 0.01*N* sodium hydroxide.

That the 6-oxygen atom cannot exist to any appreciable extent as a hydroxyl group in aqueous solution below *p*H ~ 7 is supported by the fact that only one set of isosbestic points is evident for theophylline. For if the system $>C{=}O \rightleftarrows \geqslant C\text{–}OH \rightleftarrows \geqslant C\text{–}O^-$ existed in solution, two equilibria would be involved which would give rise to two sets of isosbestic points. This was not observed in this study nor in previous studies with dioxypyrimidines.[3,4] Further, if the 6-oxygen atom existed largely as a hydroxyl group, it would be expected that the spectrum of this compound would differ significantly from that of caffeine. For example, the spectra of 2,4-diethoxypyrimidine and 4-ethoxy-2-pyrimidone are considerably different from that of uracil.[4] This interpretation, however, does not exclude the possibility that the hydroxyl form exists to a very limited extent and that ionization may indeed occur through this form.

The variation with *p*H of the spectrum of 3-methylxanthine is identical to that of 1,3-dimethylxanthine up to ~*p*H 10, and it may be deduced that they are structurally similar in this range. Above *p*H 10 it is evident from the spectrum (Fig. 1) that a second dissociation has begun to occur with 3-methylxanthine which has two potentially dissociable hydrogen atoms. The structure of the doubly-charged ion is represented best by III.

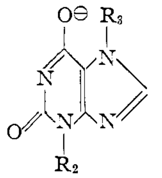
In the case of 3,7-dimethylxanthine (theobromine, R_1 = H, R_2 = R_3 = CH_3), which contains one dissociable hydrogen atom, the variation of its spectrum with *p*H is essentially similar to that for



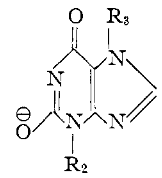
III

<*p*H 7 *p*H 10 >*p*H 13

1,3-dimethylxanthine and for the first dissociation pattern of 3-methylxanthine. The similarity is seen best by noting that each compound has two isosbestic points (*ca.* 254 and 270 mμ) and that there is a close resemblance in the 240 mμ region (between *p*H 7 and 12). Since the last two compounds most probably involve a charge on the 6-oxygen atom, it would appear that the structure for the theobromine ion above *p*H 10 is to be represented by IV. Contribution from the quinoid-type structure V, though theoretically possible, is less likely by virtue of the similarity of the theobromine spectrum to that of 1,3-dimethylxanthine. That is, V would be expected to possess an extremely large extinction coefficient.



IV

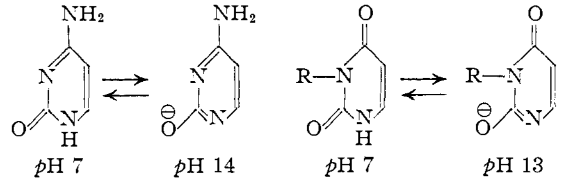


V

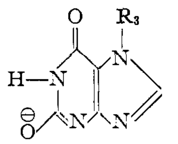
The ratio of $\epsilon_{\min}$ (244)/$\epsilon_{\max}$ (273) at *p*H 7 is 0.273. This close correspondence with the two previous compounds suggests that the 6-oxygen atom in 3,7-dimethylxanthine exists as a carbonyl group in neutral solution.

The four compounds discussed above may therefore be placed into a single class with respect to their spectral characteristics. Xanthine, 1-methylxanthine, 1,7-dimethylxanthine (paraxanthine), 7-methylxanthine and xanthosine reveal at a glance (Fig. 2) a different ionization pattern spectrally and, similarly, all may be placed into a single group.

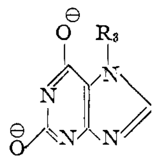
The 3-hydrogen is the only ionizable atom in 1,7-dimethylxanthine. This will give rise to either of two ions; one will have the negative charge on the 2-oxygen atom, the other on the 6. It is possible that both states contribute to the actual ion. It should be noted that the spectral pattern of this second class of compounds (Fig. 2) is entirely different from that occurring in the first class and is identical with that encountered in the pyrimidine series[4] where ionization has been ascribed to the corresponding hydrogen atom, *i.e.*



The shifts are characterized by a decrease in extinction as the *p*H increases, with a concomitant appearance of a peak at the longer wave lengths. This effect is indicated by a dotted (arrow) line in the spectrum for 1,7-dimethylxanthine (Fig. 2). It can be seen that 7-methylxanthine exhibits a strikingly similar pattern and we may therefore conclude in this case that the first ionization involves the 3-hydrogen, VI, while the second involves the 1-hydrogen, VII. These may be represented as



VI



VII

Further evidence supporting the 3,1-sequence is found in the similarity of the spectral behavior to that for the second dissociation of uracil.[4]

The changes in spectra with *p*H of 1-methyl-, 1,7-dimethylxanthine and xanthosine are essentially similar, and it is highly probable that the mode of ionization is also the same, namely, that the first dissociation involves the 3-hydrogen. The effect of placing the substituent at the 9-position (either CH_3[9] or D-ribose) is simply to shift the peak which occurs in the far ultraviolet to longer wave lengths. The same situation obtains with caffeine and isocaffeine.[9] With the latter, the methyl group of the imidazole ring is at the 9-position and the peak, occurring toward the shorter wave lengths, is more evident than in the case of caffeine.

It is apparent from the spectral variations of xanthine with *p*H that the first ionization (Fig. 2, curves for *p*H 5.92 through 9.71) involves the 3-hydrogen. The second dissociation may occur through either the 1- or 7-hydrogen atoms. Comparison with the spectrum of 1-methylxanthine at varying *p*H values[10] reveals a close similarity, and it is very likely that the second dissociation of xanthine involves the 7-hydrogen.

In the various xanthines, therefore, the sequence of the three dissociable hydrogen atoms would be: first 3, then 7, and finally 1. For example, if a 7-substituent is present in the molecule, the hydrogen atom at position 3 ionizes first and that at position 1 next.

Finally, it should be noted that the spectra of xanthine, 1-methyl- and 1,7-dimethylxanthine are nearly superimposable at *p*H values below their first ionization and are also similar (in this range) to the spectra of the four compounds in the first group discussed above. It is reasonable to conclude, therefore, that the uncharged xanthines exist in the carbonyl form.

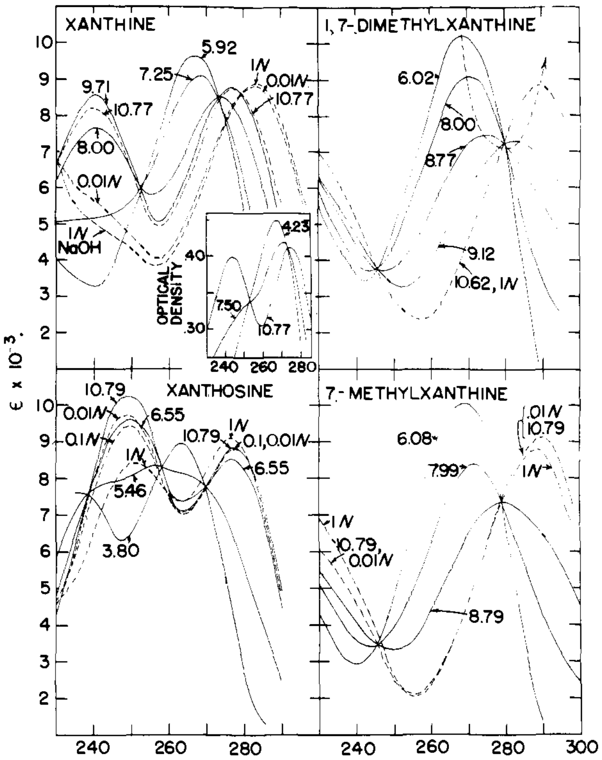
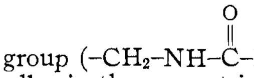


Fig. 2.—The inset shows the variation with *p*H of the spectrum of 1-methylxanthine.

Dissociation Constants and Previous Work.—In his study of the dissociation constants of various substituted xanthines, Ogston[11] has stated that the 2-oxygen atom is incapable of ionization. This view was based on (a) the work of Tafel and Dodt[12] with 1,6-dihydro-6-deoxyxanthines which, they maintained, had acidic properties attributable only to the imidazole ring and (b) on the fact that 7-methylxanthine showed only one dissociation constant[11] when titrated potentiometrically. The conclusion that the 2-oxygen atom cannot bear a negative charge is open to serious question. First, as has been pointed out by Jordan,[13] the 1,6-dihydro-6-deoxyxanthines[13] are not comparable to the xanthines themselves since in the former the ionizable group  $(-CH_2-NH-\overset{\overset{\displaystyle O}{\|}}{C}-NH-)$ is of a ureido type. Secondly, in the present investigation we have observed two dissociations spectrally in the case of 7-methylxanthine and xanthosine. It is clear, therefore, that both hydrogen atoms of the pyrimidine ring are capable of ionization and the conclusion of Ogston (reiterated by Jordan) that the 2-oxygen of 7- and 9-methylxanthine does not ionize is incorrect.

Assuming that the 7-hydrogen of 3-methyl- and 1,3-dimethylxanthine ionizes in a similar manner (I), the pK_a of the latter would be expected to be higher because of an increased electron density in the vicinity of the ionizable group.[14] The same situation may be seen to hold for 7-methyl- and 1,7-dimethylxanthine. 7- and 9-Methylxanthine represent interesting cases in that the former has a higher pK_a than xanthine while the latter has a lower value (see Table I). This may be accounted for on the basis that xanthine exhibits two tautomeric forms with their accompanying resonance structures.

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[9] J. M. Gulland, E. R. Holliday and P. F. Macrae, *J. Chem. Soc.*, 1639 (1934).

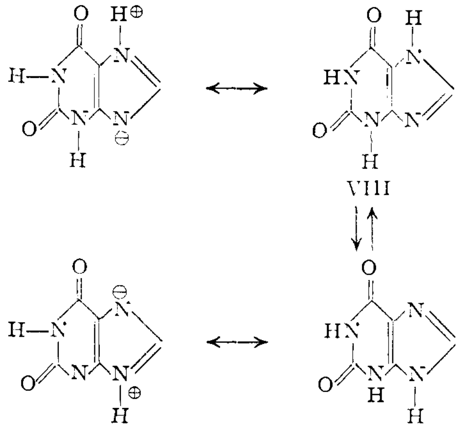
[10] This compound was unobtainable in pure form and only three curves are included in the insert of Fig. 2 to show its similarity to xanthine.

[11] A. G. Ogston, *J. Chem. Soc.*, 1376 (1935).

[12] J. Tafel and J. Dodt, *Ber.*, **40**, 3752 (1907).

[13] D. Jordan, *Ann. Rev. Biochem.*, **21**, 209 (1952).

[14] This interpretation would require the assumption that ionization occurs through the 6-OH group.



VIII

IX

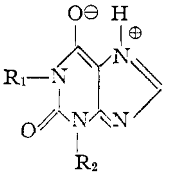
TABLE I

Compound	pK_{a1} Spectral[a]	pK_{a1} Potentiometric[11]	pK_{a2} Spectral	pK_{a2} Potentiometric[b]
Xanthine	7.53	7.7	11.63	...
1-Methylxanthine	..[c]	7.7	...[c]	12.05
3-Methyl-	8.32	8.5 (8.10)[b]	11.9	11.3
7-Methyl-	8.33	8.5 (8.30)[b]	~13	...
9-Methyl-	..	6.3 (6.25)[b]	...	...
1,3-Dimethyl-[d]	8.81	8.6	...	...
1,7-Dimethyl-	8.71	8.5	...	...
3,7-Dimethyl-	9.97	9.9	...	...
Xanthosine	5.50	6.0	~13	...

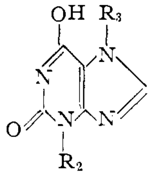
[a] $pK_a \pm 0.05$. [b] H. F. W. Taylor, *J. Chem. Soc.*, 765 (1948). [c] Due to the impurity of our sample, these pK_a's were not determined. [d] J. K. Wood, *J. Chem. Soc.*, 1839 (1906), has determined the pK_a values of xanthine and a number of methylated xanthines by observing the rate of saponification of methyl acetate in the presence of the various sodium salts.

In 7-methylxanthine only forms of type VIII can contribute to the state of the molecule. Since the seat of the negative charge is at the 9-nitrogen atom, ionization of the 3-hydrogen will occur at higher pH values. The reverse obtains with 9-methylxanthine. This explanation is also consistent with the relationship between the pK_a values of 1,7-dimethyl- and 1,9-dimethylxanthine and with the fact that pK_{a1} for xanthine lies between those for 7- and 9-methylxanthines.

On the basis of a similarity among the pK_a values of the various methylated xanthines and the ΔpK_a for water *vs.* 90% ethanol, Ogston[11] assigned zwitterionic structures for the neutral forms of most of the compounds investigated. For example, 1,3-dimethylxanthine was written as X while 3,7-dimethylxanthine (XI) was not considered to be a dipolar ion.



X



XI

The present results show that this hypothesis need not be invoked. We have shown experimentally that 1,3-dimethylxanthine cannot exist to any appreciable extent as a dipolar ion since its apparent molar volume is 143.6 ± 0.1 cc. as compared to 143.2 ± 0.1 cc. for 3,7-dimethylxanthine both at a molar concentration of 0.007590. If the former existed as a dipolar ion its partial molar volume would be significantly less than the latter due to electrostriction.[15] However, the zwitterionic hypothesis cannot be discarded on this basis alone since the dipolar ions could exist in minute and undetectable amounts.

Acknowledgment.—The authors wish to express their gratitude to Dr. George Bosworth Brown for helpful discussions and continued interest.

[15] E. Cohn and J. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 155.

[CONTRIBUTION FROM THE WELLCOME RESEARCH LABORATORIES]

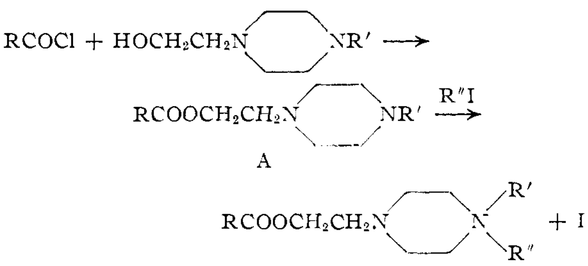
Unsymmetrically N-Substituted Piperazines. VI. Ester Derivatives as Spasmolytics[1]

BY WALTER S. IDE, EMIL LORZ AND RICHARD BALTZLY

RECEIVED JULY 27, 1953

The preparation of a series of piperazino esters, mainly derived from N-methyl-N′-hydroxyethylpiperazine, is described.

Some piperazine derivatives reported in the first paper of this series[2] exhibited a degree of spasmolytic activity. It seemed desirable therefore to examine the properties of piperazine derivatives constructed on the model of typical ester type spasmolytics. The greater part of the compounds so prepared were esters of N-β-hydroxyethyl-N′-alkyl piperazines. Data on these and on a number of the derived quaternary salts are presented in Table I. The synthetic methods involved were conventional and involved the sequence



A

B

The reactions of the acid chlorides with the aminoalcohols were conducted in inert solvents and the products isolated through their solubility in dilute

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[1] The work here reported is part of a joint project with the Pharmacology Department of these laboratories.

[2] R. Baltzly, J. S. Buck, E. Lorz and W. Schoen, THIS JOURNAL, **66**, 263 (1944).