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THE IONIZATION OF MORPHINE, HYDROXYAMPHETAMINE AND (+)-TUBOCURARINE CHLORIDE AND A NEW METHOD FOR CALCULATING ZWITTERION CONSTANTS

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1 An improved method for estimating the zwitterion constants of phenolic amines is described which involves the exploratory least-squares fit of absorbance (at a suitable wavelength) to pH, starting with estimates of pK_1 and pK_2 obtained electrometrically.

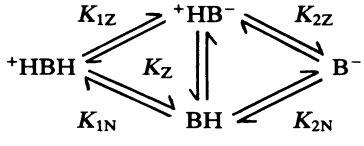
2 With the method it is possible to see that hydroxyamphetamine (α-methyltyramine) has a higher zwitterion constant than tyramine and the zwitterion constants of both compounds are lower at 37°C than at 25°C.

3 The zwitterion constant of morphine is not reduced by raising the temperature from 25° to 37°C and the effect of temperature is much greater in compounds with a primary or secondary amino group than with those containing a tertiary amino group. Some zwitterions may be stabilized by hydration and their formation will be reduced by a rise in temperature which will break up water structure.

4 From electrometric titrations with (+)-tubocurarine chloride in 0.1 M NaCl estimates of pK_1, pK_2 and pK_3 were 7.6, 8.65 and 9.65 at 25°C and 7.4, 8.6 and 9.7 at 37°C, compared with 7.8, 8.85 and 9.75 given by Perrin (1980). However, the effects of pH on absorbance show that the phenolic groups lose a proton before the ammonium group so there is extensive zwitterion formation which is probably greater at 25° than at 37°C. The *p*-phenolic group (position 13) probably ionizes first with the phenate form stabilized by hydration involving water molecules and the protonated form of the (1-) ammonium group.

Introduction

In the ionization of phenolic amines, such as morphine and tyramine, the neutral species exists as an equilibrium mixture of uncharged molecules and zwitterions. The process can be represented:



where the proton attached to nitrogen is written on the left and that attached to oxygen is written on the right. The extent of zwitterion formation will depend upon the ease with which a proton leaves the phenolic group rather than the amino group and measurements of the pK_a of phenolic quaternary ammonium salts have shown that the presence of a positive charge on a nearby nitrogen atom greatly enhances the ionization of a phenolic group (Armstrong & Barlow, 1976; Barlow & Burston, 1980).

With compounds having two phenolic groups, such as catechols, the ionization can proceed a step further but the immediate effect, particularly with *o*-diphenolic compounds such as dopamine, is that the loss of the first proton from a phenolic group is made easier, with the consequent increase in zwitterion formation. Estimates of zwitterion constants, K_Z, depend considerably on the method by which they were obtained (see below) but values of around 10 were obtained for dopamine, compared with around 2 for tyramine (Barlow & Burston, 1980). The recent report of pK_a values for (+)-tubocurarine chloride (Perrin, 1980) with the loss of the first proton ascribed to the nitrogen atom is therefore of great interest as it implies that there is no zwitterion formation.

(+)-Tubocurarine chloride (Figure 1a) contains one ammonium group, one tertiary amino group and 2 phenolic groups widely separated (in space); the pK_a values were 7.80, 8.85 and 9.75 and if the first proton is lost from the amino group, absorbance from the phenate group ($-O^-$) should not occur until the

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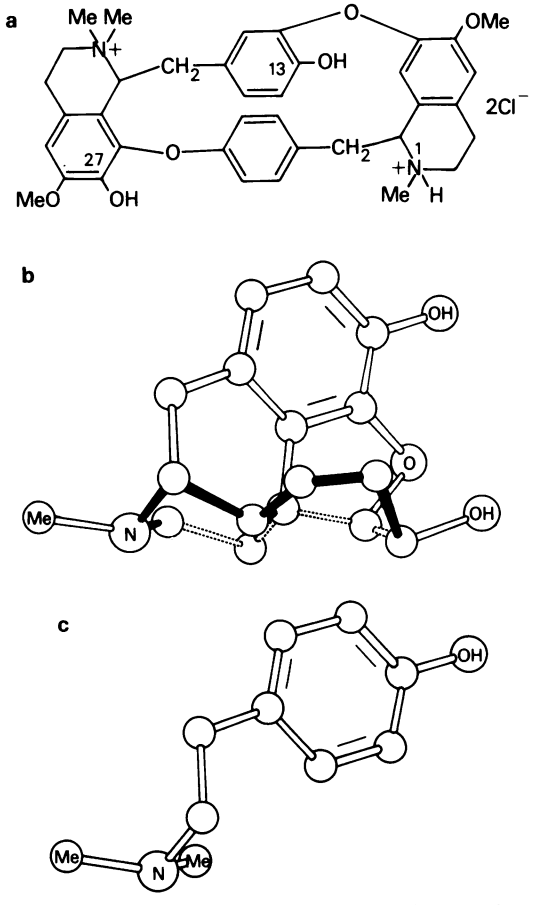

Figure 1 (a) Structure of (+)-tubocurarine chloride; (b) (−)-morphine, re-drawn from Mackay & Hodgkin (1955, A crystallographic examination of the structure of morphine, *J. chem. Soc.*, Figure 3 on page 3263). The (buckled) octahydroisoquinoline ring is drawn in a horizontal plane with the nearer edge represented by solid bonds and the further edge by dotted bonds; (c) N,N-dimethyltyramine.

second proton is lost. Results obtained by Kalow (1954) indicate appreciable absorption at 295 nM even below pH 8, however, and the matter appears to require further investigation.

Zwitterion formation with morphine also appears to require checking. Schill & Gustavii (1964) estimated $K_Z = 0.4$ and it would be interesting to know if there is a real difference between this and the value for N,N-dimethyl tyramine (K_Z about 1, Barlow & Burston, 1980), because the latter forms part of the morphine structure (Figure 1b). Hydroxyamphetamine (α-methyltyramine) is of interest because the α-methyl group should increase the strength of the amino group. From values for 2-phenethylamine and amphetamine listed in Perrin (1965) the pK_a of the group might be expected to increase by 0.2 units. This in turn might make the phenolic group more acidic and so there should be a higher proportion of zwitterions than with tyramine.

In the course of this work methods for estimating zwitterion constants have been reviewed and a new method has been evolved.

Methods

Electrometric titrations of salts of phenolic amines

These were made as described by Armstrong & Barlow (1976) but with the more accurate digital pH meter (Metrohm E 500) used by Barlow & Burston (1980). Weighed amounts of the salts were dissolved in water or 0.1 M NaCl and the pH was measured after the addition of accurately measured volumes of 0.1 M NaOH. The experiment was made under nitrogen and the temperature was 25 ± 0.1° or 37 ± 0.2°C. The relation between the total concentration of compound, C, the concentration of alkali, A, the ionization constants K_1 and K_2, the hydrogen and hydroxyl ion activities and the activity coefficient, f, is:

$$\frac{A + [H^+] - [OH^-]}{f[H^+] + 2K_2} = \frac{K_1 C}{[H^+]^2 + fK_1[H^+] + K_1K_2} \quad (1)$$

The activity coefficient was calculated from the ionic strength using Debye-Hückel parameters (Armstrong & Barlow, 1976). The ionization constants, K_1 and K_2, were calculated from pairs of experimental values of C, A, $[H^+]$ and $[OH^-]$ by the method of Britton (1942; see Albert & Sergeant, 1962) and, as in previous work, the pairs consisted of results arranged on either side of the half-neutralization point. Equation 1 is slightly different from that given by Armstrong & Barlow (1976), which contains errors in the placing of f. For titrations in water with $f \doteqdot 1$ the error is negligible; for titrations in 0.1 M NaCl with $f \doteqdot 0.79$, pK_1 was underestimated by 0.1 pH units and there were negligible effects on pK_2. As was found in previous work (Armstrong & Barlow, 1976; Barlow & Burston, 1980) the estimates of pK were not independent of concentration, in spite of the incorporation of activity coefficients into the calculations. The results were fitted to the relation $pK = pK_0 + mC^{\frac{1}{2}}$, where C is the concentration in mid-titration. The value at 10 mM, in the middle of the range of concentrations, is likely to be most accurate, though all values are restricted by the limit to which the instrument can be calibrated (± 0.01 pH units).

Ionization of (+)-tubocurarine chloride

The pK values quoted by Perrin (1980) are not accompanied by any experimental details; it seems likely that the temperature was 25°C. New measurements have therefore been made in 0.1 M NaCl at 37°C, conditions close to those in which the drug is used. The ionization can be represented:

$$^{2+}HBH_2 \rightleftharpoons {}^{+}BH_2 + H^+ \rightleftharpoons {}^{+}BH^- + H^+ \rightleftharpoons {}^{+}B^{2-} + H^+$$

and the relation between C, A, f_1 (for univalent ions), f_2 (for divalent ions), K_1, K_2, K_3 and the hydrogen and hydroxyl ion activities is given by the expression:

$$Y = \frac{C}{C-(A+[H^+]-[OH^-])} = \frac{f_1[H^+]^3 + f_2K_1[H^+]^2 + f_1f_2K_1K_2[H^+] + f_2K_1K_2K_3}{f_1[H^+]^3 - f_1f_2K_1K_2[H^+] - 2f_2K_1K_2K_3} \quad (2)$$

This can be rearranged to give

$$(Y-1)\frac{f_1}{f_2}[H^+]^2 = K_1[H^+] + f_1K_1K_2(Y+1) + K_1K_2K_3\frac{(2Y+1)}{[H^+]} \quad (3)$$

which is of the form $Z = a_1X_1 + a_2X_2 + a_3X_3$, where $X_1 = [H^+]$,

$$X_2 = f_1(Y+1) \text{ and } X_3 = \frac{(2Y+1)}{[H^+]}$$

Values of Z, X_1, X_2 and X_3 can be calculated, from C, A and pH, and the method of least-squares used to calculate the coefficients, a_1, a_2 and a_3 which give the best fit. Because all the variables, Z, X_1, X_2 and X_3 are derived from H^+ and Y it is difficult to know which fit is likely to introduce the least bias. All forms (Z on X_1, X_2 and X_3; X_1 on Z, X_2 and X_3 etc.) were made and the values of K_1, K_2 and K_3 were calculated from the coefficients, a_1, a_2 and a_3. The estimates of pK_1 so obtained were usually close but there were some differences between estimates of pK_2 obtained from the same titration by the different fitting procedures, and with pK_3 the differences were substantial.

An alternative procedure is to fit the volume of alkali added to the pH. Equation 2 can be written:

$$\frac{C}{C-(A+[H^+]-[OH^-])} = ZZ$$

where ZZ is the fraction containing terms in f_1, f_2, K_1, K_2, K_3 and $[H^+]$. Accordingly $A = C(1-ZZ) + [OH^-] - [H^+]$ and the expected volume of alkali, VA_{cal}, added to the original volume to produce the concentration A, can be calculated for particular values of pH, pK_1, pK_2, pK_3, f_1 and f_2. It is thus possible to obtain a least-squares fit of the volume of alkali added, VA_{obs}, to the pH observed by allowing pK_1, pK_2 and pK_3 to alter and finding what changes decrease $S(VA_{obs}-VA_{cal})^2$. This method requires reasonable starting estimates of pK_1, pK_2 and pK_3 and these were obtained from the previous fitting procedures (see above).

Spectroscopic measurements

Solutions of 0.1 M NaOH and 0.1 M glycine were used, as in previous work (Barlow & Burston, 1980), to prepare a suitable range of buffers which were then diluted 1 to 10 with 0.1 M NaCl. Solutions of the phenolic amines, usually 0.2 mM, were then prepared by diluting a 10 mM stock solution in water 0.5 to 25 with each of the diluted buffers. The absorbance was scanned with a Unicam SP 1800 spectrophotometer from 285–320 nm, with precise measurements at 300 nm. The solutions were kept stoppered and their pH was measured under nitrogen. The absorbance of the phenolic form was estimated from solutions in 0.1 M HCl, diluted 1 to 10 with 0.1 M NaCl; it was very small at this wavelength. The absorbance of the phenate form was estimated from similar solutions made up with 0.1 M NaOH in place of 0.1 M HCl. The ionic strength was assumed to be that of 0.1 M NaCl, because all other ions were present only in much lower concentrations, and the activity coefficient, f, was taken as 0.79. The temperatures were $25 \pm 0.2°$ and 37 ± 0.2°C.

Estimation of zwitterion constants: phenolic amines

In a previous assessment of methods for measuring zwitterion constants, Barlow & Burston (1980) divided them into: (A) the use of analogues, (B) the combination of spectroscopic and electrometric data and (C) estimates based only on spectroscopic measurements. Method (A) assumes that the microscopic ionization constants are the same as the pK_as of appropriate compounds, e.g. that pK_{1N} is the same as the pK_a of a non-phenolic amine and that pK_{1Z} is the same as the pK_a of the phenolic quaternary ammonium salt. Such assumptions may not be valid. Method (B) is complicated by the fact that, even after the incorporation of activity coefficients calculated from Debye-Hückel theory, electrometric pK_as are not strictly constant and there is some uncertainty in extrapolating from the concentrations used in the electrometric titrations (5–20 mM) to the much lower concentrations (0.2 mM) used in the spectroscopic experiments.

In theory method (C) should produce the most accurate estimates. The ratio of phenate to phenolic, R, calculated from the absorbance observed at a particular pH and that observed in extremely acid

and extremely alkaline solutions, is related to the hydrogen ion activity, H^+, the activity coefficient, f, K_Z, K_{1Z} and K_{2Z} by the expression:

$$R[H^+]^2 = \frac{fK_{1Z}}{K_Z}([H^+]K_Z - R[H^+]) + K_{1Z}K_{2Z}$$

A least-squares fit of $R[H^+]^2$ on $f[H^+]$ and $fR[H^+]$ can be made directly by calculus and used to calculate K_Z, K_{1Z} and K_{2Z}. Similary a fit of $fR[H^+]$ can be made on $f[H^+]$ and $R[H^+]^2$, likewise $f[H^+]$ can be fitted to $fR[H^+]$ and $R[H^+]^2$. These methods did not all give the same answers and it was not clear which was most likely to be correct. In the present work these methods frequently gave negative estimates of K_Z and K_{2Z} (or even of K_{1Z}).

In order to try to find out why this happened, theoretical values of absorbance and R were calculated. For a light path of 1 cm, the molar extinction coefficient $\varepsilon = [^+HBH + BH]\varepsilon_{acid} + [^+HB^- + B^-]\varepsilon_{alk}$ where ε_{acid} and ε_{alk} are the extinction coefficients for the phenolic and phenate forms respectively. The total concentration of phenolic amine =

$$[B^-]\left[\frac{[H^+]^2}{K_{1Z}K_{2Z}} + \frac{f[H^+]}{K_{2Z}}\left(1+\frac{1}{K_Z}\right) + 1\right]$$

$$= [B^-]\left[\frac{[H^+]^2}{K_1K_2} + \frac{f[H^+]}{K_2} + 1\right] = T[B^-]$$

where K_1 and K_2 are the (macroscopic) constants, obtained by electrometric titration. If Beer's Law is obeyed, the absorbance,

$$A = \left\{\left[\frac{[H^+]^2}{K_1K_2} + \frac{f[H^+]}{K_Z}\left(\frac{1}{1+K_Z}\right)\right]A_{acid} + \left[\frac{f[H^+]}{K_2}\left(\frac{K_Z}{1+K_Z}\right) + 1\right]A_{alk}\right\}/T \quad (4)$$

where A_{acid} and A_{alk} are the absorbances of the phenolic and phenate forms, respectively. It is thus possible to generate values of absorbance and pH for chosen values of K_1, K_2, K_Z, A_{acid} and A_{alk}, convert these into values of R and pH and check whether the fitting procedures gave the correct values of K_1, K_2 and K_Z.

It is necessary to be careful that with high values of pH and pK numbers do not become vanishingly small. In previous work, programmes were written in Fortran with small numbers in double-precision. In this work, calculations were made with a PET 2001 or 3032 microcomputer with programmes written in Basic and the problem was overcome by multiplying $[H^+]$ by a large number. If all pH values are reduced by the same amount, pK_{1Z} and pK_{2Z} will be reduced by this amount and K_Z will be unaffected. From theoretical values of R and pH all three methods give the same answer. Particular difficulty may be expected with fit of $fR[H^+]$ to $R[H^+]^2$ and $f[H^+]$, because R increases as $[H^+]$ decreases and the proportional change is smaller than with the other methods. All methods are very sensitive to errors in R at extreme ends of the pH range. A single overestimate at the more acid pH, for instance, can produce a negative estimate of K_Z. Because an error in the measurement of the absorbance affects the numerator and denominator in opposite ways its effect on R is exaggerated and this will be particularly marked with very small or very large values of R.

It would be better to use the absorbance itself in the fitting process. This is less sensitive to the effects of a single point and can be displayed graphically. The disadvantage is that the relation (equation 4) is complex and the fitting process must be exploratory, i.e. the difference between $S(A_{obs} - A_{cal})^2$ must be calculated for particular values of K_1, K_2 and K_Z and these are then altered so as to decrease the sum of squares. This method requires good starting estimates of K_1, K_2 and K_Z. The electrometric values of K_1 and K_2 should be somewhere near the values in the spectrophotometric experiments so K_Z can be altered so as to obtain a least-squares fit of absorbance on pH. In practice log K_Z was allowed to alter in steps until an increase or decrease produced a worse fit; the step size was then reduced and the process continued until the steps were less than 0.001 log units. The data were then examined to see what further improvement could be obtained by starting with these values of pK_1, pK_2 and log K_Z and allowing all three values to alter. The goodness of fit can be assessed from the ratio

$$\frac{S(A_{obs} - A_{cal})^2}{S(A_{obs})^2}$$

and should also be apparent from the graph of absorbance against pH.

(+)-Tubocurarine chloride

The ionization process shown above does not include the possibility that the species which has lost one proton is an equilibrium mixture, $^+BH_2 \rightleftharpoons {}^{2+}HBH^-$, and even this is an oversimplification because the proton might come from one of two different phenolic groups. The species which has lost two protons is also an equilibrium mixture, the protons could have come from the amino group and one or other of the phenolic groups or from both of the phenolic groups, i.e. $^+BH^- \rightleftharpoons {}^{2+}HB^{2-}$, though $^+BH^-$ should represent two species. It seems likely that the absorbance of the two phenate groups should be similar and it is possible to calculate the absorbance if no attempt is made to distinguish between the two phenolic groups, i.e.

$$K_{Z1} = \frac{[^{2+}HBH^-]}{[^+BH_2]} \text{ and } K_{Z2} = \frac{[^{2+}HB^{2-}]}{[^+BH^-]}$$

If C is the total concentration and A is the absorbance,

$$AC = ({}^{2+}HBH_2 + BH_2)A_{acid} + (0.5({}^{2+}HBH^- + {}^{+}BH^-) + {}^{2+}HB^{2-} + {}^{+}B^{2-})A_{alk}$$

where A_{acid} is the absorbance with both phenolic groups intact and A_{alk} is the absorbance with both ionized. It is not possible to derive the many constants simply from a least-squares fit of absorbance on pH but from the values of K_1, K_2 and K_3 obtained by electrometric titration the size of K_{Z1} and K_{Z2} can be estimated by inspection (see below).

Activity coefficients

In the electrometric titrations these were calculated, as previously, from the ionic strength, taking all ionised species into account. In the spectroscopic experiments the ionic strength was assumed to be that of the sodium chloride (0.1 M); all other species were present in much lower concentrations. For univalent ions, f was assumed to be 0.79 and for divalent ions, f_2 was taken as 0.4. In the re-evaluation of the results of Schill & Gustavii (1964) the ionic strength (0.06 M) gives f = 0.82. No attempt was made to convert zwitterion concentrations into activities.

Compounds

Morphine HCl was B.P. grade; hydroxyamphetamine HBr was a gift from Dr S.E. Smith, St. Thomas's Hospital Medical School, and had m.p. 190.2°C. (+)-Tubocurarine chloride was purchased from Sigma.

Results

The results of the electrometric titrations are shown in Table 1. For hydroxyamphetamine the interpolated values at 10 mM in 0.1 M NaCl at 37°C are 9.33 and 10.50 compared with 9.25 and 10.62 for tyramine in similar conditions (Barlow & Burston, 1980); at 25°C the corresponding values are 9.50 and 10.78, compared with 9.50 and 10.86 for tyramine. However, much lower values were obtained in experiments made in water. The results obtained with morphine (Table 1B) agree reasonably with those obtained by Schill & Gustavii (1964), though the pK_a values in water were again much lower. The insolubility of morphine base limited the range of concentrations which could be tested.

The estimates of the pK values of (+)-

Table 1 Electrometric titrations

A *Hydroxyamphetamine HBr:* $pK_a \pm$ s.e.

Conc. (mM)	pK_1	pK_2	*Number of estimates*
37°C 0.1M NaCl			
4.91	9.31 ± 0.02	10.50 ± 0.02	7
4.95	9.25 ± 0.01	10.40 ± 0.01	7
9.22	9.44 ± 0.04	10.36 ± 0.03	7
9.80	9.37 ± 0.01	10.53 ± 0.01	7
14.59	9.32 ± 0.01	10.52 ± 0.01	5
14.79	9.32 ± 0.01	10.49 ± 0.01	5
25°C, 0.1 M NaCl			
5.15	9.46 ± 0.01	10.70 ± 0.01	7
10.36	9.49 ± 0.01	10.77 ± 0.01	7
14.82	9.52 ± 0.01	10.81 ± 0.01	5
25°C, water			
5.07	8.85 ± 0.01	10.06 ± 0.02	7
10.05	9.06 ± 0.01	10.29 ± 0.02	7
14.62	9.15 ± 0.01	10.37 ± 0.01	5

These results were fitted by least squares to the expression $pK = pK_0 + mC^{\frac{1}{2}}$, with each estimate of pK weighted according to the reciprocal of the variance and where C is the concentration in mid-titration. The following equations were obtained:

37°C, 0.1 M NaCl $pK_1 = 9.14 + 0.06C^{\frac{1}{2}}$: pK_1 at 10 mM = 9.33
$pK_2 = 10.29 + 0.07C^{\frac{1}{2}}$: pK_2 at 10 mM = 10.50
25°C, 0.1 M NaCl $pK_1 = 9.36 + 0.04C^{\frac{1}{2}}$: pK_1 at 10 mM = 9.50
$pK_2 = 10.53 + 0.08C^{\frac{1}{2}}$: pK_2 at 10 mM = 10.78
25°C, water $pK_1 = 8.38 + 0.22C^{\frac{1}{2}}$: pK_1 at 10 mM = 9.07
$pK_2 = 9.63 + 0.21C^{\frac{1}{2}}$: pK_2 at 10 mM = 10.28

Table 1 Electrometric titrations *(cont.)*

B *Morphine HCl*

37°C, 0.1 M NaCl			
5.06 mM	8.16 ± 0.01	9.56 ± 0.01	7
10.43	8.18 ± 0.01	9.54 ± 0.02	5
25°C, 0.1 M NaCl			
1.54 mM	8.05 ± 0.01	9.47 ± 0.02	7
1.66	8.03 ± 0.01	9.52 ± 0.01	7
25°C, water			
3.40 mM	7.18 ± 0.01	8.69 ± 0.01	7
3.59 mM	7.22 ± 0.01	8.74 ± 0.04	3

Schill & Gustavii (1964) recorded pK_1 8.29, pK_2 9.49 at 20°C, ionic strength 0.06. Perrin (1965) lists pK_1 8.21 at 25°C. Other similar values are listed by Kaufman, Semo & Koski (1975).

C *(+)-Tubocurarine chloride*

		pK_1	pK_2	pK_3	*Ratio*
37°C 0.1 M NaCl					
5.82 mM	(i)	7.42	8.68	9.37	5.54×10^{-3}
	(ii)	7.42	8.68	negative	
	(iii)	7.42	8.63	9.81	5.32×10^{-4}
	(iv)	7.41	8.35	9.08	7.24×10^{-2}
	(v)	7.42	8.64	9.71	9.31×10^{-5}
	(vi)	7.46	8.62	9.72	4.89×10^{-5}
5.28 mM	(vi)	7.32	8.59	9.60	1.68×10^{-4}
4.90	(vi)	7.42	8.60	9.69	8.71×10^{-5}
25°C, 0.1 M NaCl					
1.76 mM	(vi)	7.60	8.68	9.63	1.15×10^{-4}
1.77 mM	(vi)	7.58	8.68	9.60	6.67×10^{-5}
1.80 mM	(vi)	7.66	8.70	9.71	2.81×10^{-5}
1.94 mM	(vi)	7.61	8.66	9.65	3.46×10^{-5}

The ratio indicates (the error sum of squares)/(the total sum of squares), i.e. indicates the fit. Methods (i) to (iv) involve the least-squares fit by calculus, method (v) involves the least-squares fit of volume of alkali added on pH by exploration with pK_1 kept fixed and pK_2 and pK_3 allowed to alter. In method (vi) all three pKs were allowed to alter. The pK_a values given by Perrin (1980) are 7.80, 8.85 and 9.75.

tubocurarine chloride (Table 1C) depend on how they are calculated and this is illustrated for one set of results in which methods (i) to (iv) are based on equation (3) above. From the values it is possible to provide starting estimates for the experimental fit of the volume of alkali added to pH first allowing only pK_2 and pK_3 to alter (v) and then (vi) allowing all three pK values to alter. This procedure was used in all subsequent experiments. The improvement in the fit is indicated by the decrease in the ratio of the error sum of squares to the total sum of squares. As with the experiments with morphine the range of concentrations which could be tested, particularly at 25°C, was limited by solubility. From the results reasonable estimates of pK_1, pK_2 and pK_3 would seem to be 7.6, 8.65 and 9.65 at 25°C and 7.4, 8.6 and 9.7 at 37°C, compared with the values 7.8, 8.85 and 9.75 given by Perrin (1980).

The spectroscopic results for morphine and hydroxyamphetamine are shown in Table 2, together with values obtained by Schill & Gustavii (1964). They used a graphical method described by Edsall, Martin & Hollingworth (1958) to calculate K_{1Z}, K_{2Z} and K_Z. In this log $R[H^+]$ is plotted against the fraction of phenate, α, and pK_{1Z} is obtained by extrapolation to $\alpha = 0$, whereas pK_{2N} is obtained by extrapolation to $\alpha = 1$. The range of values of log $R[H^+]$ is small, however, which makes extrapolation difficult and their data have been analysed by the methods used in this work. Our results (at 25°C) appear to overlap theirs (at 20°C; Table 2). The fit of absorbance to pH for morphine and hydroxyamphetamine is illustrated in Figures 2a and b. Although the zwitterion constant for hydroxyamphetamine is reduced by raising the temperature, the zwitterion constant for morphine does not seem to be.

With (+)-tubocurarine chloride there are too many variables for it to be possible to calculate zwitterion constants and pK_a values from the least-squares fit of absorbance to pH but if estimates of pK_1, pK_2 and pK_3 obtained by electrometric titration are used it is clear that there must be extensive zwitterion formation. This is illustrated in Figure 3, in

Table 2 Spectroscopic results

	pK_{1Z}	pK_1	pK_{2Z}	pK_2	K_Z	*Ratio*
A *Morphine HCl*, ionic strength 0.06, 20°C						
(Data of Schill & Gustavii (1964) re-analysed)						
(i)	8.81	(8.32)	9.17	(9.65)	0.49	1.68×10^{-3}
(ii)	8.80	(8.31)	9.14	(9.64)	0.47	1.18×10^{-3}
(iii)	8.78	(8.22)	9.02	(9.59)	0.38	2.62×10^{-4}
*	8.87	(8.31)	8.95	(9.51)	0.37	
(iv)		*8.29*		*9.49*	0.35	4.92×10^{-3}
(v)	(8.81)	8.22	(9.03)	9.62	0.42	1.81×10^{-3}
This work, 0.1 M NaCl, 25°C						
(iv)		*8.00*		*9.50*	0.46	2.60×10^{-3}
(v)	(8.37)	8.19	(9.24)	9.42	0.51	1.39×10^{-3}
0.1 M NaCl, 37°C						
(iv)		*8.12*		*9.58*	0.72	1.41×10^{-3}
(v)	(8.58)	8.21	(9.12)	9.50	0.72	5.81×10^{-4}
B *Hydroxyamphetamine HBr*, 0.1 M NaCl, 25°C						
(iv)		*9.36*		*10.53*	3.25	2.57×10^{-3}
(v)	(9.41)	9.31	(10.79)	10.89	3.87	1.04×10^{-3}
0.1 M NaCl, 37°C						
(iv)		*9.14*		*10.30*	1.91	7.68×10^{-4}
(v)	(9.30)	9.12	(10.18)	10.36	1.92	6.22×10^{-4}

Results were analysed by (i) the fit of $R[H^+]^2$ on $f[H^+]$ and $fR[H^+]$, (ii) the fit of $f[H^+]$ on $R[H^+]^2$ and $fR[H^+]$, (iii) the fit of $fR[H^+]$ on $R[H^+]^2$ and $f[H^+]$, (iv) allowing log K_Z to alter with pK_1 and pK_2 set at the values obtained electrometrically (shown in italics) and (v) allowing pK_1, pK_2 and log K_Z all to alter. The asterisk indicates the original values given by Schill & Gustavii from the graphical method of Edsall *et al.* (1958).

The goodness of the overall fit is indicated by the ratio, which is $\frac{S(R_{obs}-R_{cal})^2}{(R_{obs})^2}$ for methods (i), (ii) and (iii) and $\frac{S(A_{obs}-A_{cal})^2}{S(A_{obs})^2}$ for methods (iv) and (v). Values in parentheses have been calculated from those returned by the fitting process from the equations:

$pK_1 = pK_{1Z} - \log(1 + 1/K_Z)$; $pK_2 = pK_{2Z} + \log(1 + 1/K_Z)$: pK_{1N} and pK_{2N} can be calculated from the equations: $pK_1 = pK_{1N} - \log(1 + K_Z)$: $pK_2 = pK_{2N} + \log(1 + K_Z)$.

which the original data of Kalow are included. The lines have been fitted simply by trial, starting from the electrometric values of pK_1, pK_2 and pK_3. In spite of the uncertainty attached to the actual values of the zwitterion constants it seems likely that they are lower at 37°C than at 25°C. It is also possible to guess the microscopic dissociation constants; the pK_a of the ammonium group in $^{2+}HBH_2 = pK_1 + \log(1 + K_{Z1})$ and at 25°C this could be around 9.5; for the ammonium group in $^{2+}HB^{2-}$ the pK_a will be $pK_3 - \log(1 + 1/K_{Z2})$ which will also be around 9.5.

Discussion

The method of measuring zwitterion constants developed in this work, using the fit of absorbance to pH, starting with values of pK_1 and pK_2 determined electrometrically and then allowing them to alter, appears to have considerable advantages. In particular it does not involve transforming the experimental data (with the possible consequent introduction of bias). The fitting can also easily be seen. Accordingly it was used to re-examine the results of Barlow & Burston (1980) so as to obtain single estimates of K_Z, instead of sets obtained by different methods.

These recalculated values are shown in Table 3 and by comparing the values for tyramine with those for hydroxyamphetamine (α-methyltyramine) in Table 2 it is clearly possible to see the expected increase in zwitterion constant associated with the electron-releasing effect of the α-methyl group. The values in Table 3 also appear to confirm what was suggested in the present work, that with some compounds, e.g. morphine, temperature has little effect on K_Z, whereas with others, e.g. hydroxyamphetamine, a rise in temperature reduces K_Z. If zwitterions are stabilized by hydration their formation should be reduced by a rise in temperature and a consequent breakup of water structure and it is noticeable that the temperature effect is marked with compounds containing an $-NH_2$ or $-NHMe$ group. When these are protonated there are at least two hydrogen atoms which could form hydrogen bonds with oxygen atoms in structured water. In *m*-hydroxyphenethylmethylamine the distance between nitrogen and oxygen

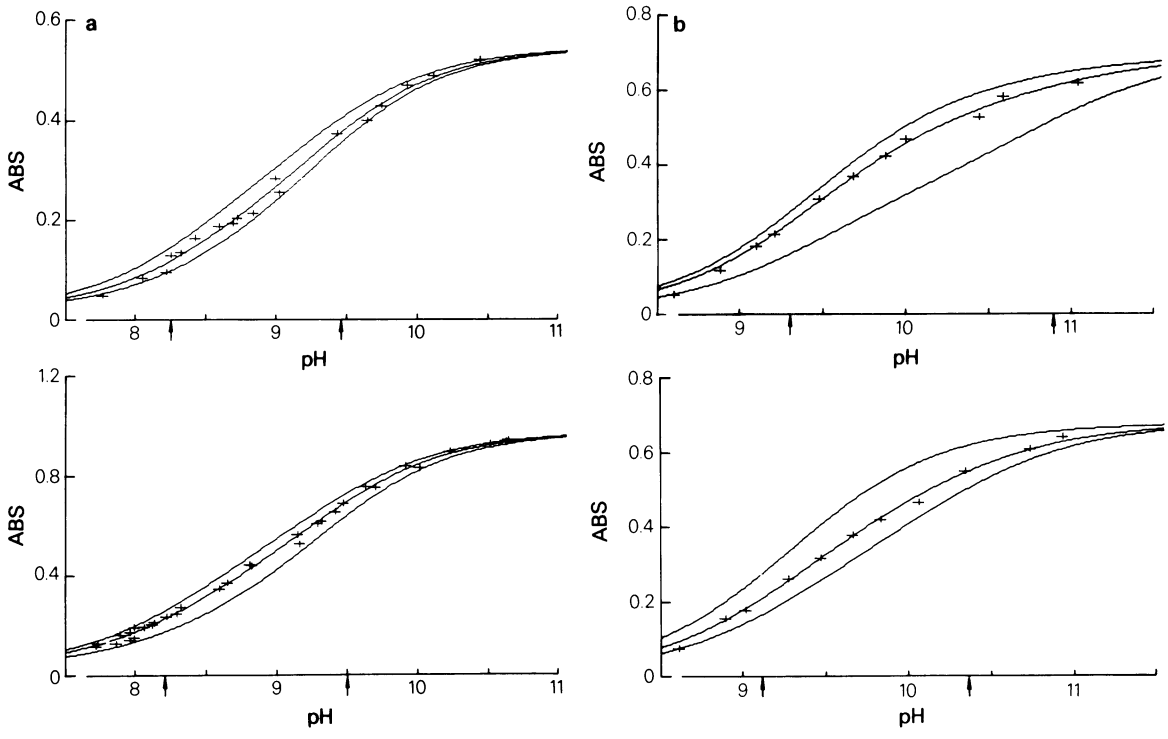

Figure 2 The effect of pH on absorbance at 300 nm. (a) For morphine at 25°C (upper panel) the lines were plotted with pK_1 8.25 and pK_2 9.46 and for the middle line, with K_Z 0.606 the ratio (indicating the incompleteness of the fit, see Table 2) was 1.6×10^{-3}; for the lower line, with K_Z 0.4 the ratio was 6.5×10^{-3}; for the top line, with K_Z 1.0 the ratio was 1.1×10^{-2}. The morphine concentration was 0.12 mM. At 37°C (lower panel) the lines were plotted with pK_1 8.21 and pK_2 9.50 and for the middle line, with K_Z 0.72 the ratio was 5.8×10^{-4}; for the lower line, with K_Z 0.4 the ratio was 9.4×10^{-3}; for the top line, with K_Z 1.0 the ratio was 3.6×10^{-3}. The morphine concentration was 0.20 mM. (b) For hydroxyamphetamine at 25°C (upper panel) the lines were plotted with pK_1 9.31 and pK_2 10.89 and for the middle line, with K_Z 3.87 the ratio was 1.0×10^{-3}; for the lower line, with K_Z 1.0 the ratio was 6.8×10^{-2}; for the top line, with K_Z 8.0 the ratio was 7.4×10^{-3}. At 37°C (lower panel) the lines were plotted with pK_1 9.12 and pK_2 10.36 and for the middle line, with K_Z 1.92 the ratio was 6.2×10^{-4}; for the lower line, with K_Z 1.0 the ratio was 1.4×10^{-3}; for the top line, with K_Z 8.0 the ratio was 3.0×10^{-2}. The hydroxyamphetamine concentration was 0.16 mM at both temperatures. The arrows (↑) indicate pK_1 and pK_2.

appears to be about 5 Å (from measurements with Dreiding models) and it is particularly easy to fit this into the Ice I lattice in which the distance between oxygen atoms which are next-nearest neighbours is 4.8 Å (Tait & Franks, 1971). It would be expected that there should be even higher zwitterion formation in *m*-tyramine but this has not been measured. By moving the hydroxyl group to the *p*-position possibilities for hydration should be greatly reduced, with a reduction in zwitterion formation. Zwitterion formation with morphine appears to be slightly less than with N,N-dimethyltyramine, which can be regarded as forming part of the morphine molecule (Figure 1). Although it may not be necessary for zwitterions to be stabilized by water, it is suggested that this can have a large effect if groups are in suitable positions and that if it occurs there is a marked reduction in zwitterion formation with a rise in temperature.

The spectroscopic results obtained with (+)-tubocurarine chloride show that Perrin's assignment of pK_1 to the loss of a proton from the amino group is incorrect; it is the phenolic groups which ionize first and there is extensive zwitterion formation. Kalow (1954) suggested that the first proton comes from the *p*-phenolic group (position 13 according to the numbering of Codding & James, 1973), although it is not possible to deduce this spectroscopically. Kalow wrote '... a methoxy group in the *o*-position is peculiar to the OH group of the double ring. This methoxy

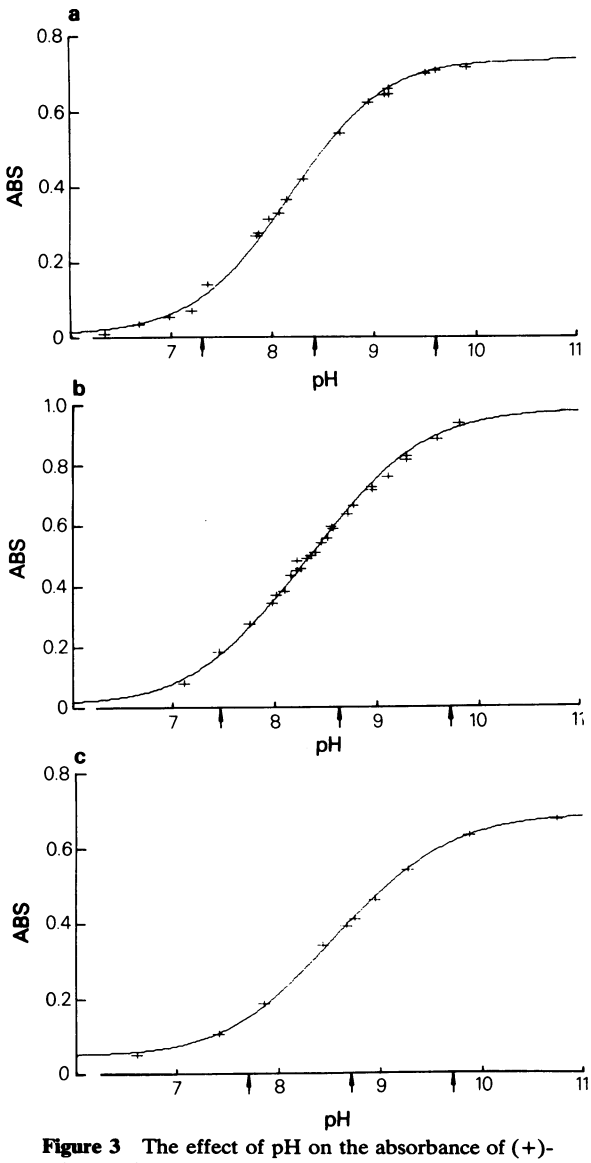

Figure 3 The effect of pH on the absorbance of (+)-tubocurarine chloride at 300 nm. (a) Shows results at 25°C and the line was plotted with pK_1 7.5, pK_2 8.4, pK_3 9.6 and K_{z1} 100, K_{Z2} 10 which gives a ratio of 7.0×10^{-4}. The (+)-tubocurarine chloride concentration was 0.08 mM. (b) Results at 37°C, the line was plotted with pK_1 7.45, pK_2 8.6, pK_3 9.7 and K_{Z1} 10, K_{Z2} 4 which gives a ratio of 5.6×10^{-4}. The (+)-tubocurarine chloride concentration was 0.10 mM. (c) Results obtained by Kalow (1954) for absorbance at 295 nm and 24°C with 0.05 mM solutions. The line was plotted with pK_1 7.7, pK_2 8.7, pK_3 9.7 and K_{Z1} 10, K_{Z2} 2 which gives a ratio of 2.9×10^{-4}. It was calculated with activity coefficients assumed to be 0.79 and 0.4, as in the present work. The arrows (↑) indicate pK_1, pK_2 and pK_3.

group must be expected to raise, as a first order effect, the pK of this latter hydroxyl'. The phenolic groups, however, have remarkably low pK_as and it is this which requires explanation, rather than the weaker acidity of the hydroxyl group *o*- to methoxy. Possibly the very high acidity (low pK_a) of the 13-hydroxyl group is due to interactions involving the proton on the 1-nitrogen atom (which at the time of Kalow's work was thought to be a quaternary ammonium group). The crystallographic studies of Codding & James (1973) with (+)-tubocurarine chloride and of Reyndolds & Palmer (1976) with the bromide show extensive hydrogen bonding involving the anions, water molecules and these OH and $^+$NH groups so it is reasonable to suppose that the zwitterion formed by loss of the first proton might be stabilized by hydration.

Some further slight support for the idea that the phenolic group attached to the tetrahydro*iso*-quinoline ring (position 27) has a pK_a between 8 and 9 comes from electrometric titrations with phenolic quaternary ammonium salt, 6-hydroxy N-methyl-tetrahydro*iso*quinoline methobromide. This was formed inadvertently in the synthesis of leptodactyline (Barlow, Bowman, Ison & McQueen, 1974) and the results of 8 titrations at 37°C in 0.1 M NaCl fitted to the equation $pK = pK_0 + mC^{\frac{1}{2}}$ gave $pK_0 = 8.92$. The correct model compound would have the hydroxyl group in the 7 position which is nearer to the quaternary ammonium group and so should be more acidic. The considerable difference in the pK_as of the phenolic groups is convenient in that it justifies the simplified treatment of the dissociation equilibria used here which ignores the effects of overlapping ionisation of the phenolic groups.

I thank Dr S.E. Smith for the sample of hydroxyamphetamine and for raising the question of its ionization and Dr C.R. Ganellin for drawing my attention to the paper by Schill and Gustavii. I am most grateful to Miss Margaret Chan for her careful and cheerful help with the pH measurements.

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Table 3 Re-calculated results of Barlow & Burston (1980), all in 0.1 M NaCl

	pK_{1Z}	pK_1	pK_{2Z}	pK_2	K_Z	*Ratio*
Tyramine						
25	(9.58)	9.44	(10.54)	10.68	2.49	1.11×10^{-3}
		9.36		*10.79*		
37	(9.48)	9.23	(10.24)	10.49	1.34	5.93×10^{-4}
		9.10		*10.57*		
N,N-dimethyltyramine						
25	(9.33)	9.03	(10.01)	10.31	0.98	6.53×10^{-4}
		9.03		*10.33*		
37	(9.42)	9.08	(10.00)	10.35	0.83	5.62×10^{-4}
		8.97		*10.35*		
m-Hydroxyphenethyldimethylamine						
25	(9.26)	8.93	(9.94)	10.27	0.86	1.62×10^{-3}
		8.87		*10.17*		
37	(9.19)	8.91	(9.93)	10.22	1.08	2.22×10^{-4}
		8.80		*10.26*		
m-Hydroxyphenethylmethylamine						
25	(9.31)	9.30	(13.65)	13.66*	63.1	3.58×10^{-4}
		9.30		*11.06*		
37	(9.18)	9.12	(10.77)	10.84	6.09	2.23×10^{-4}
		9.08		*10.90*		
5-Hydroxytryptamine						
25	(10.17)	9.68	(10.44)	10.93	0.48	1.58×10^{-4}
		9.69		*10.89*		
37	(9.94)	9.31	(10.16)	10.79	0.31	2.15×10^{-4}
		9.38		*10.73*		
Bufotenine						
37	(10.04)	9.35	(10.13)	10.82	0.26	9.67×10^{-5}
		9.29		*10.92*		

Values in italics are the electrometric values of pK_1 and pK_2 extrapolated to 0 mM: other values were calculated as in Table 2. The asterisk indicates the problem associated with measuring large values of K_Z; with the electrometric values of pK_1 and pK_2 the value of K_Z (method iv) was 28.6 and the ratio 4.88×10^{-4}.

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