[Chem. Pharm. Bull. 27(6)1366—1372(1979)]

UDC 615.31.033.076.9 : 611.1.08

Kinetics on Uptake of Drugs in Goldfish. IV.[1] Contribution of the Stationary Layer to Drug Absorption from the Gill

Yoko Sakiya and Yoshiko Miyauchi

Faculty of Pharmaceutical Science, Tokushima University of Arts and Science[2]

(Received November 6, 1978)

The contribution of the stationary layer to the absorption of benzoic acid, salicylic acid, phenol and *p*-aminoethylbenzoate from the gills of goldfish was estimated by kinetic analysis of the relationship between perfused flow to the gills and the drug absorption rate. The changes in the absorption rate constant increased as the perfusion velocity increased. A double combination model consisting of an aqueous diffusion layer in the perfusing solution and the diffusion layer on the gill membranes was used to analyze drug absorption from the membranes. Even though the molecular weights of these drugs are similar, there were variations in the permeability coefficients of the drugs through the aqueous diffusion layers, suggesting that drug absorption from the gills cannot be explained simply in terms of a static diffusion layer in the perfusing solution.

Keywords——gill of goldfish; drug absorption; unstirred layer; recirculaing perfusion method; double diffusion model; kinetic analysis

In our previous reports,[1,3,4] we described the following observations. (1) Drugs lost from an external solution due to absorption by goldfish are recovered in high yield from the body; a kinetic model was established to describe this process. (2) Absorption of the drug from the aqueous phase into goldfish and excretion of the drug from goldfish into the aqueous phase can be represented as first-order processes. (3) The absorption membrane of the goldfish is lipid-like in nature, and absorption through such a membrane is rapid in a solution in which a drug is present in nonionic form, but slow in a solution in which the bulk of the drug is in ionic form. (4) Addition of urea to the aqueous phase accelerates the absorption of drugs in ionic form.

Absorption of drugs by goldfish is thought to occur mainly through the gills. For this reason, the absorption rate is considered to be affected by the rate of water suction over the gill. Higuchi and others[5–8] pointed out the large contribution of the aqueous diffusion layer on the surface of the gastrointestinal tract to drug absorption, in addition to the distribution and diffusion of drugs on the gastrointestinal tract membrane. The thickness of the stationary layer forming the aqueous diffusion layer is affected by the surface water flow, and absorption of a drug from the gills is considered to be affected by two factors: the rate of supply of the drug solution to the gill and the thickness of the stationary water layer. In the present study, in order to examine the contribution of water flow to the absorption of salicylic acid, benzoic acid, phenol and *p*-aminoethylbenzoate from the gill, drug solution was perfused and recirculated at a definite rate from the mouth to the gill of a goldfish, and changes in absorption rate with change in the flow rate of the circulating solution were

1) Part III: Y. Sakiya, N. Umezawa and M. Hanano, *Yakugaku Zasshi*, **96**, 737 (1976).
2) Location: *Yamashiro-cho, 770, Tokushima.*
3) Y. Sakiya, N. Umezawa and M. Hanano, *Yakugaku Zasshi*, **94**, 1123 (1974).
4) Y. Sakiya, N. Umezawa and M. Hanano, *Yakugaku Zasshi*, **95**, 402 (1975).
5) A. Suzuki, W.I. Higuchi and N.F.H. Ho, *J. Pharm. Sci.*, **59**, 644 (1970).
6) A. Suzuki, W.I. Higuchi and N.F.H. Ho, *J. Pharm. Sci.*, **59** (1970).
7) N.F.H. Ho, W.I. Higuchi and J. Turi, *J. Pharm. Sci.*, **61**, 192 (1972).
8) R.G. Stehle and W.I. Higuchi, *J. Pharm. Sci.*, **61**, 1922 (1972).

measured. The results were analyzed kinetically, and the contribution of the aqueous diffusion layer is discussed.

Theoretical

Figure 1 shows a schematic diagram of the perfusion of a drug solution from the mouth to the gill of a goldfish at a constant flow rate. The drug solution (total volume, V) in the vessel passes from the mouth at a constant flow rate (F). If absorption and excretion of water are neglected, the perfusion fluid will pass out of the gill at the same flow rate (F). The mean concentration of the drug (C_o') in this outflow solution should be lower than that (C_o) in the inflow solution due to absorption from the gill. The outflowing solution will be mixed with the solution in the vessel and will recirculate as an inflow solution again. The relation between the rate of fall of drug concentration in the flow solution and the quantity of the drug on the gill can be expressed by equation (1).

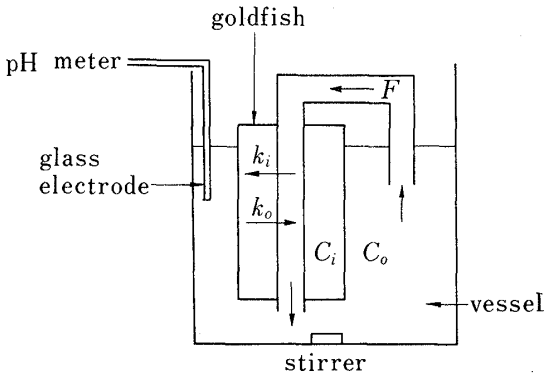

Fig. 1. Apparatus Used to Study Drug Absorption Across the Gills of Goldfish by a Recirculating Perfusion Method

C_o: Drug concentration in the vessel. C_i: Apparent drug concentration in goldfish. k_i: Apparent absorption rate constant. k_o: Apparent excretion rate constant. F: flow rate.

$$-dC_0/dt = F(C_0-C_0')/V_0 \quad (1)$$

Drug concentration in the inflow solution, C_o, has been shown experimentally to decrease by a first-order process in the initial stages of experiments, where excretion of the drug by the goldfish can be neglected. If this rate constant is taken as the apparent absorption rate constant (k_i), equation (2) is obtained, or equation (3) in terms of the apparent absorption clearance (Cl).

$$-dC_0/dt = k_i C_0 \quad (2)$$

$$-V_0\, dC_0/dt = Cl\, C_0 \quad (3)$$

$$\mathrm{Cl} = k_i V_0$$

If we consider that a perfusing solution with drug concentration C flows in at a rate $\hat{F}$ through a microvolume, dv, of part of the gill, that the drug is absorbed with an absorption rate constant $\hat{k}$, and that the liquid flows out with concentration change dc, then the mass balance can be expressed by equation (4), which can be rewritten as equation (5).

$$\hat{F}C - \hat{k}C\, dv - \hat{F}(C+dc) = 0 \quad (4)$$

$$dc/C = -\hat{k}/\hat{F}\cdot dv \quad (5)$$

For the sake of simplicity, $\hat{k}_i \cdot \hat{F}$ may be considered to be equal in all parts of the gill, and then integration of equation (5) from the inflow port to the outflow port will give equation (6),

$$\int_{C_0}^{C_0'} dc/C = -\bar{k}\, V_a/F \quad (6)$$

where $\bar{k}$ is the mean absorption rate constant of the gill and V_a is the volume of perfusing solution on the gill. By defining $\bar{k}V_a$ as the intrinsic absorption clearance, $\hat{\mathrm{Cl}}$, equation (6) can be rewritten as equation (7).

$$ln\, C_0'/C_0 = -\hat{\mathrm{Cl}}/F \quad (7)$$

This equation (7) is the same as that obtained from the parallel tube model[9] in organ perfusion. Consequently, the effective mean concentration in the gill, $\bar{C}$, is indicated by the logarithmic mean in equation (8).

9) K.S. Pang and M. Rowland, *J. Pharmacokinetics and Biopharm.*, **5**, 625 (1977).

$$\bar{C} = (C_0 - C_0')/\ln(C_0/C_0') \quad (8)$$

The absorption rate is then given by equation (9).

$$-V_0\, dC_0/dt = \hat{Cl}\cdot\bar{C} \quad (9)$$

The relationship between the apparent clearance, Cl, and intrinsic clearance, $\hat{Cl}$, is expressed by equations (10) and (11).

$$\hat{Cl} = -F\ln(1-Cl/F) \quad (10)$$

$$Cl = F(1-e^{-\hat{Cl}/F}) \quad (11)$$

The effect of the stationary layer on drug absorption has been analyzed by the two diffusion layer model.[5] Absorption of a drug is assumed to occur by diffusion between two layers; an aqueous diffusion layer with a permeability coefficient of p_1 and a membrane parenchymal layer with a permeability coefficient of p_2. By taking the partition coefficient between the aqueous layer and the membrane as K in a stationary state, the permeability coefficient p of the two diffusion layers can be expressed by equation (12).

$$1/p = 1/p_1 + K/p_2 \quad (12)$$

By averaging equation (12) for the whole of the gill, and by defining equations (13) and (14) as the intrinsic clearances when the permeability of each diffusion layer reaches the steady state, equation (15) is obtained.

$$p_1\cdot V_a = Cl_1 \quad (13)$$

$$p_2\cdot V_a/K = Cl_2 \quad (14)$$

$$1/Cl = 1/Cl_1 + 1/Cl_2 \quad (15)$$

In the present series of experiments, clearance was not measured at the stationary state, but the change of drug concentration in the perfusate was slow, and the data were analyzed on the assumption that equation (15) is applicable, *i.e.*, that a pseudo-steady state is established.

The thickness (δ) of the aqueous diffusion layer is known to change with flow rate (F), diffusion constant of the drug (D), viscosity of the perfusate (ν), and the shape of the membrane surface. The surface of the gill is complex, and the presence of a specific diffusion layer formed by secretion from the gill into the perfusate can be expected by analogy with the surface of the small intestine. It would be difficult to find the relationship between the thickness of the aqueous diffusion layer and the flow rate for goldfish gill and, as a rough approximation, an attempt was made to correlate the change in the thickness of a diffusion layer for flow over a plane surface. When a flat board is placed in parallel with water flow, the thickness of the diffusion layer at a distance, x, from the end of the board where the flow first strikes can be expressed by equation (16).[10]

$$\delta \approx D^{1/3}\nu^{1/6}\sqrt{x/F} \quad (16)$$

If the effect of the shape of the gill and that of the end of the outflow region are neglected, the mean thickness of the water diffusion layer for the gill as a whole can be obtained by integration of equation (16) over the length of the water path in the gill, L.

$$\bar{\delta} \approx \frac{D^{1/3}\nu^{1/6}}{\sqrt{F}\cdot L}\cdot\int_0^L\sqrt{x}\cdot dx = 2/3\, L^{1/2}\cdot\frac{D^{1/3}\nu^{1/6}}{\sqrt{F}} \quad (17)$$

The permeability coefficient of the water diffusion layer, p_1, is given by the thickness of the diffusion layer, the diffusion constant of the drug, and the surface area (A) of the gill; it can be expressed by equation (18).

$$p_1 = A\cdot D/\delta \quad (18)$$

It can be seen from equations (17) and (18) that a proportional relationship is established between the square root of the flow rate and the permeability coefficient of the water diffusion

10) V.G. Levich, "Physicochemical Hydrodynamics," Translated by Scripa Technica, Inc., Prentice-Hall, Inc., 1962, p. 59.

layer or the absorption clearance (Cl_1) when the permeability is rate-determining in the water diffusion layer. As a result, a plot of the reciprocal of intrinsic clearance on the ordinate against the square root of the flow rate of the perfusion liquid on the abscissa will give a straight line. From the intercept on the ordinate, Cl_2, *i.e.*, the clearance when permeation through the membrane is the rate-determining step, can be obtained; the size of the contribution of the water diffusion layer is given by the slope.

Experimental

Goldfish——*Carassius auratus*, weighing 13±2 g, was used.

Materials——Salicylic acid (SA, mp 158—159°), benzoic acid (BA, mp 121—122°), phenol (Ph, mp 40—41°), *p*-aminoethylbenzoate (*p*-ABAE, mp 88—90°) and urea (U, mp 132—133°) were used. Dichloroethane was distilled at 83—84° before use. All other reagents were of special grade and were obtained commercially.

Preparation of Drug Solutions——SA, BA and Ph were each dissolved in water to 1 mM concentration; *p*-ABAE was used as a 0.1 mM solution. Another series of solutions was prepared by addition of 0.5 M U to each of these solutions. The solutions were adjusted to pH 4.0 for SA, pH 4.0 and 5.0 for BA (different brands), and pH 6.0 for Ph using diluted HCl or diluted NaOH solution. *p*-ABAE solution was used for absorption experiments without pH adjustment.

Experimental Procedure——Polyvinyl tubing was inserted into the mouth of a goldfish and the other end of the tubing was placed in the drug solution. In the experiment on absorption of SA and BA, the fish body was covered with a rubber bag. The drug solution was recirculated at a constant flow rate from the mouth to the gill, using a perfusion pump (model SJ-1210, Mitsumi Sci. Ind., Inc.). The flow rate was in the range of 0.9—11.0 ml/min, at three different levels. The drug solution (70 ml) was maintained at 25° in a 200 ml flask during the experiment using a thermostatic bath, and the pH of the solution was maintained by the addition of dilute HCl or dilute NaOH solution. A pH-meter was used to check the pH of the drug solution, which was agitated with a stirrer during the experiment. A point 5 min after the start of perfusion was taken as 0 time, and 1 ml aliquots of the drug solution in the flask were collected at 0, 2, 4, 6, 8, 10, 20, and 30 min.

Analytical Method——Analytical procedures for SA, BA and Ph in each sample were conducted as described in the previous papers.[1,3,4] *p*-ABAE was determined from the UV absorption of the sample solution diluted with 2 ml of distilled water. The absorbance was read at 287 nm. Throughout these experiments, UV absorption was measured with a Hitachi 200-20 spectrophotometer.

Results and Discussion

Drug absorption through the gills of goldfish has been examined by the perfusion of drug solutions, using low concentrations (1 mM solutions of BA, SA and Ph, and 0.1 mM *p*-ABAE) to avoid a physiological reduction in absorption rate with time. It was confirmed that each goldfish could resume normal movements in fresh water after completion of the experiments. It has been reported[1] that drug excretion from the body of a goldfish occurs during such tests, and an equation was proposed taking the rate of excretion into consideration. In the present experiment, however, where the amount of drug absorption into the goldfish is small and where the concentration of the drug taken up into the body of the goldfish is small, excretion of the drug can be neglected. The concentration

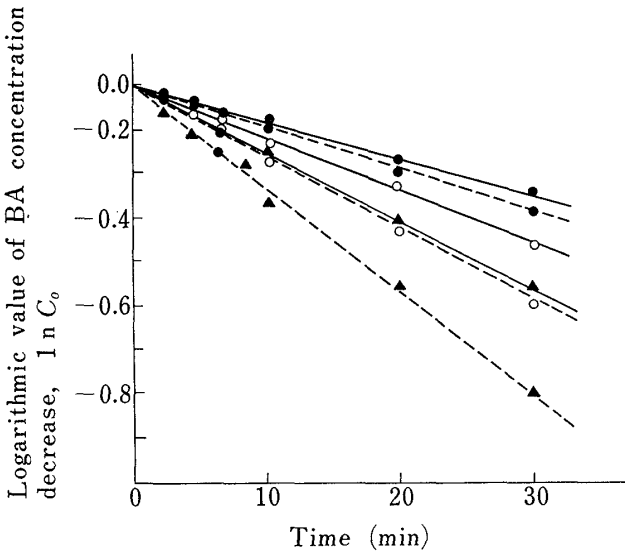

Fig. 2. Semi-logarithmic Plots of BA Concentration in Perfusing Solution Recirculating Across the Gills of Goldfish at Different Flow Rates at pH 4.0 and 25°

The initial concentration of BA was 1 mM. —●—, —○— and —▲— are with 0.5 M U. —●—, —○— and —▲— indicate flow rates of 2, 4 and 11 ml/min, respectively. Solid and dotted lines were calculated by the least-squares method. For other conditions, see in Fig. 1 and Table I.

of each drug in the perfusing solution fell at a first-order rate during the experiments, and the drugs were found to be absorbed by a first-order process. An example is shown in Fig. 2, for the time courses of BA concentration in perfusing solutions of BA and of BA plus U.

The apparent absorption rate constant was calculated by the least-squares method from a plot of the logarithm of the concentration of the drug against time. By obtaining the mean rate constant from the calculated value (k_i), regarding the body weight as a simple weight, the average apparent absorption rate constant, $\bar{k}_i$, was calculated as follows,

$$\sum(k_i W_i)\sum(1/W_i) = \bar{k}_i$$

where W represents the body weight of a goldfish (g). By substituting this into equation (3), the apparent absorption clearance was obtained. By substituting the apparent absorption clearance into equation (10), the intrinsic absorption clearance was calculated. These values for each drug are listed in Table I. It is clear that the apparent absorption rate

Table I. Kinetic Parameters of BA, SA, Ph and *p*-ABAE Uptake across the Gills of Goldfish from Recirculating Solution at Different Flow Rates at 25°

Sample solution	pH	n	Flow rate (ml/min)	Apparent absorption rate constant $\times 10^3$, (min^{-1}) mean	± SEM	Apparent absorption clearance[a] (ml/min) mean	± SEM	Intrinsic absorption clearance[b] (ml/min)
BA	4.0	7	2	8.791	1.012	0.615	0.071	0.735
		17	4	12.640	1.268	0.885	0.089	1.000
		13	11	15.936	1.257	1.116	0.088	1.177
BA+U	4.0	8	2	9.825	0.996	0.688	0.070	0.843
		7	4	16.506	2.072	1.155	0.145	1.363
		11	11	24.300	2.389	1.701	0.167	1.848
BA	5.0	7	2	3.771	0.266	0.264	0.019	0.283
		7	4	4.318	0.316	0.302	0.022	0.314
		7	11	4.871	0.461	0.341	0.032	0.346
SA	4.0	7	2	2.820	0.373	0.198	0.026	0.207
		8	4	3.909	0.489	0.274	0.034	0.284
		7	11	5.461	0.415	0.382	0.029	0.389
SA+U	4.0	8	2	3.200	0.199	0.224	0.014	0.238
		8	4	4.503	0.458	0.315	0.032	0.328
		11	11	7.129	0.506	0.499	0.035	0.512
Ph	6.0	7	0.9	5.318	0.478	0.372	0.033	0.480
		8	4	7.652	0.666	0.536	0.047	0.575
		7	11	9.204	0.662	0.644	0.046	0.664
Ph+U	6.0	8	0.9	4.667	0.639	0.327	0.044	0.406
		8	4	6.723	0.592	0.471	0.041	0.501
		10	11	7.515	0.690	0.526	0.048	0.539
p-ABAE	—	7	0.9	4.183	0.535	0.293	0.037	0.354
		7	4	9.422	1.057	0.660	0.074	0.721
		8	11	13.058	1.268	0.914	0.089	0.954
p-ABAE+U	—	7	0.9	3.181	0.326	0.223	0.023	0.256
		7	4	6.003	0.728	0.420	0.051	0.444
		8	11	7.180	0.839	0.503	0.059	0.515

BA: benzoic acid, SA: salicyclic acid, *p*-ABAE: *p*-aminobenzoic acid ethyl ester, Ph: phenol, U: urea (0.5 M). The concentration of drug solution was 1 mM, except for 0.1 mM p-ABAE.
a) and *b*) were calculated by introducing an apparent absorption rate constant into eq. 3 and an apparent absorption clearance into eq. 10, respectively. n: number of experiments.

constants increased as the perfusion velocity increased, indicating that the velocity of perfusion over the gills substantially influences the absorption of the drugs through the gills. As indicated above, the perfusion velocity affects the difference of drug concentration between

the tips of the gills and also the thickness of the stationary water layer on the absorption surface. Both can affect the apparent absorption clearance. The thickness of the stationary water layer is reduced as the flow rate increases, and drug absorption by diffusion in the layer falls. In order to examine the effect of diffusion, it was assumed that the effect of the stationary layer can be neglected at maximal perfusion velocity, and the intrinsic absorption clearance at the maximal perfusion velocity was substituted into equation (11) to calculate the apparent absorption clearance at each flow rate. The relationship between the apparent absorption clearance thus calculated and the apparent absorption clearance determined is shown in Fig. 3 for BA. The apparent absorption clearance was smaller than the calculated value, so that the diffusion of drugs in the stationary layer does affect the absorption of the

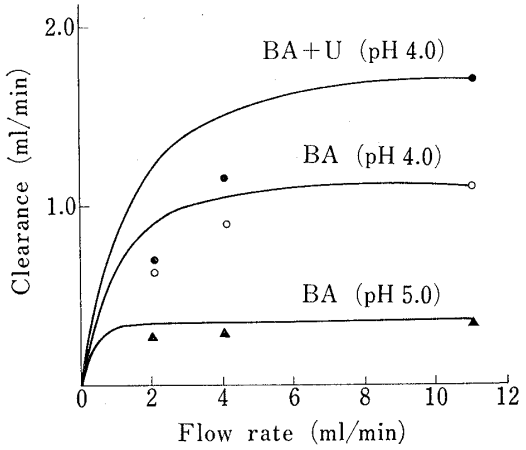

Fig. 3. Decrease of the Apparent Absorption Clearance with Decreasing Flow Rate of Perfusate on the Gill, Suggesting the Effect of Diffusion in the Stationary Water Layer on the Absorption of BA

Data from Table I; solid lines represent the apparent absorption clearances calculated by introducing the intrinsic absorption clearance at maximum flow rate into eq. 11. For other conditions, see Table I.

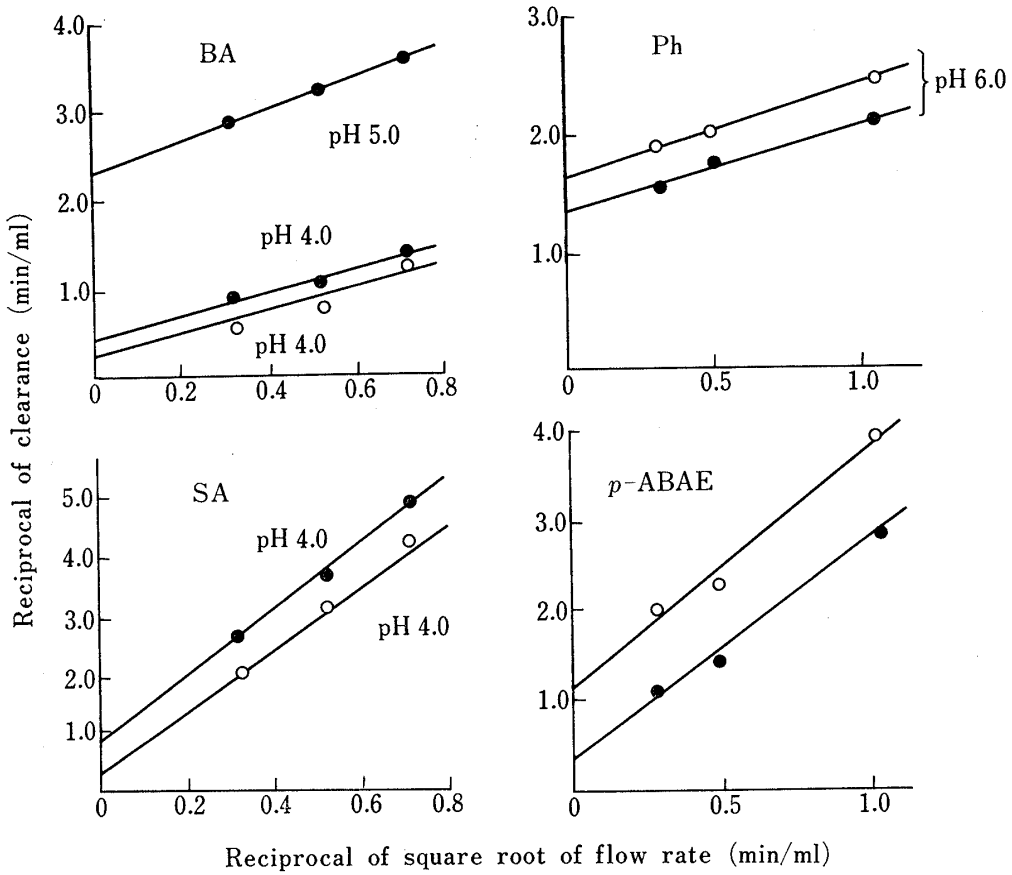

Fig. 4. Relationship between the Reciprocals of Intrinsic Absorption Clearance and the Square Root of Flow Rate for SA, BA, Ph and *p*-ABAE in the Presence or Absence of U

Data taken from Table I; solid lines are the regression lines.
—●—: without U. —○—: with 0.5 M U.
For other conditions, see Table I.

drug. The same results were obtained with other drugs. From equations (17) and (18), based on the double combination model of the aqueous diffusion layer in the perfusion liquid and the diffusion layer on the gill membranes, the reciprocal of the intrinsic absorption clearance was plotted against the reciprocal of the square root of the perfusion velocity. All the test drugs gave almost linear relationships (Fig. 4), confirming that the double combination model is applicable for drug absorption through membranes. It also appears that the theoretical equation for the thickness of the diffusion layer on a flat plate can be applied to drug absorption through the gills of goldfish. Figure 4 also shows that the presence of U affects drug penetration through the membranes. In the cases of BA and SA, the plots indicate that the presence of U assists the penetration of drugs through the membrane. At a pH of 4.0 with BA solution, U enhanced penetration approximately 5-fold. The values at pH 4.0 were about 7 times higher than those at pH 5.0, and it appears that drugs can pass through the membrane more easily in molecular form than in ionic form. The penetration of Ph and *p*-ABAE through the membrane is inhibited by the addition of U. The extent of the inhibition was about 1.2 times with Ph and about 2.4 times with *p*-ABAE. It has already been reported[3] that the effect of U on the absorption of Ph in goldfish was almost negligible under normal conditions. Thus, U promotes the absorption of anionic organic compounds such as BA and SA through the gills, while with non-ionic compounds, such as Ph and *p*-ABAE, this does not occur. In the present experiments, we found that the absorption of BA and SA (ionic forms) through the body surface could not be neglected. About 13.4% (mean of 9 samples) was absorbed within 30 min in the case of 1 mM BA solution (pH 5.0) and about 4.0% (mean of 6 samples) in the case of 1 mM SA solution (pH 4.0), but no absorption was noted with Ph or *p*-ABAE (non-ionic forms). The slope of the plot in Fig. 4 in the presence of U was the same as that in its absence ($p<0.05$), so that U had no significant effect upon the diffusion of the drug in the stationary layer. There was no significant difference ($p<0.05$) in the case of BA between pH 4.0 and 5.0. The slopes of the plots for SA in the presence and absence of U were significantly different from those of all the other drugs ($p<0.05$). The slopes in the cases of Ph and *p*-ABAE were significantly different from each other ($p<0.05$). However, no other significant differences between slopes were noted with other drugs ($p<0.05$). A significant difference between the slopes could mean that there were differences in the permeability coefficients of these drugs in aqueous diffusion layers, even though their molecular weights are similar. It appears that drug absorption through the gill structure of goldfish cannot be explained simply in terms of static diffusion layers in the perfusiong solution. Further studies are necessary to develop a more suitable theoretical model.

Acknowledgement The authors are grateful to Dr. Manabu Hanano, Tokyo University, for valuable advice during this work.