Talanta, Vol. 32, No. 2, pp. 159–165, 1985
Printed in Great Britain.

0039-9140/85 $3.00 + 0.00

ANALYTICAL DATA

STUDIES ON FLUORESCEIN—II*

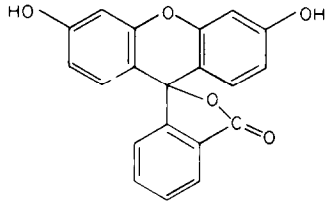
THE SOLUBILITY AND ACID DISSOCIATION CONSTANTS OF FLUORESCEIN IN WATER SOLUTION

HARVEY DIEHL and RICHARD MARKUSZEWSKI†
Department of Chemistry, Iowa State University, Ames, Iowa, 50011, U.S.A.

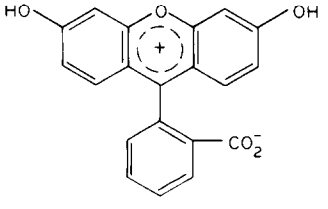
(*Received* 4 *April* 1984. *Accepted* 14 *September* 1984)

Summary—The solubility of yellow fluorescein and of red fluorescein as a function of pH has been measured in water at ionic strength 0.10. The pH of minimum solubility is the same for both, 3.28. The intrinsic solubility, defined as the solubility of the undissociated species, H_2Fl, and assumed to be constant and independent of pH, was calculated from the observed solubilities on the low-pH side of the minimum: $S_{i,yellow} = 3.80 \times 10^{-4} M$: $S_{i,red} = 1.45 \times 10^{-4} M$. The first dissociation constants were evaluated from the intrinsic solubilities and the observed solubilities on the low-pH side: both fluoresceins yielded the same value, $pK_{H_3Fl} = 2.13$. In using the observed solubilities on the high-pH side of the minimum to evaluate the intrinsic solubility and the second dissociation constant it was necessary to modify the existing theoretical approach by taking into account the presence of the fully dissociated anion. Appropriate mathematical treatments were devised to handle the more complex equations. Both fluoresceins yielded the same value for the second dissociation constant, $pK_{H_2Fl} = 4.44$. Both fluoresceins give the same yellow colour in saturated solution and the results just reported for the pH of minimum solubility and for the dissociation constants also indicate that for each of the three prototropic forms of fluorescein present in solution, H_3Fl^+, H_2Fl, and HFl^-, only one structure exists.

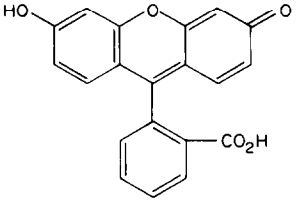
During 1980 we advanced structures for the three solid forms of fluorescein,[1] *viz.* for the colourless solid the lactone structure (**I**), for the red solid the *p*-quinonoid structure (**III**), and for the yellow solid a zwitterion structure with a positive charge distributed over the oxygen-bearing ring (**II**). We have now measured the solubilities of the yellow and red solids



I Lactone structure, colourless form



II Zwitterion structure, yellow form



III *p*-Quinonoid structure, red form

as functions of pH. As pointed out in the earlier paper, the colourless lactone solid exists in solution only in dry organic non-polar solvents such as dioxan and, on contact with water, changes quickly to the yellow zwitterion form; it therefore plays no part in the present discussion.

Fluorescein is characterized by three acid dissociation constants. For clarity, and to emphasize the amphoteric nature of fluorescein, we have adopted the designation H_2Fl for fluorescein (the "free acid") and for the respective dissociation constants the symbols:

Reaction	*Constant*	
$H_3Fl^+ = H^+ + H_2Fl$	$K_{H_3Fl} = [H^+][H_2Fl]/[H_3Fl^+]$	(1)
$H_2Fl = H^+ + HFl^-$	$K_{H_2Fl} = [H^+][HFl^-]/[H_2Fl]$	(2)
$HFl^- = H^+ + Fl^{2-}$	$K_{HFl} = [H^+][Fl^{2-}]/[HFl^-]$	(3)

We have determined the first two of the three acid dissociation constants from data on the solubilities as a function of pH, for both the yellow and red solids, by a modification of the procedure of Krebs and Speakman.[2] The concentration of fluorescein in the various saturated solutions was determined by mea-

*Part I—*Talanta*, 1980, **27**, 937.
†Present address: Ames Laboratory, Iowa State University, Ames, Iowa 50011, U.S.A.

suring the fluorescence of an aliquot brought to pH 11.

As in the earlier paper, we worked with fluorescein prepared from diacetylfluorescein and verified the purity of the materials by titration with alkali; we thus avoided certain faults which adversely affected certain earlier studies.

EXPERIMENTAL

Materials

Diacetylfluorescein, yellow fluorescein and red fluorescein were prepared as described earlier.[1]

Buffers with pH-values in the range 1–6 were prepared from 0.10*M* hydrochloric acid, potassium hydrogen phthalate and potassium hydroxide. Dilutions were made with 0.10*M* potassium chloride to maintain the ionic strength. The pH of the buffer solutions was measured before and after saturation with the fluorescein, with a Corning Model 10 pH-meter, with a Beckman glass electrode and a saturated calomel electrode as reference electrode. The pH-meter was standardized with a standard buffer solution of pH 4.01, prepared from potassium hydrogen phthalate according to the NBS specification.

Preparation of calibration graphs

Highly purified yellow and red fluorescein were dried at 100–110° for 3 hr, and 100.0 mg of each were dissolved in, and diluted to exactly 1 litre with, 0.10*M* potassium hydroxide. Stock solutions containing 1.0 ppm of fluorescein were prepared by diluting 1.00 ml of these solutions to 100.0 ml with 0.10*M* potassium hydroxide. Volumes of 1, 3, 6, 10, 15 and 20 ml of these solutions were taken and diluted to 100.0 ml with 0.10*M* potassium hydroxide for fluorimetric measurement. The calibration curves for the yellow and red fluoresceins were identical.

Measurement of fluorescence

Relative fluorescence was measured with a Turner Model 110 Fluorometer. A Corning No. 5850 filter was used as a primary filter to isolate the blue portion of the excitation radiation. A combination of a Corning Yellow 2A-15 filter and a Wratten N. D. Filter 10 per cent 1.00 was used as the secondary filter. The calibration graphs were measured at three different sensitivity settings of the fluorometer. This, in combination with variations in the size of aliquots and final volumes, provided maximum accuracy.

Solubility measurements

Approximately 25 ml of each buffer solution was placed in a 600-ml plastic bottle provided with a screw cap. Into each bottle sufficient red or yellow fluorescein was added to ensure saturation of the resulting solution. Bottles and suspensions were then shaken mechanically for 72 hr at room temperature, 23.0 ± 0.5°. Each solution was subsequently filtered through a sintered-glass crucible, and the final pH was measured. A 5.00-ml aliquot of each solution was diluted to 100.0 ml with potassium hydroxide and the relative fluorescence of the solution was measured. The results are given in Table 1.

EVALUATION OF THE INTRINSIC SOLUBILITY OF THE YELLOW AND RED FLUORESCEINS

As will be seen on examination of Table 1 and Fig. 1, the solubilities of both the yellow and red forms of fluorescein exhibit a minimum at pH 3.28. The solubility increases on the low-pH side owing to protonation to form H_3Fl^+ [equation (1)] and on the high-pH side owing to dissociation to form HFl^- [equation (2)]. Such amphoteric behaviour is typical of zwitterions, and the dissociation constants can be evaluated from the solubility data by a method originated by Krebs and Speakman.[2]

The solubility of the free acid, the *intrinsic solubility*, S_i, is assumed to be constant and independent of pH:

$$S_i = [H_2Fl] \quad (4)$$

The solubility at any pH below the minimum is given by

$$S_j = [H_3Fl^+] + [H_2Fl] \quad (5)$$

and that at pH values above the minimum, S_h, is given by

$$S_h = [H_2Fl] + [HFl^-] \quad (6)$$

It is also assumed that over the pH range immediately above the minimum the concentration of Fl^{2-} is

Table 1. Solubility of yellow and red fluorescein as a function of pH; ionic strength 0.10

	Yellow fluorescein		Red fluorescein	
Initial pH of buffer	Final pH	Concentration, M	Final pH	Concentration, M
1.00	1.10	7.22×10^{-3}	1.05	2.41×10^{-3}
1.49	1.53	2.11×10^{-3}	1.51	6.92×10^{-4}
2.00	2.07	7.82×10^{-4}	2.05	2.59×10^{-4}
2.28	2.33	6.62×10^{-4}	2.35	2.50×10^{-4}
2.64	2.69	5.29×10^{-4}	2.65	1.87×10^{-4}
2.98	3.01	4.81×10^{-4}	3.01	1.69×10^{-4}
3.40	3.39	3.85×10^{-4}	3.43	1.50×10^{-4}
3.90	3.92	4.45×10^{-4}	3.92	1.62×10^{-4}
4.35	4.37	5.84×10^{-4}	4.36	2.17×10^{-4}
4.90	4.90	1.40×10^{-3}	4.90	5.06×10^{-4}
5.17	5.18	2.15×10^{-3}	—	—
5.35	5.34	3.31×10^{-3}	5.35	1.25×10^{-3}
5.55	5.53	4.72×10^{-3}	5.55	1.90×10^{-3}
6.04	6.03	1.80×10^{-2}	6.06	6.95×10^{-3}

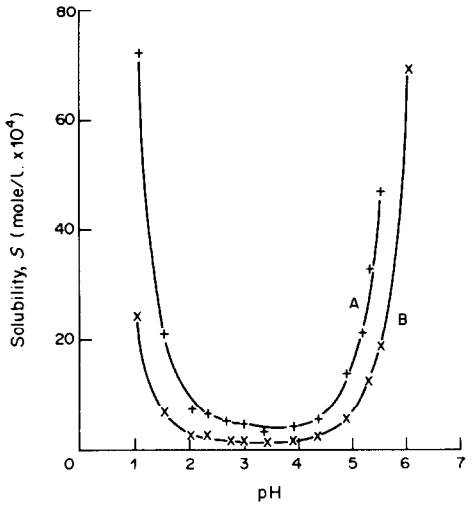


Fig. 1. Solubility of yellow fluorescein (curve A) and of red fluorescein (curve B) as a function of pH at 23°.

negligible (the validity of this assumption is examined below).

Combination of equations (1) and (4) yields

$$K_{H_3Fl} = [H^+]S_i/(S_j - S_i) \quad (7)$$

Rearrangement of equation (7) gives

$$S_j = S_i + [H^+]S_i/K_{H_3Fl} \quad (8)$$

and

$$-\log[(S_j/S_i) - 1] = -pK_{H_3Fl} + pH \quad (9)$$

According to equation (8) a plot of S_j *vs.* $[H^+]$ yields a straight line, the intercept of which at $[H^+] = 0$ is S_i. According to equation (9) the plot of $-\log[S_j/S_i) - 1]$ *vs.* pH yields a straight line of unit slope, the intercept of which is pK_{H_3Fl}.

Similarly, combination of equations (2) and (4) yields

$$K_{H_2Fl} = [H^+](S_h - S_i)/S_i \quad (10)$$

Rearrangement of equation (10) gives

$$S_h = S_i + K_{H_2Fl}S_i/[H^+] \quad (11)$$

or

$$\log[(S_h/S_i) - 1] = -pK_{H_2Fl} + pH \quad (12)$$

According to equation (11) a plot of S_h *vs.* $1/[H^+]$ yields a straight line, the intercept of which at $1/[H^+] = 0$ is S_i. According to equation (12) a plot of $\log[(S_h/S_i) - 1]$ *vs.* pH gives a straight line of unit slope, the intercept of which is $-pK_{H_2Fl}$.

Thus, the intrinsic solubility can be evaluated from two sets of data, on the low-pH side of the minimum, equation (8), and on the high-pH side, equation (11). For yellow fluorescein, the two approaches yielded somewhat different values, as shown in Table 2. Least-squares treatment of the 6 data points on the low-pH side yielded $S_j = 3.795 \times 10^{-4} + 0.0579[H^+]$ with R (correlation coefficient) = 0.997 (Fig. 2 curve A); hence $S_{i,yellow} = 3.80 \times 10^{-4}M$, and $K_{H_3Fl(yellow)} = 6.55 \times 10^{-3}$. For red fluorescein, the corresponding values were $S_j = 1.45 \times 10^{-4} + 0.0175[H^+]$, $R = 0.994$ (Fig. 1, curve B), $S_{i,red} = 1.45 \times 10^{-4}M$, and $K_{H_3Fl(red)} = 8.30 \times 10^{-3}$.

Least-squares treatment of the data on the high-pH side of the minimum, equation (11), yielded intercepts which varied with the number of data points used: $3.23 \times 10^{-4}M$ ($n = 4$), $3.49 \times 10^{-4}M$ ($n = 5$), $3.12 \times 10^{-4}M$ ($n = 6$), $3.22 \times 10^{-7}M$ ($n = 7$), $-0.347 \times 10^{-4}M$ ($n = 8$); although the successive plots (as n increased) were linear with high correlation coefficients (Fig. 3 curve A, for $n = 7$, for which least-squares gave $S_h = 3.22 \times 10^{-4} + 1.309 \times 10^{-9}/[H^+]$; $R = 0.9962$), only a likely average (disregarding

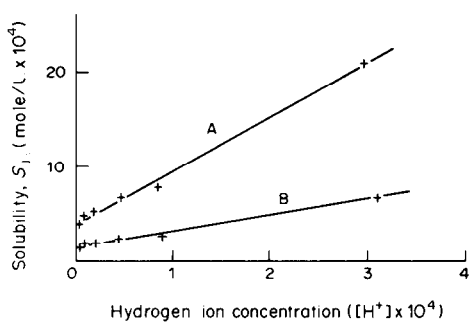


Fig. 2. Determination of the intrinsic solubility, S_i, of yellow fluorescein (curve A) and of red fluorescein (curve B) from solubility data on the low-pH side of the minimum solubility [equation (8)].

Table 2. Values for the intrinsic solubility, S_i, of yellow and red fluorescein (ionic strength 0.10)

Data used	Yellow fluorescein, 10^{-4}M	Red fluorescein, 10^{-4}M
Low-pH side of minimum: equation (8)	3.80	1.45
High-pH side of minimum: equation (11)*	3.27†	1.21†
equation (16)§		
first treatment*	3.42	1.24
second treatment*	3.48	1.30

*By least-squares treatment.
†Variable, depending on the number of data points included; see text.
§Correction made for the presence of Fl^{2-}.

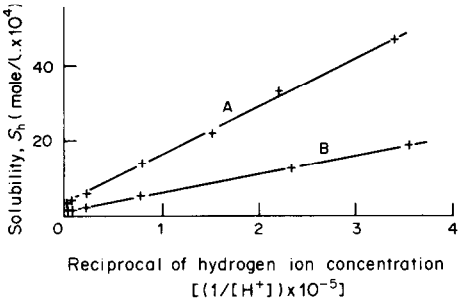


Fig. 3. Determination of the intrinsic solubility, S_i, of yellow fluorescein (curve A) and of red fluorescein (curve B) from solubility data on the high-pH side of the minimum solubility [equation (11)].

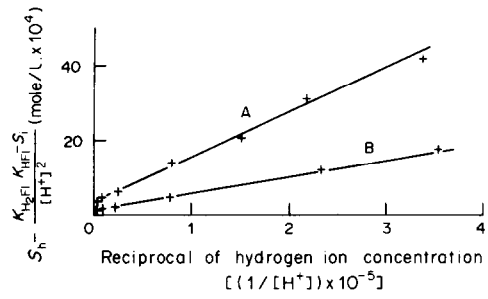


Fig. 4. Determination of the intrinsic solubility, S_i, of yellow fluorescein (curve A) and of red fluorescein (curve B) from solubility data on the high-pH side of the minimum solubility, with correction applied for the presence of Fl^{2-}; [equation (16), second approximation treatment].

$n = 8$) could be chosen: $S_{i,yellow} = 3.27 \times 10^{-4} M$ (Table 2). In general, the inclusion of the data at pH 6 (6.03 for yellow and 6.06 for red fluorescein) seriously distorted the results; it was suspected that at this pH (well above the pH, 5.5, at which the neutral form is completely dissociated) the systems did not conform to the assumptions made, so these data were not used in obtaining results by the unmodified Krebs and Speakman procedure.

For red fluorescein, corresponding treatment again yielded a value for the intercept which varied with the number of data included: $1.24 \times 10^{-4} M$ (4), $1.23 \times 10^{-4} M$ (5), $1.16 \times 10^{-4} M$ (6), $0.018 \times 10^{-14} M$ (7), *e.g.*, Fig. 2, curve B (for $n = 6$, for which least-squares gave $S_h = 1.16 \times 10^{-4} + 4.97 \times 10^{-9}/[H^+]$, $R = 0.99965$); again only a likely average (omitting the data for pH 6.06) could be chosen: $S_{i,red} = 1.21 \times 10^{-4} M$ (Table 2).

The variability and the drift in the values for the intrinsic solubilities obtained on the high-pH side of the minimum indicated that some additional factor was involved.

Since we knew from other work that the difference between the second and third dissociation constants is small (about 1.5–1.8 pK units) we realized that a significant amount of the doubly-charged anion must be present and of course increases with rising pH. The solubility on the high-pH side is then given by

$$S_h = [H_2Fl] + [HFl^-] + [Fl^{2-}] \qquad (13)$$

Introduction of equations (1)–(3) into (13) and assuming the solubility of the undissociated species to be constant and independent of pH, so that $[H_2Fl] = S_i$, gives

$$S_h = S_i\{1 + K_{H_2Fl}/[H^+] + K_{H_2Fl}K_{HFl}/[H^+]^2\} \qquad (14)$$

This is an awkward form for evaluation of S_i, and we adopted an approximation approach. The values best known at the time for the three constants in the third term within the braces were used to calculate the value of this term at each experimental pH, and this was applied as a correction to the solubility observed:

$$S_h - K_{H_2Fl}K_{HFl}S_i/[H^+]^2 = S_i + K_{H_2Fl}S_i/[H^+] \qquad (15)$$

For yellow fluorescein, the constants used in this treatment were p$K_{H_2Fl} = 4.73$, p$K_{HFl} = 6.36$, $S_{i,yellow} = 3.80 \times 10^{-4} M$. The correction was zero at the data points near the minimum but became significant at higher pH-values. The plots of $S_h - K_{H_2Fl}K_{HFl}S_i/[H^+]^2$ *vs.* $1/[H^+]$ were linear and led to an essentially constant value for the intrinsic solubility as the number of sets of data included was increased. The value obtained was $S_{i,yellow} = 3.42 \times 10^{-4} M$, Table 2. This value was then used in equation (12), as described in the next section, to obtain a new value for the second dissociation constant; this new value (p$K_{H_2Fl} = 4.52$) and the corrected S_i value were then used in a second approximation. The plot was linear (Fig. 3, curve A) and least-squares treatment yielded $S_h - K_{H_2Fl}K_{HFl}S_i/[H^+]^2 = 3.48 \times 10^{-4} + 1.198 \times 10^{-8}/[H^+]$, giving $S_{i,yellow} = 3.48 \times 10^{-4} M$, practically the same as that from the first approximation.

For red fluorescein, the constants used in the first approximation treatment were p$K_{H_2Fl} = 4.73$, p$K_{HFl} = 6.36$, $S_{i,red} = 1.16 \times 10^{-4} M$, and gave $S_{i,red} = 1.24 \times 10^{-4} M$ (Table 2). This value was used in equation (12) to obtain p$K_{H_2Fl} = 4.52$, and the second approximation treatment gave a linear plot (Fig. 4, curve

Table 3. Values for the dissociation constants, K_{H_3Fl} and K_{H_2Fl}, of yellow and of red fluorescein as determined from solubility data (ionic strength 0.10)

Constant	Yellow fluorescein	Red fluorescein
pK_{H_3Fl} low-pH side of minimum equation (9)[a]	2.15	2.11
pK_{H_2Fl} high-pH side of minimum equation (12)[b]	4.44[c,d]	4.42[e,d]
equation (18)	4.45[f]	4.43[f]

[a] By least-squares with slope forced to be equal to 1.000.
[b] From intercept on pH-axis.
[c] Including the data at pH 6.03.
[d] By least-squares; same value obtained by forcing slope to be 1.000.
[e] Including data point at pH 6.06.
[f] Slope of least-squares line was zero; direct averaging of the value on the ordinate of the plot gave: yellow fluorescein, pK 4.43, red fluorescein, pK 4.42.

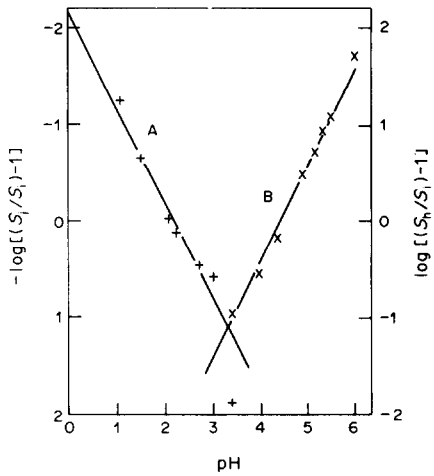


Fig. 5. Evaluation of the dissociation constants, K_{H_3Fl} and K_{H_2Fl}, of yellow fluorescein. Curve A, from solubility data on the low-pH side; $-\log[(S_j/S_i) - 1]$ *vs.* pH [equation (9)]; curve B, from solubility data on the high-pH side; $\log[(S_h/S_i) - 1]$ *vs.* pH [equation (12)]. For both straight lines the slope has been forced to be 1.000 in the least-squares treatment. The intersection point, pH 3.29, is the pH of minimum solubility.

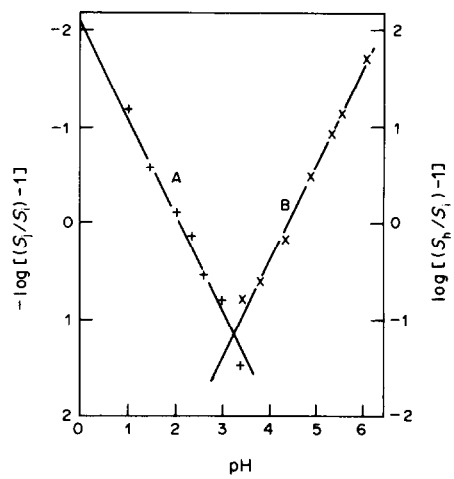


Fig. 6. Evaluation of the dissociation constants, K_{H_3Fl} and K_{H_2Fl}, of red fluorescein. Symbols and method as for Fig. 5. The point of intersection, pH 3.27, is the pH of minimum solubility.

B): $S_h - K_{H_2Fl}K_{HFl}\ S_i/[H^+]^2 = 1.30 \times 10^{-4} + 4.42 \times 10^{-9}/[H^+]$. Thus $S_{i,red} = 1.30 \times 10^{-4}M$ (Table 2) was again little changed from the first approximation.

EVALUATION OF THE FIRST AND SECOND DISSOCIATION CONSTANTS OF YELLOW AND RED FLUORESCEIN

To obtain values for the dissociation constants, it is convenient to place the two plots, $-\log[(S_j/S_i) - 1]$ *vs.* pH [equation (9)] and $\log[(S_h/S_i) - 1]$ *vs.* pH [equation (12)] on the same graph, as in Fig. 3 for yellow fluorescein and Fig. 4 for red fluorescein. The values obtained are given in Table 3.

For both fluoresceins, the values for the intrinsic solubility used in equation (9) were those found by evaluating the data on the low-pH side as described above: $S_{i,yellow} = 3.80 \times 10^{-4}M$; $S_{i,red} = 1.45 \times 10^{-4}M$. Least-squares treatment of data from equation (9) gave for both yellow fluorescein (Fig. 5, curve A) and red fluorescein (Fig. 6, curve A) straight lines of slope close to the theoretical value of one, and intercepts on the pH-axis which were not greatly changed by the number of sets of data included. We also used a least-squares treatment in which the slope was forced to be 1.000, and consider the values so obtained to be the best:

Number of sets of data included	5	6	7	Forced slope
Yellow fluorescein				
Intercept on pH-axis	2.212	2.231	2.155	2.152
Slope	1.048	1.019	1.2416	1.000
R	0.9900	0.99049	0.975	
Red fluorescein				
Intercept on pH-axis	2.090	2.090	2.212	2.113
Slope	0.9961	0.9955	1.0429	1.000
R	0.9713	0.9853	0.9910	

The astonishing feature is that yellow fluorescein and red fluorescein yield practically the same value for the first dissociation constant. We believe that the various protonated species in water exist in only one structural form and accordingly average the values 2.152 and 2.113, and propose

$$K_{H_3Fl} = 7.41 \times 10^{-3};\ pK_{H_3Fl} = 2.13 \qquad (16)$$

To evaluate the second dissociation constant from the data on the high-pH side of the minimum, both equations (12) and (17) were used; for both equations a value must be selected for the intrinsic solubility (Table 2). When the S_i value was that obtained by equation (11), the values for K_{H_2Fl} [the intercept of equation (12)] varied with the number of data points included, for both fluoresceins. On the other hand, when the S_i value was that obtained by equation (15), second approximation, concordant values were obtained, with little variation with the number of sets of data included, and incorporated the high-pH data smoothly.

Thus application of equation (12) to yellow fluorescein, with use of $S_{i,yellow} = 3.48 \times 10^{-4}M$ and least-squares treatment forcing the slope to be 1.000 for all 8 sets of data in Fig. 5 curve B, gave $pK_{H_2Fl} = 4.43$. Similarly for red fluorescein, with $S_{i,red} = 1.30 \times 10^{-4}M$ and all 7 sets of data from Fig. 6 curve B, and a forced slope of 1.000, $pK_{H_2Fl} = 4.42$.

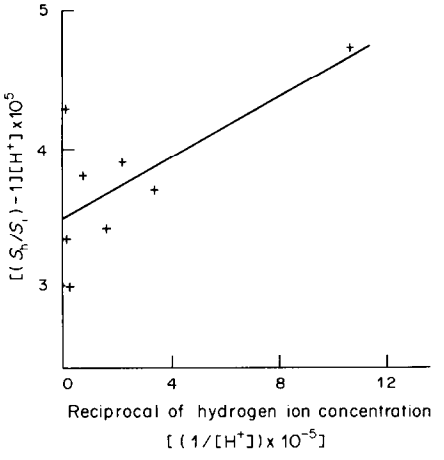


Fig. 7. Evaluation of the second dissociation constant, K_{H_2Fl}, of yellow fluorescein from solubility data on the high-pH side of the minimum solubility, with correction for the presence of the doubly-charged anion [equation (17)].

Equation (12) is based on the assumption that only the neutral species and the singly-charged anion are present, although a correction for the presence of the double-charged anion was made in arriving at the value of the intrinsic solubility used. A more rigorous treatment followed next, starting with the assumptions in equations (13)–(15). Equation (15), on rearrangement, yields

$$[(S_h/S_i) - 1][H^+] = K_{H_2Fl} + K_{H_2Fl}K_{HFl}/[H^+] \quad (17)$$

A plot of $[(S_h/S_i) - 1][H^+]$ *vs.* the reciprocal of the hydrogen-ion concentration for yellow fluorescein, again using $S_{i,yellow} = 3.48 \times 10^{-4}M$, is shown in Fig. 7; least-squares treatment gave $[(S_h/S_i) - 1][H^+] = 3.496 \times 10^{-5} + 1.103 \times 10^{-11}/[H^+]$, for all 8 sets of data ($R = 0.68511$), *i.e.*, $K_{H_2Fl} = 3.496 \times 10^{-5}$. The slope of almost zero makes equation (17) an interesting function, since the left-hand side is essentially independent of pH. A consequence of this is a low correlation coefficient of the least-squares line [for yellow fluorescein, $R = 0.01739$ for 4 sets of data, increasing to $R = 0.1492$ (6 sets), and to $R = 0.68511$ (8 sets, including the set at pH 6)]; thus, not surprisingly, the eight values for $[(S_h/S_i) - 1][H^+]$ yielded, on simple averaging, an intercept of 3.757×10^{-5}, and thus $pK_{H_2Fl} = 4.43$. Note that equations (12) and (17) yielded the same value for the second dissociation constant from the data for yellow fluorescein. Basically, then, the approach in equations (13)–(15) and (17) appears correct.

Application of equation (17) to red fluorescein, using $S_{i,red} = 1.30 \times 10^{-4}M$, yielded a linear plot (Fig. 8); least-squares treatment gave $[(S_h/S_i) - 1][H^+] = 3.690 \times 10^{-5} + 5.73 \times 10^{-12}/[H^+]$, so $pK_{H_2Fl} = 4.33$. As with yellow fluorescein, the slope was close to zero; a simple averaging of the seven values for $[(S_h/S_i) - 1][H^+]$ yielded 3.841×10^{-5}, *i.e.*, $pK_{H_2Fl} = 4.42$.

As reported above and in Table 3, equations (12) and (17) yielded the same values for the second dissociation constant for both the yellow and red fluoresceins. We therefore reiterate our belief that only one structure is present in the various prototropic forms of fluorescein existing in water solution and accordingly we average the results for both fluoresceins and propose $K_{H_2Fl} = 3.63 \times 10^{-5}$ ($pK_{H_2Fl} = 4.44$).

Comment on the pH of minimum solubility

Addition of equations (9) and (12) gives

$$\log[(S_h - S_i)/(S_j - S_i)] = 2pH - pK_{H_3Fl} - pK_{H_2Fl} \quad (18)$$

At the point of minimum solubility, the disproportionation of the undissociated species, the only species theoretically present, would produce equal amounts of the cation and the singly-charged anion:

$$2H_2Fl = H_3Fl^+ + HFl^- \quad (19)$$

and

$$S_j = S_h \quad (20)$$

At this pH, the left-hand side of equation (18) becomes zero, and

$$pH_{min.soly} = \tfrac{1}{2}(pK_{H_3Fl} + pK_{H_2Fl} \quad (21)$$

For the values presented in equations (16) and (18), $pH_{min.soly} = \tfrac{1}{2}(2.13 + 4.44) = 3.28$.

RESULTS AND DISCUSSION

The U-shaped curves of solubility as a function of pH, Fig. 1, for the yellow and red fluoresceins, are not expected to be symmetrical about the pH of min-

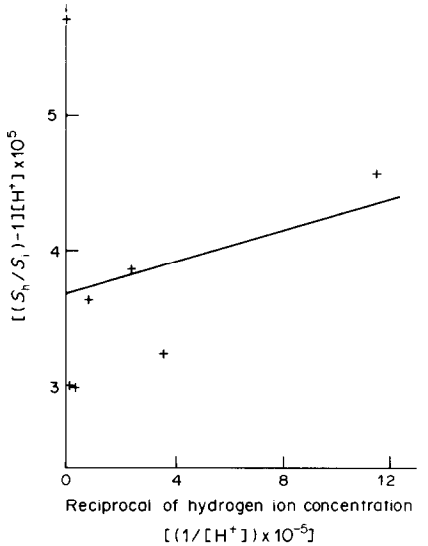


Fig. 8. Evaluation of the second dissociation constant, K_{H_2Fl}, of red fluorescein from solubility data on the high-pH side of the minimum solubility, with correction for the presence of the doubly-charged anion [equation (17)].

imum solubility, inasmuch as the branches are defined by different equations [(8) and (11)] and constants. The intrinsic solubility, however, is given the same definition, equation (4), in both equations and is assumed to be a constant independent of pH. Thus, the evaluation of the intrinsic solubility by equations (8) and (11), the first phase of the Krebs and Speakman treatment, would be expected to yield the same values, but failed to do so for the solubilities of the two fluoresceins. The treatment on the low-pH side of the minimum was straightforward, the linear least-squares treatment of the plots, Fig. 2, curves A and B, having high correlation coefficients, and the intercepts and slope showing essentially no variation with the number of sets of data used. The values found were: $S_{i,yellow} = 3.80 \times 10^{-4}M$; $S_{i,red} = 1.45 \times 10^{-4}M$ (Table 2). These values, when used in conjunction with the observed solubilities of the yellow and the red fluoresceins in the second phase of the Krebs and Speakman treatment [equation (9)] gave an identical value for the first dissociation constant $K_{H_3Fl} = 7.41 \times 10^{-3}$ ($pK_{H_3Fl} = 2.13$) (Table 3).

For both fluoresceins the data on the high-pH side of the minimum, however, gave by equation (11) significantly lower values for the intrinsic solubilities than those obtained from the data on the low-pH side, and moreover, gave values which shifted with the number of data points used. A similar effect was observed for the values of the second dissociation constants [equation (12), Krebs and Speakman second phase]. The supposition that the results were being distorted by the presence of appreciable amounts of the doubly-charged anion, Fl^{2-}, proved to be correct. The values for pK_{H_2Fl} and pK_{HFl} differ by only 1.5–1.8 and the dissociation of HFl^- is significant even at pH 4.5; at pH 5.5 it is completely dissociated and it is not surprising that the system does not conform to the assumption of the Krebs and Speakman approach.

The new theory, in which all three species (H_2Fl, HFl^- and Fl^{2-}) are assumed to be present [equations (13)–(17)], proved to be successful. Because of the nature of the equations involved, it was necessary to evaluate the intrinsic solubility by an approximation procedure. In practice, the second approximation was all that was necessary and the results from use of equation (15) conformed nicely to the theory and smoothly incorporated the data at high pH (pH > 6, data discarded in the simpler approach). The values found were $S_{i,yellow} = 3.48 \times 10^{-4}M$; $S_{i,red} = 1.30 \times 10^{-4}M$ (Table 2).

The second phase, the evaluation of the second dissociation constant, proved interesting. From the value for the intrinsic solubility obtained by use of equation (15) with the presence of Fl^{2-} taken into account, the same value for the second dissociation constant was obtained by both equation (12) (Krebs and Speakman) and equation (17) (new approach), for both yellow fluorescein and red fluorescein (Table 3); $pK_{H_2Fl} = 4.44$.

That sets of data on the solubility of two solid forms of the same chemical material should yield identical values for the pH of minimum solubility and for the two sets of dissociation constants is remarkable. The conclusion is that only one *structure* exists for fluorescein in water solution, for all three (and probably four) prototropic forms. Because the solutions at all pH values are yellow, the structure is probably the zwitterion structure **II**, with a positive charge located on the central oxygen-bearing ring and with protons and negative charges distributed about the periphery as determined by the prevailing pH.

REFERENCES

1. R. Markuszewski and H. Diehl, *Talanta*, 1980, **27**, 937.
2. H. A. Krebs and J. C. Speakman, *J. Chem. Soc.*, 1945, 593.