Fig. 3. Calibration of Molecular Weight

SDS-polyacrylamide gel electrophoresis of a mixture containing five kinds of reference proteins was carried out under the same conditions as before, and the SDS gel was stained with Coomassie brilliant blue(R-250). Each point is the mean of three experiments.

The calibration of molecular weight was performed by SDS-polyacrylamide gel electrophoresis with a mixture containing five reference proteins; trypsin inhibitor (T_1), bovine serum albumin(BSA) and RNA-polymerase(α , β and β') (Fig. 3). The molecular weights of the main components in F_2 fraction were estimated to be in the range of 22000—30000.

We could observe many bands on SDS gels stained with Coomassie brilliant blue (R-250) in electrophoresis of both F_1 and F_3 fractions, but we failed to distinguish clearly a peak of D-[5- 3 H]-glucose-binding membrane proteins on each electrophoretic pattern, and peaks of II or III could not be matched to those of F_1 and F_3 fractions on SDS gel. The radioactivity in each gel slice was very low in the present experiments, leading to relatively high errors and poor reproducibility of results, so labelled glucose which has a higher specific activity is required. Moreover, the conditions of solubilization of membrane proteins and polyacrylamide gel electrophoresis require improvement in order to avoid a decrease in D-[5- 3 H]glucose-binding with membrane protein.

Consequently, it was found that the main D-[5- 3 H]glucose-binding components in the F_2 fraction consisted of five membrane proteins, including glycoprotein, with a molecular weight range of 22000—30000. However, further purification of the F_2 fraction will be necessary to characterize and confirm the existence of glucoreceptors on or in the B cell membrane in rat islets of Langerhans.

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Simultaneous Determination of Acid Dissociation Constants and True Partition Coefficients by Analysis of the Apparent Partition Coefficients. II. Dibasic Acid and Diacidic Base

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The simultaneous determination of acid dissociation constants (pK_a) and true partition coefficients (P) of a dibasic acid and a diacidic base was achieved by analyzing the pH dependence of the apparent partition coefficient (P_a). Equations derived theoretically show a parabolic relation of P_a to $(H^+)^{-1}$ and (H^+) for a dibasic acid and a diacidic base, respectively, (H^+) being the proton concentration. Theoretical considerations indicate

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that the present method is especially useful for the case where the pK_{a1} and pK_{a2} values are similar in magnitude. Experimental confirmation was obtained for a typical dibasic acid and a typical diacidic base.

Keywords—dissociation constant; partition coefficient; structure-activity relationship; octanol; dissociation in organic phase; UV spectra; correlation coefficient in least-squares method; dibasic acid; diacidic base

In the preceding paper of this series²⁾ we presented a new method for obtaining the acid dissociation constant pK_a and the true partition coefficient P simultaneously by analyzing the pH dependence of the apparent partition coefficient (P_a). This procedure is simple and offers various advantages;²⁾ it is especially useful for the study of quantitative structure-activity relationships (QSAR). In this paper we report examples of the application of this technique to the proton dissociation equilibria in an aqueous medium of a dibasic acid AH_2 and a diacidic base BH_2^{2+} .

Experimental

The experimental technique used for measuring apparent partition coefficients, P_a , was the same as reported in our previous paper.²⁾ Pure octanol purchased from E. Merck Co., which was purified by repeated rectifications, was used as an organic phase throughout all the experiments. As the aqueous phase, two kinds of buffer solution were employed: 1 M (1 mol dm⁻³) HCl-KCl for pHs 1.89–2.53, and 1 M HCl-CH₃-COONa-NaCl for pHs 2.64–5.81. The ionic strength of solutions was adjusted to 0.5 with KCl and NaCl for the former and the latter, respectively.

Samples used were 6059S [6(*R*),7(*R*)-7-[2(*R*)-carboxy-2-(4-hydroxyphenyl)acetamido]-7-methoxy-3-[[1-(1-methyl-1H-tetrazol-5-yl)thio]-methyl]-8-oxo-5-oxa-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid disodium salt] (I) and *o*-tolidine (II). The former is a β -lactam antibiotic having strong antibacterial activity, synthesized and developed by Shionogi Research Laboratory.³⁾ These compounds each contain two functional groups, whose pK_a values are similar in magnitude, and so they are very suitable for the purpose of this work (vide infra). A pure sample of 6059S was kindly supplied by Dr. Narisada of our laboratory. A sample of *o*-tolidine provided by Dr. Ogawa in our laboratory was repeatedly sublimed (mp 131°). The elementary analysis data for the purified material agreed well with the calculated values. The details of the method for measuring the apparent partition coefficient have already been described.²⁾ All the measurements were performed at ~23°. In view of the solubilities of the two samples, an aqueous buffer solution of 6059S was partitioned with *n*-octanol, but *o*-tolidine in *n*-octanol was partitioned with buffer solution. The concentration of the solute was measured by a ultraviolet (UV) spectral method.

Results and Discussion

Theoretical Considerations

For the case of the proton dissociation equilibria of AH_2 , written as $AH_2 \rightleftharpoons AH^- + H^+$ (Step A1) and $AH^- \rightleftharpoons A^- + H^+$ (Step A2), the relation of P_a and P values is given by Eq. 1.²⁾

$$\frac{1}{P_a} = \frac{1}{P} + \frac{K_{a1}^A}{P} \left[\frac{1}{(H^+)} \right] + \frac{K_{a1}^A K_{a2}^A}{P} \left[\frac{1}{(H^+)} \right]^2 \quad (1)$$

Here, K_{a1}^A and K_{a2}^A are the dissociation constants for the equilibria of Step A1 and Step A2,

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respectively, and the proton concentration of buffer solution is given by (H^+) . On the other hand, Eq. 2 can be applied to the proton dissociation equilibria of BH_2^{2+} , $BH^+ \rightleftharpoons B + H^+$ (Step B1) and $BH_2^{2+} \rightleftharpoons BH^+ + H^+$ (Step B2).²⁾

$$\frac{1}{P_a} = \frac{1}{P} + \frac{1}{PK_{a1}^B}[(H^+)] + \frac{1}{PK_{a1}^B K_{a2}^B}[(H^+)^2] \quad (2)$$

The dissociation constants corresponding to the equilibria of Step B1 and Step B2 are expressed as K_{a1}^B and K_{a2}^B , respectively.⁴⁾

For the case where the proton dissociation equilibrium due to Step A2 or Step B2 is ignored we have already presented a detailed discussion.²⁾ Here, the direct application of Eqs. 1 and 2 for the determination of the two-step proton dissociation constants and the P values will be discussed. Note that these equations are particularly useful for the case where the values of the first and the second dissociation constants are very similar. The reason is as follows. When the values of pK_{a1}^A and pK_{a2}^A are considerably different, the proton dissociation equilibrium due to Step A2 can be neglected in the pH region where the proton dissociation of Step A1 occurs predominantly. However, in the pH region of Step A2 the equilibrium due to Step A1 is almost completely shifted to dissociation. This is not consistent with the assumption made in deriving the Eq. 1, as was discussed in detail in the previous paper.^{2,4)} Therefore, direct application of Eq. 1 to evaluate the pK_{a1}^A and pK_{a2}^A is not valid for the case where the two dissociation constants are quite different. Of course, these considerations are also applicable to Eq. 2, which describes the proton dissociation of Step B1 and Step B2.⁵⁾

Experimental Results

For the above reasons, compounds having two similar dissociation constants were selected. These are 6059S as a dibasic acid and *o*-tolidine as a diacidic base. The observed values of P_a as a function of pH of buffer solutions are collected in Table I. The data in Table I are also

Fig. 1. The Parabolic Relation given by Eq. 1 for 6059S in *n*-Octanol-buffer Systems

Fig. 2. The Parabolic Relation given by Eq. 2 for *o*-Tolidine in *n*-Octanol-buffer Systems

- 4) In the course of the derivation of Eqs. 1 and 2, it was assumed that the unionized species alone exists in the organic solvent phase, and also that the activity coefficient f for all the species is 1. See the text and footnotes 10 and 12 of our previous paper²⁾ for detailed discussions.
- 5) *n*-Octanol is frequently employed for QSAR studies,²⁾ and our experiments indicate that it is also a convenient solvent for the purpose of pK_a determination of acids or bases.

TABLE I. Apparent Partition Coefficients (P_a) of 6059S and *o*-Tolidine as a Function of pH, and Their Dissociation Constants and log P Values

6059S		<i>o</i> -Tolidine	
pH ^{a)}	P_a ^{a)}	pH ^{a)}	P_a ^{a)}
1.890	0.2154	3.860	14.13
2.321	0.1486	3.893	15.93
2.462	0.1233	3.934	18.49
2.527	0.1098	3.960	19.59
2.638	0.9408×10^{-1}	4.120	31.61
2.821	0.6904×10^{-1}	4.200	38.84
3.060	0.3270×10^{-1}	4.310	52.47
3.053	0.3207×10^{-1}	4.379	53.83
3.178	0.2797×10^{-1}	4.550	74.41
3.267	0.1936×10^{-1}	4.730	100.5
3.390	0.1266×10^{-1}	4.830	113.4
3.460	0.9908×10^{-2}	4.911	125.9
3.490	0.8766×10^{-2}	5.018	144.8
3.537	0.7453×10^{-2}	5.140	160.2
		5.290	180.1
		5.530	203.0
		5.809	240.8
log $P = -0.58 \pm 0.12$		log $P = 2.34 \pm 0.03$	
$pK_{a1}^A = 2.48 \pm 0.18$		$pK_{a1}^B = 4.69 \pm 0.06$	
$pK_{a2}^A = \begin{cases} 3.24 \pm 0.10 \\ 3.24^{b)} \pm 0.10 \end{cases}$		$pK_{a2}^B = \begin{cases} 3.90 \pm 0.06 \\ 3.89^{b)} \pm 0.06 \end{cases}$	

a) Although the last figure of P_a and pH values is not reliable, the calculations of $(P_a)^{-1}$, $(H^+)^{-1}$ and (H^+) in Eqs. 1 and 2 were done using the values in this table.

b) These values were derived from the hypothetical proton concentration at the minimum point of the least-squares fitted curves in Figs. 1 and 2. The other values were obtained by direct analyses of Eqs. 1 and 2. See the text for details. The errors were calculated using the estimated standard errors of the partial regression coefficients.²⁾

illustrated in Figs. 1 and 2. It is clear from the figures that all the points fall on a smooth parabolic curve that is given by $(1/P_a) = 3.926 + 1.269 \times 10^{-2} (H^+)^{-1} + 7.313 \times 10^{-6} (H^+)^{-2}$ for 6059S (Fig. 1) and $(1/P_a) = 4.631 \times 10^{-3} + 2.267 \times 10^2 (H^+) + 1.782 \times 10^6 (H^+)^2$ for *o*-tolidine. The correlation coefficient (r) and the standard deviation (s), respectively, are 0.9993 and 1.8395 for the former, and 0.9995 and 0.0007 for the latter. It is noteworthy²⁾ that in the case of Eqs. 1 and 2 three physical constants are obtained from one parabolic relation, so that for determining the constants accurately the correlation coefficient of the least-squares fitted curve is required to be very high, and the number of data points should be large. The technique described in the appendix in our first paper²⁾ of this series is suitable for evaluating a_0 , a_1 , and a_2 of the parabolic equation $y = a_0 + a_1x + a_2x^2$. The values of pK_{a1} , pK_{a2} , and log P thus obtained are listed in Table I. K_{a2} can also be evaluated from the relation $K_{a2}^A = -(H^+)_{\min}^h/2$ or $K_{a2}^B = -2(H^+)_{\min}^h/(H^+)_{\min}^h$, $(H^+)_{\min}^h$ being the hypothetical proton concentration at the minimum point of the least-squares fitted curve of Eqs. 1 and 2. The K_{a2} values thus obtained are also included in Table I. In practice, the following points should be taken into consideration in the experimental planning in order to obtain accurate physical constants. As is clear from Eqs. 1 and 2, the value (intersection at the ordinate) corresponding to $(H^+)^{-1} = 0$ for Eq. 1 or $(H^+) = 0$ for Eq. 2 is equal to P^{-1} and is always positive. Now our experience indicates that if insufficient experimental data points in the neighborhood of the pH values of $(H^+)^{-1} \approx 0$ (for Eq. 1) or $(H^+) \approx 0$ (for Eq. 2) are obtained, the least-squares fitted curve sometimes intersects the ordinate with a negative value (see also footnote 26a in Ref. 3). To avoid this, and to obtain a good least-squares parabolic curve, P_a measurements in the above pH region are necessary. Of course, P_a measurement should not be carried out in the pH region where the relative amount of dianion ($A^{=}$) or dication (BH_2^{2+}) becomes very large, particularly for the *n*-octanol-water

system, in order to avoid error due to the dissociation effect in the organic solvent phase.²⁾

Now let us turn to the pK_a and $\log P$ values listed in Table I. In the case 6059S there are two COOH groups: one is the ring carboxylic group at the 2nd position and the other is in the side chain group at the 7th position. It was reported that the basicity of the 1-methyl tetrazole group is weak,⁶⁾ and proton addition to the ring nitrogen atoms does not occur in the pH region studied here.¹⁰⁾ $pK_{a1}^A=2.49$ could be reasonably assigned to the dissociation of the ring COOH group at the 2nd position. Our other data¹¹⁾ on pK_a 's measured by the UV and partition coefficient methods for 6059S analogs that have only the COOH group at the 2nd positions as a dissociation center always gave a pK_a value close to 2.5. In addition, reports indicate that the pK_a value of the above COOH of cephalosporins^{10,12,13,15)} is between 2.2 and 2.9, while in the case of penicillins^{14,15)} the value is in the range of 2.5—2.9. These results support the assignment of $pK_{a1}^A=2.49$ to the carboxy group at the 2nd position. Accordingly the value of $pK_{a2}^A=3.24$ should be attributed to the COOH of the type $-\text{CH}(\text{COOH})-\text{CO}-$, in the side chain at the 7th position. It is likely that the acidity of the COOH group is increased by the electron-attracting nature of the $\text{C}=\text{O}$ group at the nearest-neighbour position. The value of $\log P=-0.59$ of 6059S is, of course, for its neutral species. This compound is very soluble in water, so the $\log P_a$ value is much less than zero.

In the case of *o*-tolidine there are two NH_2 groups at the *p*,*p*'-positions. It is well known that the two benzene rings of the biphenyl are not coplanar, being considerably twisted, and also that the *o*-methyl group with respect to *p*- or *p*'- NH_2 exerts some steric influence upon the resonance effect between the amino and the benzene π -electrons.¹⁶⁾ These effects mean that the basicities of the two NH_2 groups at the *p*- and *p*'-positions are quite similar. On this basis, and also in comparison with the dissociation constants of benzidine,¹⁸⁾ which were reported to be $pK_{a1}^B=4.97$ and $pK_{a2}^B=3.75$,¹⁹⁾ and $pK_{a1}^B=4.65$ and $pK_{a2}^B=3.43$,²⁰⁾ the values obtained here for *o*-tolidine (see Table I) appear to be reasonable. Next, let us consider the value of

- 6) Conversely, it is known that for the 1H-tetrazoles (tetrazolic acids) the acid dissociation shown below occurs in the pH region studied here.⁷⁻⁹⁾ This is due to the increase of the electronegativity of the conjugated ring system caused by the nitrogen atoms.

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 16) Note that although the *ortho*- CH_3 group may affect the NH_2 group, the degree of steric hindrance is small,^{17,20)} so the dissociation constants of *o*-tolidine would be of the same order as those of benzidine. However, the finding that the difference between pK_{a1}^B and pK_{a2}^B of *o*-tolidine (~ 0.91) is smaller than that of benzidine (1.22) can reasonably be interpreted as being due to some steric effect of the *ortho*- CH_3 group.
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$\log P=2.35$ for *o*-tolidine in the *n*-octanol-water system. The $\log P$ value of *o*-toluidine was reported as 1.29.²¹⁾ Thus, the $\log P$ value of *o*-tolidine can be estimated as 2.58 (twice 1.29) or 2.19 (twice 1.29 minus twice 0.193).²²⁾ These calculated values are reasonable in magnitude compared with that obtained here. In conclusion, it appears that the true partition coefficient, pK_{a1} , and pK_{a2} of 6059S or *o*-tolidine can be evaluated in terms of Eqs. 1 and 2 under the assumptions mentioned in the text.

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Spectrophotometric Determination of Bromazepam¹⁾

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Bromazepam (7-bromo-1, 3-dihydro-5-(2-pyridyl)-2H-1, 4-benzodiazepin-2-one) was determined by formation the chelate compound with iron(II) followed by extraction from the organic phase into an aqueous solution. The absorption spectrum of the chelate compound in aqueous solution showed maximum absorbance at 580 nm and the absorbance was stable for about 5 hr at room temperature.

The calibration curve was linear from 0.2 to 50 $\mu\text{g/ml}$ of bromazepam and the sensitivity of the procedure was 0.2 $\mu\text{g/ml}$ of bromazepam.

This procedure was applied to the determination of bromazepam in rat blood. The recovery of added bromazepam from rat blood ranged from 96 to 100.5%, with a mean recovery of 99.1%.

Keywords—bromazepam; 1,4-benzodiazepine derivatives; iron(II) chelate compound; spectrophotometric determination; thin-layer chromatography; rat blood

Introduction

Bromazepam³⁾ was synthesized by Fryer *et al.*⁴⁾ and has been clinically evaluated for the control of the obsessive compulsive neurosis.⁵⁾ Bromazepam can be hydrolyzed with strong mineral acid or decomposed thermally to yield an aromatic primary amine which can be determined by the Bratton-Marshall procedure⁶⁾ or by gas-liquid chromatography.⁷⁾

Sabation *et al.*⁸⁾ reported that ferrous ions formed purple complexes with pyridyl benzodiazepin-2-ones; these compounds have a basic dipyridyl type bond structure which has excellent

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