Infrared and Ultraviolet Studies on the Tautomeric Equilibria in Aqueous Medium between Monoanionic Species of Uracil, Thymine, 5-Fluorouracil, and Other 2,4-Diketopyrimidines

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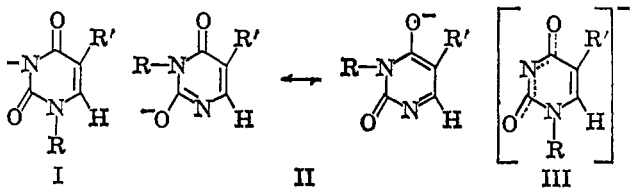
Infrared spectra have been recorded in aqueous medium, in the range of valence-bond vibration frequencies, 1750–1250 cm.$^{-1}$, of the monoanions of thymine, 1-methylthymine, 3-methylthymine, 5-fluorouracil, and the photodimers of uracil and thymine; the latter were employed as alkali-stable models of the monoanions of 5,6-dihydrouracil and 5,6-dihydrothymine. The infrared data demonstrate unequivocally that the monoanions of thymine and 5-fluorouracil each consist of an equilibrium mixture of two tautomeric forms corresponding to dissociation of the N-1 or the N-3 protons. The relative contents of the two forms, calculated from the extinctions of the characteristic bands of each, were in reasonably good agreement with the values calculated from the electronic absorption spectra under the same conditions. With the aid of solvents of lower dielectric constant, consisting of dioxane–water mixtures, it was found possible to shift the equilibrium between the two tautomeric anions in favor of the less polar form, in agreement with expectations and checked by means of both the infrared and ultraviolet spectra. An empirical analysis of the infrared spectra of the two types of monoanions has been carried out and their structures have been formulated on the basis of band assignments.

It was initially proposed by Nakanishi, *et al.*,[1] that the singly ionized form of uracil in aqueous medium may consist of an equilibrium mixture of two tautomeric forms. The very broad, asymmetric, long wave length ultraviolet band of the uracil monoanion[2] was ascribed to the superposition of two bands, each representing one of two tautomeric forms corresponding, respectively, to proposed structures for the monoanions of 1-methyluracil (1-MeU$^-$, I) and 3-methyluracil (3-MeU$^-$, II).[3] The presumed structure of the monoanion I, which assumed the negative charge to be located on the less electronegative ring nitrogen, and implicitly postulated the absence of resonance delocalization of the π-electrons in the potentially conjugated bond system, was based[1] on an analogous conception of Spinner[4] for the anion of 2-ketopyrimidine, as well as on the absence of a bathochromic shift in the long wave length ultraviolet absorption band of 1-MeU on dissociation of the proton on N-3.[2] Subsequently Wempen and Fox,[5] on the basis of a study of the ultraviolet spectra of 6-halogeno derivatives of uracil, and of the ultraviolet data of Berens and Shugar[6] for 5-halogenouracils (in particular 5-fluorouracil), interpreted the monoanion spectra of both groups of compounds in terms of the proposal of Nakanishi, *et al.*,[1] *viz.* the differences in location of the long wave length ultraviolet absorption bands of the 5- and 6-substituted derivatives being ascribed to a shift in tautomeric equilibrium towards form I (R = H) by a 5-halogeno substituent and to form II (R = H) by a 6-halogeno substituent.[7]



I II III

I, III, R = CH_3; R′ = H (1-MeU$^-$)
R = H; R′ = H (1-HU$^-$)
R = CH_3; R′ = CH_3 (1-MeT$^-$)
R = H; R′ = CH_3 (1-HT$^-$)
R = H; R′ = F (1-HFU$^-$)
II, R = CH_3; R′ = H (3-MeU$^-$)
R = H; R′ = H (3-HU$^-$)
R = CH_3; R′ = CH_3 (3-MeT$^-$)
R = H; R′ = CH_3 (3-HT$^-$)
R = H; R′ = F (3-HFU$^-$)

Our interest in this problem arose during the course of an initial systematic investigation on the infrared spectra in aqueous medium of some common pyrimidine derivatives[8] and attendant difficulties associated with attempts to interpret the spectra of such compounds as uracil and thymine in alkaline medium (pH 12) in terms of the existence of a single monoanionic species. The present communication provides more direct evidence for the presence of an equilibrium mixture of two monoanions in solutions of 2,4-diketopyrimidines, based on a study of the characteristic infrared bands of aqueous solutions of the monoanionic forms of thymine and its 1- and 3-methyl derivatives, and of 5-fluorouracil, as well as on the shift in tautomeric equilibria in aqueous dioxane solutions with lower dielectric constants. An empirical analysis of the infrared spectra of the anions in relation of their structure has also been carried out and demonstrated, among

(1) K. Nakanishi, N. Suzuki, and P. Jamazaki, *Bull. Chem. Soc. Japan*, **34**, 53 (1961).
(2) D. Shugar and J. J. Fox, *Biochim. Biophys. Acta*, **9**, 199 (1952).
(3) The following abbreviations are used in this text: U, T, and FU are the neutral forms of uracil, thymine, and 5-fluorouracil, while U$^-$, T$^-$, and FU$^-$ are the corresponding monoanions; 1-HU$^-$ and 3-HU$^-$ are the two possible monoanions of uracil, resulting from dissociation of the N-3 and N-1 protons, respectively, with similar connotations for T and FU; 1-MeU, 3-MeU, and 1,3-DMeU are the mono- and dimethyl derivatives of U, and similarly for T and FU.
(4) E. Spinner, *J. Chem. Soc.*, 1232 (1960).
(5) I. Wempen and J. J. Fox, *J. Am. Chem. Soc.*, **86**, 2474 (1964).
(6) K. Berens and D. Shugar, *Acta Biochim. Polon.*, 10, 25 (1963).
(7) It should be noted that A. Giner-Sorolla and A. Bendich, *J. Am. Chem. Soc.*, **80**, 5744 (1958), had earlier drawn attention to the fact that the ultraviolet spectrum of 6-trifluoromethyluracil monoanion was strikingly similar to that of the monoanion of 3-methyluracil, *i.e.*, to form II (R = H).
(8) K. L. Wierzchowski, E. Litonska, and D. Shugar, in preparation.

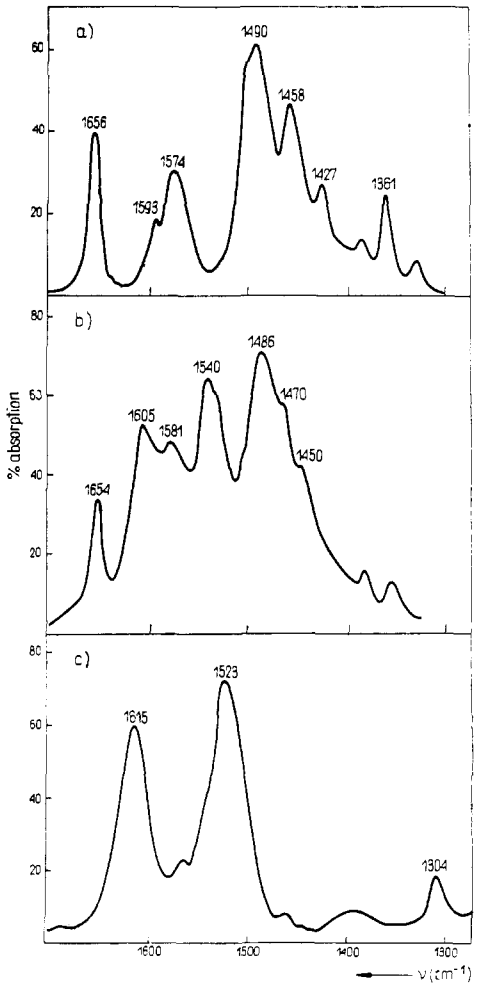


Figure 1. Infrared spectra in the region 1700–1300 cm.$^{-1}$, embracing the characteristic frequencies of the ring and valence vibrations, of the monosodium salts of: (a) 1-methylthymine, c = 0.121 M, l = 25 μ; (b) thymine, c = 0.116 M, l = 50 μ; (c) 3-methylthymine, c = 0.11 M, l = 35 μ; all in 0.01 N NaOD in D_2O.

others, that the structure proposed by Nakanishi, *et al.*,[1] for the type I anions is inconsistent with the experimental data and that this structure is more correctly represented by the resonance hybrid III. Finally, it will be seen from what follows that the existence in 2,4-diketopyrimidines of an equilibrium mixture of two tautomeric monoanions is a frequently encountered phenomenon which must be taken account of in any investigations of their structures by optical methods.[9]

Results and Discussion

Infrared Spectra of Monoanions of Thymine (T^-), 1-Methylthymine (1-MeT$^-$), and 3-Methylthymine (3-MeT$^-$) in D_2O. In Figure 1 are presented the infrared spectra of the monosodium salts of thymine and its two monomethylated derivatives in 0.01 N NaOD in D_2O in the spectral range 1700–1300 cm.$^{-1}$, where one may expect to encounter the characteristic frequencies of the ring and substituent valence vibrations. Even a cursory examination of the three spectra reveals that the spectrum of T^- (Figure 1b) represents reasonably well the superposition of the spectra of the two species 1-HT$^-$ and 3-HT$^-$,[10,11] closely resembling 1-MeT$^-$ (Figure 1a) and 3-MeT$^-$ (Figure 1c). This is perhaps clearer from an examination of Table I, which lists all the principal bands in 1-MeT$^-$ and 3-MeT$^-$ against the corresponding ones in T^-. The agreement is highly satisfactory, particularly if we bear in mind the small potential differences which may result from the replacement of a proton by a methyl group.

Table I. Frequencies (cm.$^{-1}$) of Principal Infrared Bands in 1-MeT$^-$ and 3-MeT$^-$ Compared with the Corresponding Bands in the Equilibrium Mixture of the Two Tautomeric Anions Represented by T^-, *i.e.*, 1-HT$^-$ and 3-HT$^-$

T^-	1-MeT$^-$	3-MeT$^-$
1654	1656	...
1605	...	1615
1581	1593, 1574	...
1540	...	1523
1486	1490	...
1470	1458	...
1450	1427	...
1384	1384	...
1357	1361	...

Assuming from the above that the monoanion T^- consists of an equilibrium mixture of two anionic forms, it is possible to calculate the relative contents of the two from the extinction (ϵ^A) of the clearly resolved band at 1654 cm.$^{-1}$ ascribed to the anion 1-HT$^-$, with respect to the extinction of the corresponding 1656-cm.$^{-1}$ band in the spectrum of 1-MeT$^-$. This presupposes that the substitution of a methyl group for the proton on the N-1 ring nitrogen does not appreciably affect the extinction of the 1656-cm.$^{-1}$ band, an assumption which seems reasonable in view of the small difference in its frequency as between T^-, *i.e.*, 1-HT$^-$ (1654 cm.$^{-1}$), and 1-MeT$^-$ (1656 cm.$^{-1}$). Consequently $\epsilon^A_{T^-}:\epsilon^A_{1\text{-MeT}^-}$ = fractional content of 1-HT$^-$ in the mixture of the two monoanions, ~0.4.[13]

Calculation of Relative Concentrations of Tautomeric Monoanions from Ultraviolet Spectra. The procedure adopted by Nakanishi, *et al.*,[1] and Wempen and Fox[5] for calculation of the relative concentrations of the two tautomeric monoanions made use of a "wing correction," based on the assumed symmetry of the long wave length absorption bands of the type II

(9) In a recent publication, L. B. Clark and I. Tinoco, Jr., *J. Am. Chem. Soc.*, 87, 11 (1965), ascribe to uracil at pH 14 the structure 3-HU$^-$. Apparently they were unaware of earlier work[2] which demonstrated unequivocally that at pH 14 uracil is partially in the form of the doubly dissociated anion; nor were they aware of the results of Nakanishi, *et al.*,[1] and Wempen and Fox.[5]

(10) Strictly speaking one should refer to 1-DT$^-$ and 3-DT$^-$, but this is avoided in order to maintain uniformity in nomenclature throughout the text.

(11) The pK_a values for 1-MeT and 3-MeT have not hitherto been recorded. These were therefore measured spectrophotometrically both in H_2O and D_2O. For 1-MeT the values found were 10.3 and 10.5, respectively, and for 3-MeT, 10.7 and 11.0. The corresponding values for thymine were also measured and found to be 9.9 (*cf.* ref. 2) and 10.2. Since 0.01 N NaOD corresponds to a pD of 12.4,[12] it follows that in this medium about 98% of 1-MeT exists in the form 1-MeT$^-$, about 94% of 3-MeT is in the form 3-MeT$^-$, while all the thymine is in the form T^-.

(12) K. Mikkelsen and S. O. Nielsen, *J. Am. Chem. Soc.*, 64, 632 (1960).

(13) The concentrations employed for recording the infrared spectra are much higher than those normally used in ultraviolet spectroscopy. It was, however, found that the equilibrium between the two tautomeric monoanions was independent of the concentration, at least in the range herein employed (see Experimental Section).

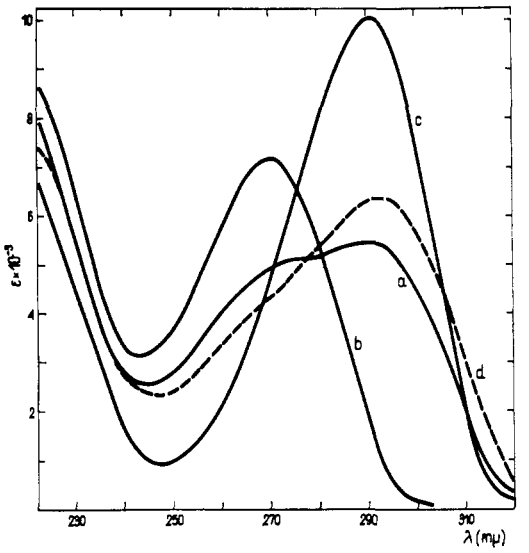


Figure 2. Ultraviolet spectra in 0.01 *N* NaOH of: (a) thymine, consisting of a 1:1 mixture of 1-HT$^-$ and 3-HT$^-$; (b) 1-methylthymine; (c) 3-methylthymine; and (d) thymine at 90°.

monoanions. This is, however, true only to a first approximation. From Figure 2 it can be seen that the long wave length band of 3-MeT$^-$ is, in fact, asymmetrical, as it is also for that of 3-MeU$^-$.[2]

A detailed analysis of the ultraviolet absorption spectrum of T$^-$ showed that the absorption maximum of 3-HT$^-$ is shifted about 40 Å. to the red of the corresponding band in the spectrum of 3-MeT$^-$. Furthermore the absorption band of 1-HT$^-$ is 50 Å. to the blue of that in 1-MeT$^-$. It follows that substitution of a methyl group for a proton results in substantially different effects on each of the two anions. Consequently a calculation of the extinctions of the anions from the simple relationship[1,5] $\epsilon_T/\epsilon_{3\text{-HT}^-} = \epsilon_{3\text{-MeT}}/\epsilon_{3\text{-MeT}^-}$ is not fully valid.

Applying the approximation that $\epsilon_{1\text{-HT}^-} = \epsilon_{1\text{-MeT}^-}$ and $\epsilon_{3\text{-HT}^-} = \epsilon_{3\text{-MeT}^-}$ (taking into account the wave length shifts cited in the previous paragraph), and treating the ultraviolet spectrum of T$^-$ as a simple, two-component mixture, the calculated proportion of 1-HT$^-$ comes out to be close to 0.5 (Figure 2). This is to be compared with the value 0.4 obtained from the infrared data above. The agreement is not too bad if it is recalled that both in the infrared and ultraviolet spectra it was necessary to assume that the extinctions of 1-HT$^-$ and 3-HT$^-$ correspond to those of 1-MeT$^-$ and 3-MeT$^-$, respectively.

Solvent-Induced Shift in Tautomeric Equilibrium. The foregoing interpretation of the infrared spectrum of the thymine anion as the superposition of the spectra of the two tautomeric species 1-HT$^-$ and 3-HT$^-$ was further substantiated by an examination of the spectrum of T$^-$ in 0.01 *N* alkali in dioxane–water, 3:1 (v./v.). Under these conditions a marked shift in equilibrium was observed both in the infrared and ultraviolet spectra.

By comparison with the infrared spectrum of the monosodium salt of T in 0.01 *N* NaOD in D_2O (Figure 3a), that in 75% dioxane exhibited a marked weakening, or almost complete disappearance, of the bands ascribed above (Table I) to 1-HT$^-$, together with a pronounced enhancement in the apparent ex-

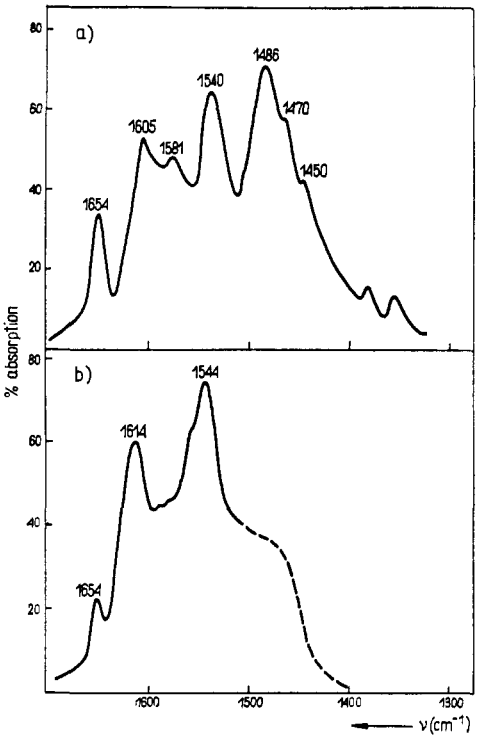


Figure 3. Infrared spectra of the monosodium salt of thymine in: (a) 0.01 *N* NaOD in D_2O, c = 0.116 *M*, l = 50 μ; (b) in 0.01 *N* NaOD in 75% dioxane in D_2O, c = 0.027 *M*, l = 195 μ. (Note: In this, and subsequent figures, the dashed portion of an absorption spectrum indicates that measurements in this region are uncertain because of high solvent absorption.)

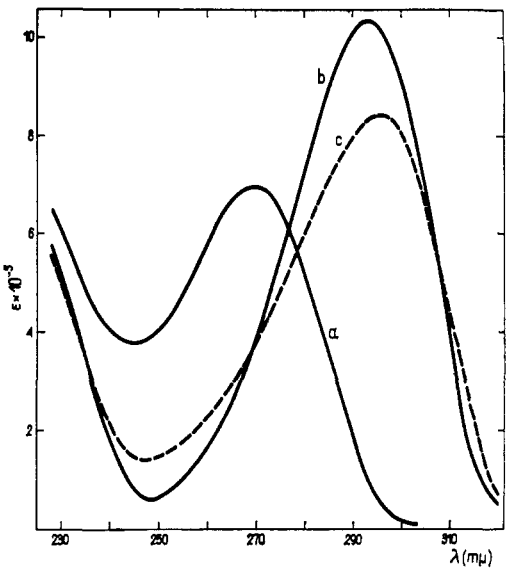


Figure 4. Ultraviolet spectra in 0.01 *N* NaOH in 75% dioxane–H_2O: (a) 1-methylthymine, (b) 3-methylthymine, and (c) thymine, consisting of a 1:3 mixture of 1-HT$^-$ and 3-HT$^-$.

tinctions (ϵ^A) of the 1614- and 1544-cm.$^{-1}$ bands assigned to 3-HT$^-$ (Figure 3b). Calculation of the tautomeric equilibrium, as described above, showed that under these conditions the fraction of 1-HT$^-$ was equal to 0.25 as compared to 0.5 in aqueous medium.

In the ultraviolet spectrum of T$^-$ in 75% dioxane–0.01 *N* NaOH, the location and shape of the long wave length absorption band (Figure 4, curve c) will be seen to approach that of 3-MeT$^-$ (Figure 4, curve b),

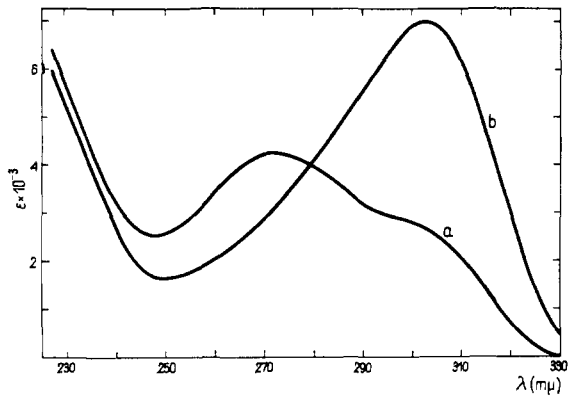


Figure 5. Ultraviolet spectrum of the monosodium salt of 5-fluorouracil in 0.001 *N* NaOH: (a) in H_2O, consisting of a mixture of 1-HFU⁻ (63%) and 3-HFU⁻ (37%); (b) in 67–85% aqueous dioxane, where the proportion of 1-HFU⁻ to 3-HFU⁻ is 1:3.

owing to the diminution in the former of the short wave length slope of the broad T⁻ band (Figure 2, curve a) due to 1-HT⁻. These modifications clearly point to a net increase in the proportion of the form 3-HT⁻ in aqueous dioxane, in agreement with the infrared data. Furthermore, a calculation of the tautomeric equilibrium point from the ultraviolet data for T⁻, 1-MeT⁻, and 3-MeT⁻ in 75% dioxane–0.01 *N* NaOH (Figure 3) gives a ratio for the two tautomeric anions as 1-HT⁻/3-HT⁻ = 1:3, in agreement with that obtained from the infrared data above. In view of what was said above, the excellent agreement prevailing in this case may be fortuitous.

The dielectric constant of 75% aqueous dioxane, $\epsilon^{20°} = 16$, is considerably lower than that for water, $\epsilon^{20°} = 80.4$, so that in the former solvent the tautomeric equilibrium point should be shifted in favor of the less polar form of the two anions. And, in fact, it is 3-HT⁻, the relative concentration of which is greater in 75% dioxane than in water, which possesses a distribution of charge density that is more equally dispersed throughout the molecule than in 1-HT⁻ (see discussion below on the structure of the two anions from infrared data).

It was also found possible to shift the tautomeric equilibrium point, albeit to a lesser degree, by an increase in temperature in aqueous 0.01 *N* NaOH (Figure 2, curve d). Here again, as in aqueous dioxane, the shift was in the direction to be expected from the known decrease in the dielectric constant of water with increase in temperature ($\epsilon^{90°} = 68$). The validity of this result from a quantitative point of view may, however, be questioned since the tautomeric equilibrium point itself is probably also a function of temperature.

Solvent-Induced Tautomeric Equilibrium Shift for 5-Fluorouracil. An analogous examination of the effect of solvent on the tautomeric equilibrium point was carried out for FU⁻ which, in 0.001 *N* NaOH,[6] was presumed to consist predominantly of the form 1-HFU⁻.[5] From calculations based on the ultraviolet spectra of FU⁻ and its two N-monomethyl derivatives,[14]

(14) 1-MeFU and 3-MeFU were prepared on a small scale, because of lack of sufficient starting material, by treatment of FU in aqueous medium with dimethyl sulfate, separation of the methylation products by paper chromatography, and calculation of their extinction coefficients by a comparison method (see Experimental Section).

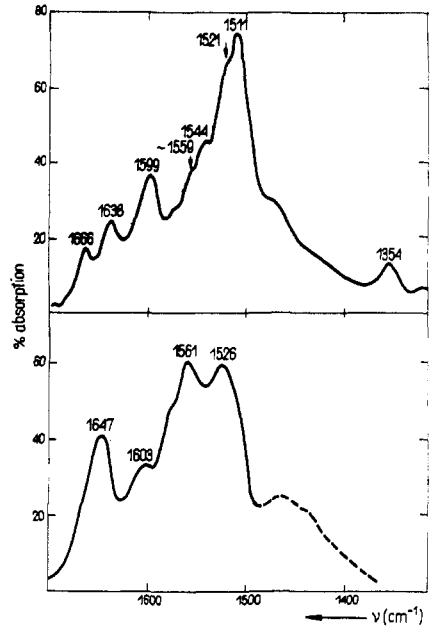


Figure 6. Infrared spectrum of monosodium salt of 5-fluorouracil in 0.001 *N* NaOD: (a) in D_2O, $c = 0.15$ *M*, $l = 30$ μ; (b) in 67% dioxane in D_2O, $c = 0.05$ *M*, $l = 100$ μ.

the proportion of 1-HFU⁻ in FU⁻ was found to be about 0.63 and decreased to about 0.20 in 85% dioxane, 0.001 *N* NaOH (Figure 5). Solubility difficulties made it possible to record the infrared spectrum of FU⁻ in a maximum concentration of only 67% aqueous dioxane. However, the proportion of 1-HFU⁻ even in this solvent, calculated from the ultraviolet data, was found in fact to be the same (Figure 5).[15]

Adequate quantities of 1-MeFU and 3-MeFU for recording the infrared spectra of their monoanions were not available. Consequently the origins of the various bands in FU⁻ were ascertained by comparing its spectrum with that of T⁻ and from observations on the modifications in extinction due to a change in solvent from 0.001 *N* NaOD in D_2O to 0.001 *N* NaOD in 67% dioxane in D_2O (Figure 6a, b). In this way it was deduced that the bands 1666, 1599, and 1511 cm.⁻¹ (Figure 6a) in FU in 0.001 *N* NaOD, the intensities of which are appreciably diminished in 67% dioxane (Figure 6b, in which will be noted the absence of a resolved band at 1666 cm.⁻¹, while those at 1599 and 1511 cm.⁻¹ correspond to 1603 and 1526 cm.⁻¹, respectively), may be assigned to 1-HFU⁻. The bands 1638 and 1599 cm.⁻¹ in 0.001 *N* NaOD, and with enhanced intensities in 67% dioxane, where they are located at 1647 and 1561 cm.⁻¹, were assigned to 3-HFU⁻. The general features of the deduced spectra of the two monoanionic forms of FU therefore closely resemble those of the corresponding anions of thymine, notwithstanding that their frequencies and extinctions are not fully analogous (*cf.* Figures 3 and 6; see Table II). Particularly striking is the decrease in extinction of the highest frequency band in 1-HFU⁻,

(15) It is known that the ionization of weak acids decreases in H_2O–dioxane mixtures; however, as might be anticipated, this effect disappears in the presence of a strong base: R. P. Ball and R. R. Robinson, *Trans. Faraday Soc.*, 57, 965, 1961. In agreement with this, it was found both in the present instance, as well as in the case of thymine in dioxane–water referred to above, that the concentrations of alkali employed were sufficient to give quantitatively the monoanionic forms.

Table II. Principal Infrared Band Frequencies, ν (± 2 cm.$^{-1}$), and Apparent Molecular Extinction Coefficients, ϵ^A ($\pm 10\%$), of Monoanionic Species in D_2O, and (in Parentheses) in Dioxane-D_2O

3-MeT$^-$		T$^-$		1-MeT$^-$		1-MeU$^-$		FU$^-$	
ν	ϵ^A	ν	ϵ^A	ν	ϵ^A	ν	ϵ^A	ν	ϵ^A
								1666, 1638 (1647)	185, 280 (470)
		1654 (1654)	310 (195)	1656	800	1638	900		
1615	1030	1605 (1614)	550 (760)						
		1581	515	1593sh	340	1598	495	1599 (1603)	450 (360)
				1574	565	1580	495	1559sh, 1544 (1561)	460, 600 (780)
1523	1540	1540 (1544)	745 (1120)						
								1521sh, 1511 (1526)	1100, 1350 (800)
		1486[a]	865	1490	1370	1499	1360		
		1470	590	1458	870	1450	910		
		1450	330	1427	420	1425	470		
		1384		1384					
		1357		1361	390	1363	440	1354	175
				1330		1325	265		

[a] In the range 1500–1300 cm.$^{-1}$; high solvent absorption made it impossible to record the bands in dioxane-D_2O.

1666 cm.$^{-1}$ (ϵ^A = 185 for a 63% content of 1-HFU$^-$ in the mixture of anions) as against the extinction of the 1654-cm.$^{-1}$ band in 1-HT$^-$ (ϵ^A = 340 for a 50% content of this anion in the mixture).

It did not prove feasible to assess more accurately the equilibrium distribution of the two monoanionic species in FU$^-$ from the infrared data, largely because of the fact that none of the principal strong bands was sufficiently well resolved. Nonetheless the observed solvent modifications in extinction of the groups of bands assigned to the individual anions are in good agreement with the equilibrium shift established by means of the ultraviolet spectral data under identical conditions.

Structure of Tautomeric Anions. A comparison of the infrared spectra of the neutral, diketo forms of 1-MeT and 3-MeT, or their uracil analogs (Figure 7; Table III), with those for the corresponding anions

Table III. Principal Band Frequencies, ν (± 2 cm.$^{-1}$), and Apparent Molecular Extinction Coefficients, ϵ^A ($\pm 10\%$), of Various N-Methylated 2,4-Diketopyrimidines in Dimethyl Sulfoxide

1-MeU		1-MeT		3-MeT		1-MeU·H_2	
ν	ϵ^A	ν	ϵ^A	ν	ϵ^A	ν	ϵ^A
1734	280						
						1717 sh	
1703	990	1701	1290	1709	855	1702	1560
1682	1765	1679	1480	1665	990	1691	1340
1657 sh		1655 sh		1637	1070		
						1498	480
						1445	305
						1398	170
1375	340	1367	170			1373	480
1328	320	1330	340			1326	130
		1206	160			1289	155
						1266	245
						1236	810
						1192	185

(Figure 1; Table II), indicates that dissociation of a proton is accompanied by a pronounced modification of electron density distribution in the ring and substituents. The clearest manifestation of this is the absence, in the anion spectra, of the band group at about 1700 cm.$^{-1}$ which, in the spectra of the diketo forms, corresponds to a superposition of the $\nu(C_2{=}O)$, $\nu(C_4{=}O)$, and $\nu(C_5{=}C_6)$ frequencies.[8] As regards the anionic forms of the 3-N methyl derivatives, this is in logical accord with the proposed resonance structure (II) of their anions, implying extensive equalization of the π-electron density along the bond system (O$=$C$_4-$C$_5=$C$_6-$N$_1=$C$_2-$O)$^-$. But this cannot apply to the proposed structure of the 1-N methyl derivatives on the assumption that the negative charge is located on N-3, *i.e.*, I,[1] for this implies the absence of appreciable delocalization of the π- and n-electrons, and would require the presence in the anion spectra of the partially perturbed carbonyl frequencies of the diketo forms.

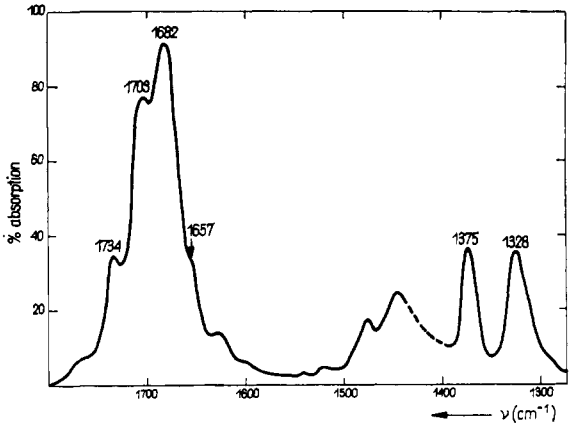


Figure 7. Infrared spectra of neutral, diketo, form of 1-methyluracil in dimethyl sulfoxide, c = 0.28 M, l = 25 μ.

Since the characteristic frequencies of the neutral forms are absent in the spectra of 1-MeU$^-$ and 1-MeT$^-$, and in view of the appearance in this latter of several strong bands in the 1650–1450-cm.$^{-1}$ region, a more plausible structure for these anions is the resonance hybrid III, involving resonance delocalization of the π- and n-electrons in the bond system (O$=$C$_2-$N$_3=$C$_4-$O)$^-$. We now proceed to an examination of this proposal by means of an empirical analysis of the infrared spectra of anions of this type with the aid of simpler, structurally similar, systems.

Infrared Spectra of Photodimers of Uracil and Thymine As Analogs of 5,6-Dihydrouracil and 5,6-Dihydrothymine. Undoubtedly the simplest model of the proposed resonance system of conjugated bonds, (O$=$C$-$N$=$C$-$O)$^-$, in the anion 1-MeU$^-$ (or 1-

Table IV. Infrared Band Frequencies, ν (in cm.$^{-1}$ ± 2 cm.$^{-1}$), and Molar Extinction Coefficients, ϵ^A (±10%), for Monoanionic Forms of 1-Methyluracil and 1-Methylthymine, and of the Monomer Units of Uracil and Thymine Dimers, with Their Assignments

T⁻-Dimer[a]		U⁻-Dimer[a]		1-MeU⁻		1-MeT⁻		
ν	ϵ^A	ν	ϵ^A	ν	ϵ^A	ν	ϵ^A	Assignment
				1638	900	1656	800	ν (C_5=C_6)
1603	380	1603	410	1598, 1580	495, 495	1593sh, 1574	340, 565	ν^{B_1}(C=N)
1537	1240	1536	1150	1499	1360	1490	1370	ν^{B_1}(C=O)
1442	1240	1436	850	1450	910	1458	870	ν^{A_1}(C=O)
1328	440	1315	330					ν^{A_1}(C=N)

[a] For the reasons presented in the text, ϵ^A values for the dimers have been calculated in terms of the monomer units, and are therefore directly comparable with those for the other pyrimidines.

MeT⁻) is the anion of the corresponding 5,6-dihydro derivative (IVa). However, the anions of 2,4-diketo-5,6-dihydropyrimidines are much too unstable[16] to permit the recording of their infrared spectra. By contrast the monoanions of the 5,5′ : 6,6′ photodimers of U and T (IVb) are fully stable at ambient temperatures.[17,18] Furthermore, since both in the dihydropyrimidines,[16] and in the pyrimidine components of the dimers,[17] it is the N-3 hydrogen which first ionizes, it was unnecessary to examine the spectra of the unavailable dimers of 1-MeU or 1-MeT.

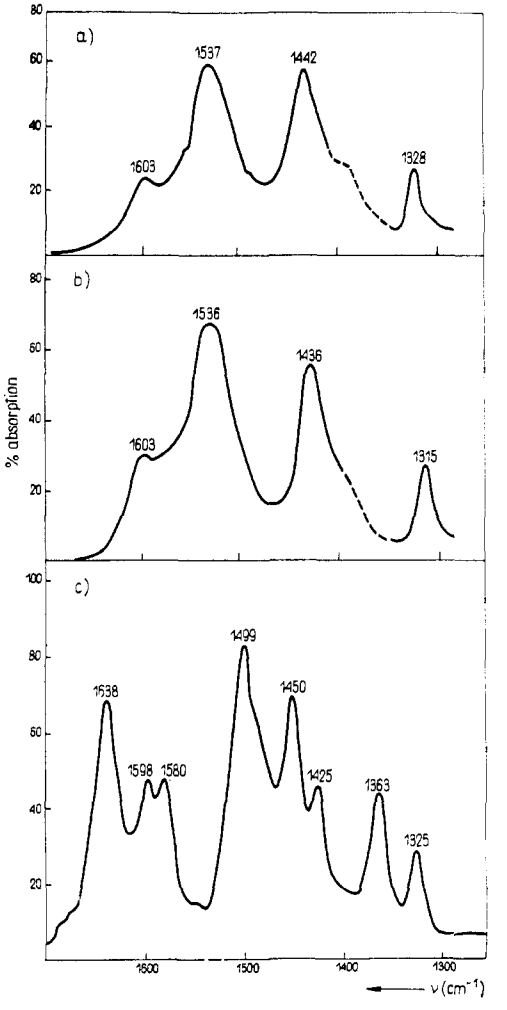
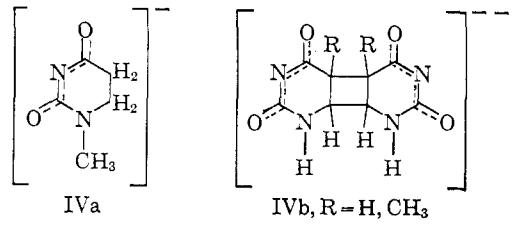


Figure 8. Infrared spectra of the monoanionic forms of: (a) thymine photodimer in 1 *N* NaOD in D_2O, c = 0.13 *M*, l = 25 μ; (b) uracil photodimer in 1 *N* NaOD in D_2O, c = 0.17 *M*, l = 25 μ; (c) 1-methyluracil in 0.1 *N* NaOD in D_2O, c = 0.228 *M*, l = 25 μ. (Note: The high p*K* values for the photodimers[17] necessitated the use of 1 *N* NaOD in order to obtain the monoanionic forms.)



The infrared spectra of the monoanions of the U-dimer and T-dimer in aqueous medium (D_2O, pD 14) in the spectral range 1750–1250 cm.$^{-1}$ are represented in Figure 8. The dimer samples employed[17] consisted of a mixture of stereoisomers of unknown composition formed photochemically.[19]

The dynamic interaction between the resonant bond systems (O=C-N=C-O)⁻ with their adjacent localized bonds may, to a first approximation, be assumed negligible, and, since these systems in the dimer monoanion are further separated by the cyclobutane bonds, the effect of isomerism on the frequencies of interest here may be ignored. Hence each delocalized bond system may be treated as one possessing C_{2v} symmetry, and the four possible valence oscillations for this system should show up as strong bands because of the large variations in dipole moments accompanying the vibration of a bond system with delocalized π- and n-electrons.

The infrared spectra of both dimer anions lack the two very strong carbonyl bands, 1722 and 1687 cm.$^{-1}$, exhibited by the neutral form of U-dimer in KBr,[20] but they absorb intensely at about 1500 and 1400 cm.$^{-1}$ (Figure 8; Table IV). In the U-dimer anion these bands are at 1536 ($\epsilon^A \sim 1150$) and 1436 cm.$^{-1}$ ($\epsilon^A \sim 850$)[21] and are accompanied by two weaker, although still intense, bands at 1603 ($\epsilon^A \sim 415$) and 1315 cm.$^{-1}$ ($\epsilon^A \sim 330$).[22] On the basis of the assumptions made in the previous paragraph, these four bands are

(16) C. Janion and D. Shugar, *Acta Biochim. Polon.*, 7, 309 (1960).

(17) A. Smietanowska and D. Shugar, *Bull. Acad. Polon. Sci. Classe II*, **9**, 375 (1961).

(18) E. Sztumpf and D. Shugar, *Biochim. Biophys. Acta*, **61**, 555 (1962).

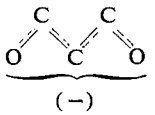
(19) D. L. Wulff and G. P. Fraenkel, *ibid.*, **51**, 332 (1961).

(20) The neutral forms of the dimers are highly insoluble in water.

(21) In accordance with point a of the preceding paragraph, the $\pi\epsilon^A$ values are calculated for the monomer units of the dimers (see Table IV).

(22) Under the experimental conditions employed, bands corresponding to CH_3 and CH deformations, and stretching frequencies of single bonds, could not be resolved from background noise.

assigned to the four basic stretching frequencies of the delocalized bond system $(O{=}C{=}N{=}C{=}O)^-$. The two very intense bands 1536 and 1436 cm.$^{-1}$ correspond, respectively, to the asymmetric and symmetric vibrations dominated by the vibrational energies of the most polar bonds, *i.e.*, $C{=}O^-$. The two remaining weaker and more widely split bands, 1603 and 1315 cm.$^{-1}$, would then correspond to the asymmetric and symmetric vibrations of the two adjacent C=N bonds. The same general characteristics are exhibited by the spectrum of the acetylacetone anion,[23] and the anion of dimedone, a cyclic 1,3-diketone with an analogous conjugated bond system[24]



The 1482-cm.$^{-1}$ band in the latter, interpreted as an asymmetric vibration $\nu \sim \nu(C{=}O)^-$, has an $\epsilon^A \sim 2000$, hence about 50% greater than that of the corresponding band in the anion of the dimer. By contrast the bands assigned to the vibrations of the adjacent C=C bonds are less intense, split to a lesser degree, and are at a lower resultant frequency, 1404 cm.$^{-1}$, as compared to 1459 cm.$^{-1}$ ($\frac{1}{2}$(1603 + 1315)) in the spectrum of the dimer anion. This implies, in agreement with the differences in electronegativity between O, N, and C, a more pronounced difference in polarity and π-electron density between the C=O and C=C bonds in the dimedon anion than between the C=O and C=N bonds in the dimer anion. Undoubtedly a fraction of the negative charge is located on N-3 in the dimer anion.[25]

Spectra and Structure of Anions of 1-Methyl-2,4-diketopyrimidines. We may now proceed to an examination of these spectra, which may be considered as those of the dimer anion resonance conjugated bond system, perturbed by the $C_5{=}C_6$ bond potentially conjugated with it. The presence of this bond is known to result in a bathochromic shift of the long wave length $\pi \rightarrow \pi^*$ absorption band from about 2350 Å. for the monoanions of 5,6-dihydropyrimidines[14] to 2650 Å. in the spectrum of 1-MeU$^-$. In the infrared spectra of the neutral, diketo, forms of 1-MeU, 1-MeT, and their dihydro derivatives (including the UU and TT dimers), there are no marked differences in the location of the two carbonyl frequencies,[8] from which it is apparent that the $C_5{=}C_6$ bond does not appreciably affect the π-electron density of the carbonyl groups in 1-MeU and 1-MeT. Additional support for this is provided by the frequency of the ν(C=C) band in 1-MeU, 1657 cm.$^{-1}$, hence in the spectral range expected for weakly coupled C=O and C=C bonds, and by its low extinction, indicating the absence of polarization of the bond. In the short wave length spectral region embraced herein, both 1-MeU$^-$ and 1-MeT$^-$ exhibit strong bands (Figures 1 and 8) at 1638 ($\epsilon^A \sim 900$) and 1656 cm.$^{-1}$ ($\epsilon^A \sim 800$), respectively, which are absent in the spectra of the corresponding dimer anions. Apart from the foregoing difference the spectra of 1-MeU$^-$ and 1-MeT$^-$ closely resemble those of the dimers, all containing strong bands at approximately 1600, 1500, and 1450 cm.$^{-1}$ (*cf.* Figures 1 and 8). However, in the long wave length region there are several strong bands not observed in the dimer anion spectra but present in the spectra of the neutral forms of the 1-methyl derivatives.[8]

Assignment of the origins of the 1656- and 1638-cm.$^{-1}$ bands in 1-MeU and 1-MeT was considerably simplified by the observation that the corresponding band in 1-HFU$^-$, ~1666 cm.$^{-1}$, is much less intense ($\epsilon^A \sim 300$). This suggested that the bond responsible is the $C_5{=}C_6$. In the anions 1-MeU$^-$ and 1-MeT$^-$ this bond, if its double-bond character is basically unaffected as a result of its resonance conjugation with the principal resonance conjugated bond system, should at least be highly polarized; it should therefore exhibit a high extinction in the infrared. In 1-HFU$^-$, on the other hand, the inductive effect of the highly electronegative fluorine on C-5, which is directly opposed to the direction of polarization of $C_5{=}C_6$ owing to its conjugation with the system $(O{=}C{=}N{=}C{=}O)^-$, will diminish the effect of the latter; hence the extinction of the band corresponding to $\nu(C_5{=}C_6)$ in 1-HFU$^-$ should be appreciably diminished. This conclusion derives indirect support from observations on the photochemical reactivity of monoanions such as 1-HU$^-$ and 1-HFU$^-$. Both 1-MeU$^-$ at pH 12 and 5-fluorouridine at pH 9.5 (almost fully dissociated form, pK = 7.7[6]) undergo photochemical hydration at the 5,6-bond much the same as their neutral forms,[26] indicating that the properties of the unsaturated $C_5{=}C_6$ bond are retained in the ionized molecules.[27]

The remaining bands in both N-1-substituted anions, in the spectral region where the corresponding dimer anions have been shown to exhibit the bands characteristic of the bond system $(O{=}C{=}N{=}C{=}O)^-$, may be analogously interpreted (Table III), bearing in mind the evidence presented above for the relatively low degree of conjugation of this bond system with $C_5{=}C_6$. It would then follow that the most intense bands, 1499 ($\epsilon^A \sim 1360$) and 1450 cm.$^{-1}$ ($\epsilon^A \sim 910$), in 1-MeU$^-$ (or 1490 and 1458 cm.$^{-1}$ in 1-MeT$^-$) are due to the out-of-phase and in-phase oscillations in which the dominant vibrational energy contributions are those of the most polar bonds, *viz.* $C{=}O^-$. At about 1600 cm.$^{-1}$, where the dimer of U$^-$ exhibits a band assigned to the antisymmetric vibration ν^{B_1}(C=N), there are two equally intense bands, 1598 and 1580 cm.$^{-1}$ (in 1-MeT$^-$ the absorption in this region is somewhat complicated by the superposition of a third band, so that the intensity ratio of the 1592 and 1574 cm.$^{-1}$ bands is altered). Both of these bands are assigned to ν^{B_1}(C=N) on the basis of the observed splitting of the antisymmetric frequencies ν^{B_1}(C=C) in the anion of acetylacetone.[23] It was not found feasible, at least for the present, to identify in the spec-

(23) K. L. Wierzchowski and D. Shugar, *Spectrochim. Acta*, 21, 943 (1965).

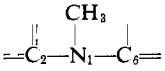
(24) E. Litońska, K. L. Wierzchowski, and D. Shugar, in preparation.

(25) M. Tsuboi, Y. Kyogoka and T. Shimanouchi, *Biochim. Biophys. Acta*, 55, 1 (1962), present the structure of the uridine monoanion with the negative charge on N-3, although they correctly suggest that dissociation of the N-3 proton is accompanied by charge delocalization.

(26) For 1-MeU$^-$ at pH 12, photochemically induced addition of water to the $C_5{=}C_6$ bond may be observed only at low temperature, since at room temperature the base-catalyzed dehydration reaction is very rapid: M. Fikus and D. Shugar, in preparation.

(27) This is in agreement with the findings of B. I. Sukhorukov, V. I. Poltev, and L. A. Blumenfeld, *Biophysics* (USSR) (English Transl.), 9, 266 (1964), who showed that the apparent pK of the singlet excited state in the reaction $U^* + H_2O \rightleftharpoons 1\text{-}HU^{-*} + H_3O^+$ is only 0.5 pK unit above that for the ground state.

trum of 1-MeU$^-$ (or 1-MeT$^-$) the second ν^{B_1}(C=N) frequency corresponding to the in-phase vibration of both bonds, because of the appearance in the region 1400–1300 cm.$^{-1}$ of several strong peaks with frequencies and extinctions comparable to those of bands appearing in the spectra of U-dimer and 1-MeU·H_2. It is very likely that two of these bands are due to the bond system



which would not be expected to be appreciably perturbed by dissociation of the N-3 proton.

In general the foregoing empirical analysis of the infrared spectra of the monoanions of 1-R-2,4-diketopyrimidines (R = H, Me) provides an interpretation consistent with the proposed resonance structure (III) of these molecules.[28]

The spectra of the type III monoanions closely resemble those of the parent diketones, except for a small hypsochromic shift. Hence the delocalization of π- and n-electron density in the bond systems (O=C_2=N_3=C_4=O)$^-$ and N_1–H (or CH_3) of diketopyrimidines leads only to a small increase in the energy of the first singlet $\pi \rightarrow \pi^*$ transition. A similar situation is found for some monoazines.[29]

Finally the tautomeric equilibrium point between the two monoanionic species II and III, determined by the ratio of the microscopic dissociation constants K_{II} and K_{III}, which is therefore independent of pH,[30] is seen to be dependent on external factors such as the solvent, as well as on internal factors such as the nature of the substituent on C-5 and/or C-6. In addition to the examples cited in the above text, we have found from an examination of the spectra of other 2,4-diketopyrimidines that 5-carbethoxyuracil and 5-nitrouracil appear to ionize almost uniquely to type II monoanions. This matter is the subject of further study.

Experimental Section

The dioxane employed was Schardt spectral grade. D_2O was Norsk Hydro, purity 99.98%. Thymine was Calbiochem A grade, chromatographically homogeneous; 5-fluorouracil was a gift of Dr. R. Duschinsky of Hoffman–LaRoche Inc., and the Cancer Chemotherapy National Service Center, Bethesda, Md.

1-Methyluracil and 1-methylthymine were prepared according to the method of Hilbert and Johnson.[31]

3-Methylthymine. This was prepared by a method similar to that previously employed for 3-methyl-5-bromouracil.[6] To 800 mg. of 1-acetylthymine, prepared as described by Spector and Keller,[32] was added 15 ml. of anhydrous ether saturated with diazomethane. After 24 hr. at room temperature, a second 15-ml. portion of diazomethane-saturated ether was added, followed by 100 ml. of ether. Following another 24 hr. insoluble material was filtered off and the filtrate was brought to dryness. The resulting yellow oily residue was dissolved in 100 ml. of water, the pH was brought to 9 with ammonia, and the solution was extracted with chloroform. The chloroform extract was brought to dryness and the residue was dissolved in ethanol and precipitated by addition of ether. The product, 600 mg., was found chromatographically to be contaminated with about 20% 1,3-dimethylthymine. Crystallization from ethanol gave 170 mg. (28%) of chromatographically homogeneous product, m.p. 202° (lit.[33] m.p. 202–205°).[34]

Ultraviolet Extinction Coefficients of Neutral and Anionic Forms of 1-Methyl-5-fluorouracil and 3-Methyl-5-fluorouracil. To a solution of 6.5 mg. of 5-fluorouracil in 2 ml. of water was added 20 μl. of dimethyl sulfate and the mixture was agitated vigorously for 30 min. Ascending paper chromatography with Whatman No. 1 and *n*-butyl alcohol–formic acid–water (77:10:13, v./v./v.) demonstrated four spots with R_f values of 0.47, 0.60, 0.70, and 0.80. The first of these corresponded to 5-fluorouracil and the last to authentic 1,3-dimethyl-5-fluorouracil.[15] Spectral examination, at neutral and alkaline pH, of the eluate of the spot with R_f 0.60 showed that it must be 1-methyl-5-fluorouracil, by comparison with 1-methyluracil and 1-methyl-5-bromouracil.[2,6] The spot with R_f 0.70 was similarly identified as 3-methyl-5-fluorouracil. If the reaction time is prolonged to 1 hr., the only product is 1,3-dimethyl-5-fluorouracil.

About 1 mg. of the reaction mixture was chromatographed as above, the spots with R_f 0.60 and 0.70 were eluted, and the acid (pH 2, neutral form) and alkaline (pH 11, monoanionic form) spectra were recorded. The molar extinctions of the neutral forms were calculated from the extinction of 5-fluorouracil, taking advantage of the observation that the ratios of the extinctions of the neutral forms of the methylated derivatives of uracil to uracil itself,[2] and of the methylated derivatives of 5-bromouracil to bromouracil itself,[6] are the same, assuming that this also applies to the fluorouracil analogs. The extinction coefficients for the alkaline forms were calculated from the optical density ratios of the neutral and alkaline eluates above.

	pH 2		pH 11	
Compd.	Max.	Max.	Max.	Max.
1-Methyl-5-fluorouracil	274 mμ	8.4×10^3	271 mμ	6.2×10^3
3-Methyl-5-fluorouracil	266 mμ	6.2×10^3	292 mμ	8.2×10^3

As a check on the foregoing, the extinction coefficient for 1,3-dimethyl-5-fluorouracil was calculated from that for 5-fluorouracil, to give a value of 7.5×10^3 at 274 mμ (lit.[35] 7.4×10^3 at 274 mμ).

Infrared spectra were recorded on a Hilger H800 spectrophotometer with NaCl optics and the ultraviolet spectra with a Unicam SP700 and Hilger UVISPEK. Variable path length cuvettes with CaF_2 windows were used for the infrared spectra; this made it possible, following recording of the infrared spectra, to decrease the path length and record the ultraviolet spectra of the same solutions without change of concentration. It proved possible in this way to verify that the tautomeric equilibrium point between the two types of mono-

(28) As regards the proposal of Spinner,[4] utilized by Nakanashi, *et al.*,[1] to postulate structure I for the anion 1-MeU$^-$ (and 1-HU$^-$), this was based on a rather fragmentary analysis of the infrared spectrum of 2-ketopyrimidine, which will be discussed in detail elsewhere.[8]

(29) S. F. Mason, *J. Chem. Soc.*, 1253 (1959).

(30) We are indebted to one of the referees for helpful comments on this point.

(31) G. E. Hilbert and T. B. Johnson, *J. Am. Chem. Soc.*, **52**, 2001 (1930).

(32) L. B. Spector and E. B. Keller, *J. Biol. Chem.*, **232**, 185 (1958).

(33) T. B. Johnson and S. H. Clapp, *J. Biol. Chem.*, **5**, 56 (1908).

(34) We are indebted to Dr. Barbara Zmudzka for the preparation of this compound.

(35) M. Fikus, K. L. Wierzchowski, and D. Shugar, *Photochem. Photobiol.*, 4, 521 (1965).

anions was virtually independent of concentration, so that the infrared and ultraviolet data were quantitatively comparable. Concentrations were determined from the known extinction coefficients of the diketo forms of the various compounds.[2,6]

For the infrared spectra in D_2O solution, path lengths varied from 15 to 50 μ. The cuvettes were calibrated by means of the known band extinction of the carbonyl group of acetone. Nonetheless the evaluations of molar extinctions (ϵ^A) from the measured path lengths were limited to an accuracy of ± 5–10%.

The shift in equilibrium point of the thymine monoanion equilibrium mixture as a function of temperature was measured in cuvettes fitted with ground stoppers in a specially constructed thermostated carriage fitted to the Hilger UVISPEK.

Acknowledgments. We are indebted to the World Health Organization for financial support of this work through the Department of Biophysics, University of Warsaw.

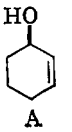
Steroids. CCLXXXIII.[1] Stereochemically Controlled Simmons–Smith Methylenation of Homoallylic Alcohols of Low Reactivity. A New Synthesis of 10α-Testosterone

R. Ginsig[2] and A. D. Cross

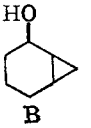
Contribution from the Research Laboratories, Syntex, S. A., Apartado 2679, Mexico, D. F. *Received June 28, 1965*

Employing published reaction conditions, no Simmons–Smith methylenation of steroid 5(10)-en-3α-ols occurred. A modification of the reaction method is reported which gave stereospecific α-face addition in up to 85% yields. Based on this new procedure a novel synthesis of 10α-testosterone (Ia) was achieved. With several reagents known to effect cleavage of cyclopropanes the 5α,10α-methylene steroids afforded principally 5α-substituted methyl derivatives, thus foiling attempts to prepare 19-substituted 10α-testosterone analogs.

In the few years which have elapsed since Simmons and Smith first described the over-all addition of methylene to double bonds by treatment of the olefin with methylene diiodide and a zinc–copper couple,[3] numerous syntheses of cyclopropanes by this method have been described.[4] Concurrently, Simmons and his co-workers studied the reaction mechanism observing the formation of ethers as reaction by-products and the operation of a large steric effect.[5] Also of interest were the observations by Winstein and others that the addition of methylene to the double bond of cyclic allylic and homoallylic alcohols takes place stereospecifically *cis* to the hydroxyl group (A → B), the stereochemical control being lost on acetylation of the alcohol.[6]

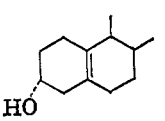


A



B

As part of a broad program in these laboratories aimed at developing new synthetic routes to steroids of abnormal configuration,[7] we required an efficient chemical synthesis of 10α-androst-4-en-3-ones. A synthesis of 10α-testosterone (Ia) involving a photochemical transformation, then six chemical steps, had already been reported.[8] A purely chemical route to 10α-androstan-2-ones was subsequently described involving methyl Grignard addition to estr-1(10)-en-2-ones.[9] The recent findings that metal hydride reductions of steroid 5(10)-en-3-ones (readily available from estrone 3-ethers by Birch reduction) give predominantly 5(10)-en-3α-ols (C)[10,11] suggested a simple chemical synthesis of 10α-androst-4-en-3-ones according to the scheme C → F.



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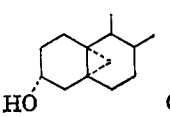

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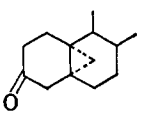

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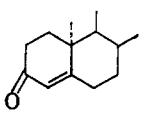

F

Accomplishment of the synthesis depended on stereochemical control by the 3α-hydroxy group in the Sim-

(1) Steroids. CCLXXXII: J. A. Edwards, H. Carpio, A. Cruz, and M. J. Teran, *J. Org. Chem.*, in press.

(2) Syntex Postdoctoral Fellow, 1963–1964, Mexico.

(3) H. E. Simmons and R. D. Smith, *J. Am. Chem. Soc.*, 80, 5323 (1958); 81, 4256 (1959).

(4) See, for example, N. T. Castelluci and C. E. Griffin, *ibid.*, 82, 4107 (1960); E. F. Ullman and W. J. Fanshawe, *ibid.*, 83, 2379 (1961); A. C. Cope, S. Moon, and C. H. Park, *ibid.*, 84, 4843 (1962), and later papers in this series; R. S. Boikess and S. Winstein, *ibid.*, 85, 343 (1963); W. D. Kumler, R. Boikess, P. Bruck, and S. Winstein, *ibid.*, 86, 3126 (1964).

(5) E. P. Blanchard and H. E. Simmons, *ibid.*, 86, 1327 (1964); H. E. Simmons, E. P. Blanchard, and R. D. Smith, *ibid.*, 86, 1347 (1964); *cf.* also G. Wittig and F. Wingler, *Chem. Ber.*, 97, 2146 (1964).

(6) S. Winstein, J. Sonnenberg, and L. De Vries, *J. Am. Chem. Soc.*, 81, 6523 (1959); S. Winstein and J. Sonnenberg, *ibid.*, 83, 3235 (1961); E. J. Corey and R. L. Dawson, *ibid.*, 85, 1782 (1963); W. G. Dauben and G. H. Berezin, *ibid.*, 85, 468 (1963); W. G. Dauben and A. C. Ashcraft, *ibid.*, 85, 3673 (1963); P. Radlick and S. Winstein, *ibid.*, 86 1866 (1964).

(7) J. A. Edwards, P. Crabbé, and A. Bowers, *ibid.*, 85, 3313 (1963); J. A. Edwards, H. Carpio, and A. D. Cross, *Tetrahedron Letters*, 3299 (1964); A. D. Cross, J. A. Edwards, P. Crabbé, H. Carpio, and E. Denot, to be published.

(8) R. Wenger, H. Dutler, H. Wehrli, K. Schaffner, and O. Jeger, *Helv. Chim. Acta*, 45, 2420 (1960).

(9) M. Torigoe and J. Fishman, *Tetrahedron Letters*, 1251 (1963).

(10) S. G. Levine, N. H. Eudy, and E. C. Farthing, *ibid.*, 1517 (1963).

(11) A. D. Cross, E. Denot, R. Acevedo, R. Urquiza, and A. Bowers, *J. Org. Chem.*, 29, 2195 (1964).