Biochemical Pharmacology, Vol. 37, No. 8, pp. 1519–1523, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

EFFECT OF PERFUSED RAT MANDIBULAR-GLAND pH_I ON THE RATIO OF PROCAINAMIDE CONCENTRATION IN SALIVA TO THAT IN VENOUS EFFLUENT*

KIKUO IWAMOTO,† JUN WATANABE, YASUSHI KANAI, SATOSHI MIZUNO and SHOJI OZEKI

Department of Hospital Pharmacy, Shimane Medical University, Izumo 693; and Department of Biopharmaceutics, Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya 467, Japan

(*Received* 1 *May* 1987; *accepted* 11 *September* 1987)

Abstract—The saliva to venous-effluent concentration ratio (S/E ratio) for procainamide (PA) was determined and compared with the ratio calculated by using the intracellular pH value of glandular cells. Exposed mandibular gland was perfused *in situ* with Krebs–Ringer bicarbonate buffer containing PA (10–100 μg/ml) and acetylcholine (ACh, 0.1 to 10 μM) or pilocarpine (10 μM). These perfusion conditions maintained almost normal physiological function of the mandibular gland throughout the perfusion period of 60 min, since the salivary Na^+ and K^+ concentrations were kept at almost constant levels, comparable with those reported *in vivo*, and the salivary flow, pH and protein level were also stabilized. Under fixed stimulation conditions with 1 μM ACh or 10 μM pilocarpine, the perfusate PA concentration ranging from 20 to 100 μg/ml did not affect the S/E ratio (approximately 0.3). There was a negative correlation between the S/E ratio and salivary pH when stimulated with 0.1 to 10 μM ACh. However, Matin's equation [S. B. Matin *et al.*, *Clin. Pharmac. Ther.* **16**, 1052 (1974)] employing venous effluent and salivary pH values did not explain fully these observed ratios. In contrast, Borzelleca's model [J. F. Borzelleca and J. W. Putney, *J. Pharmac. exp. Ther.* **174**, 527 (1970)] for salivary drug transport using intracellular pH of the mandibular gland cells predicted S/E ratios relatively close to the observed values when the gland was perfused at pH 7.4 or 8.0.

The essential prerequisite for saliva utilization in therapeutic drug monitoring is the presence of a consistent correlation between drug concentrations in plasma (or serum) and saliva over a broad concentration range. Matin *et al.* [1] propose that the saliva-to-plasma concentration ratio (S/P ratio) for weakly acidic or basic compounds can be predicted from a modified pH-partition (between plasma and saliva) hypothesis. On the other hand, Borzelleca and Putney [2] suggest that the intracellular pH in the epithelial cells of the salivary gland, rather than the plasma (or serum) pH, may limit the salivary transport of many drugs. They propose that the luminal side, rather than the serosal side, of the epithelial cellular membrane in the salivary gland may play an important role as a lipid barrier. However, there has been no reevaluation of Matin's equation by demonstrating the importance of intracellular pH of the salivary glands in studies on salivary excretion of drugs.

A certain *in situ* technique which permits modification of the perfusion and/or stimulation conditions for the salivary gland was required to clarify the effect of the intracellular pH of the glandular tissue on the S/P or saliva to venous-effluent concentration ratio (S/E) of drugs. The present work was designed to examine the effect of the intracellular pH value of the perfused mandibular gland on the S/E ratio for procainamide by changing pH or stimulation conditions in the perfusate. Procainamide was chosen as a model drug because its salivary excretion had been proven to obey the pH-partition hypothesis [3–6].

MATERIALS AND METHODS

Materials. Procainamide (PA) hydrochloride was donated by the Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). Chemicals were purchased from the following sources: acetylcholine (ACh) chloride from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); pilocarpine hydrochloride from the Hoei Pharmaceutical Co. (Osaka, Japan); 5,5-dimethyl-2,4-oxazolidinedione (DMO) and Brij-35 (polyoxyethylene ethers of fatty alcohols) from the Tokyo Kasei Chemicals Co. (Tokyo, Japan); polyvinylpyrrolidone (PVP) from the Katayama Chemicals Co. (Nagoya, Japan); and bovine plasma albumin from the Sanko Pure Chemicals Co. (Tokyo, Japan). All other chemicals and reagents used were of analytical grade.

Animals. Male Wistar rats (300–400 g, 9- to 11-weeks-old) were purchased from the Shizuoka Laboratory Animal Farm (Hamamatsu, Japan) and fed *ad lib.* Shortly after anesthesia with pentobarbital (50 mg/kg, i.p.), each rat was tracheotomized, and a salivary duct was catheterized. Body temperature

* This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Sciences and Culture of Japan.

† Address all correspondence to: Dr. Kikuo Iwamoto, Director of Pharmacy, Shimane Medical University, 89-1, Enya-cho, Izumo 693, Japan.

was kept at 37° by means of thermostatic heating pads placed under the supine rat.

Perfusion of mandibular gland to collect venous effluent and saliva samples. After cannulating (PE-10 tubing, Clay Adams, Tokyo, Japan) one (A) of the paired duct orifices of the mandibular gland in the same manner as reported previously [6], the gland was exposed carefully while keeping the blood circulation intact; the sublingual gland was tied off. After cannulating (PE-10 tubing) the submental artery (B) retrogradely, the anterior facial vein (C) was cannulated (PE-60 tubing) and all other small blood vessels or their branches were tied off in order to perfuse the gland from B to C without recirculation.

Krebs–Ringer bicarbonate buffer solution containing 5% PVP, oxygenated with 95% O_2–5% CO_2 and kept at 37°, was the main perfusion medium in the present study. The pH of this buffer was adjusted to pH 6.8, 7.4 or 8.0; the concentration of PA was prepared at 10, 20, 30, 50 or 100 μg/ml (as base). Each perfusion medium contained ACh (0.1, 1 or 10 μM) or pilocarpine (10 μM) as a stimulant for salivation. The perfusion flow rate was maintained at 1.5 ml/min by a SJ-1215 peristaltic pump (Atto Co., Nagoya, Japan). The pH of the venous effluent was monitored by a microelectrode GS-195C (Toa Dempa Co., Nagoya, Japan) throughout the experiment. After a lag time of 15 min to attain a steady salivary flow, both the venous effluent (from C) and the saliva (from A) samples, pooled for 5 min, were collected periodically over another 45 min.

Determination of intracellular pH of the mandibular glandular cells. The mandibular gland was perfused for 30 min with Krebs–Ringer bicarbonate buffer solution (pH 6.8, 7.4 or 8.0) containing 0.2 mg/ml of DMO and 10 mg/ml of inulin. The gland was then isolated gently from the body and homogenized in distilled water (1 to 20) with a Potter–Elvehjem-type Teflon homogenizer. DMO concentrations in the effluent (0.2 ml collected in the last 10 min of the above perfusion) ($C_{E,DMO}$) and in the tissue homogenate (0.5 ml) ($C_{T,DMO}$) were determined by slightly modifying the method of Butler [7]. After adding distilled water (0.2 and 0.5 ml to the effluent and homogenate respectively) and 1 ml of 5 M NaH_2PO_4 solution, the mixture was extracted with 6 ml of ether by vigorous shaking for 15 min. The separated ether layer (5 ml) was re-extracted with 4 ml of pH 9.0 borate buffer. The DMO concentration of the resultant aqueous layer was determined by the absorbance difference at 215 and 220 nm. Inulin concentrations in the effluent ($C_{E,inulin}$) and in the homogenate ($C_{T,inulin}$) were determined by the method of Iwamitsu *et al.* [8] with the exceptions that the sample size was 0.1 ml for both venous effluent and homogenate and that the absorbance measurement was carried out 20 min after adding 1 ml of 1% Brij-35. Intracellular pH (pH_I) was then determined by the following equation [9, 10],

$$pH_I = 6.13 + \log\left(\frac{V_{DMO} - V_{inulin}}{V_T - V_{inulin}} \times (1 + 10^{pH_E - 6.13}) - 1\right) \quad (1)$$

where V_T is the total water content in the gland tissue (ml/g gland) which was estimated from the difference in the tissue weight before and after complete desiccation and V_{DMO} ($=C_{T,DMO}/C_{E,DMO}$) and V_{inulin} ($=C_{T,inulin}/C_{E,inulin}$) are DMO and inulin space (ml/g gland) respectively.

Measurement of salivary pH and flow rate. Both salivary pH and flow rate were measured by methods reported previously [6].

Determination of salivary protein and Na^+ *and* K^+ *levels.* The protein level was determined by the method of Lowry *et al.* [11] using bovine plasma albumin as standard. The Na^+ or K^+ concentration was determined by flame analysis using an AA-630-12 analyzer (Shimadzu Seisakusho Co., Kyoto, Japan) after appropriate dilution.

Determination of PA in venous effluent and saliva. Both venous effluent (0.2 ml) and saliva (0.05 ml) samples were analyzed for PA and its metabolite, *N*-acetylprocainamide (NAPA), by essentially the same method reported previously [6], except that NaCl was not used and 4 ml of 0.1 M HCl was used for the re-extraction from the benzene layer. The coefficients of variation for the assay of PA (1–100 μg/ml) and NAPA (0.1 to 10 μg/ml) were less than *ca.* 5 and 8% respectively. Trace amounts of NAPA were not detected in either venous effluent or saliva. The extent of binding of PA to PVP in the perfusate was evaluated by the conventional equilibrium dialysis method [6].

RESULTS

Integrity of the perfused mandibular gland. Salivary flow rates, Na^+ and K^+ levels, pH values, and protein levels were monitored to check the viability of the *in situ* perfused gland. All of these variables were close to their steady-state levels with both stimulants after 15 min of perfusion. The salivary flow rates with 1 μM ACh (N = 20) and 10 μM pilocarpine (N = 15) were 93.0 ± 19.5 and 77.7 ± 20.8 μl/min/g gland respectively. The mean Na^+ and K^+ levels when stimulated with 1 μM ACh were 14.0 ± 4.2 and 33.7 ± 1.5 mM, respectively, whereas when stimulated with 10 μM pilocarpine they were 10.6 ± 3.5 and 32.3 ± 4.4 mM respectively. These data, which are characteristic of the mandibular saliva, suggested to us that the perfusion and stimulation conditions were suitable for investigating the *in situ* salivary drug excretion mechanism.

Effects of perfusate stimulant dose, pH, and PA level on the S/E ratio of PA. As shown in Fig. 1, increasing the ACh dose (0.1 to 10 μM) raised the salivary pH and reduced the saliva to venous-effluent concentration (S/E) ratio of PA, when perfused at 10 μg/ml. Individual S/E data (Y) against individual salivary pH values (X) revealed a negative correlation [$Y = -0.37X + 3.2$, $r = -0.755$ ($N = 48$), $P < 0.01$].

Figure 2 shows the effect of the perfusate PA level on the S/E ratio during stimulation with 1 μM ACh or 10 μM pilocarpine. With each stimulation condition, a similar tendency in the S/E ratio was observed; no S/E ratio differed from others except at 10 vs 100 μg/ml PA with ACh. Furthermore, there was no significant difference between the S/E ratios

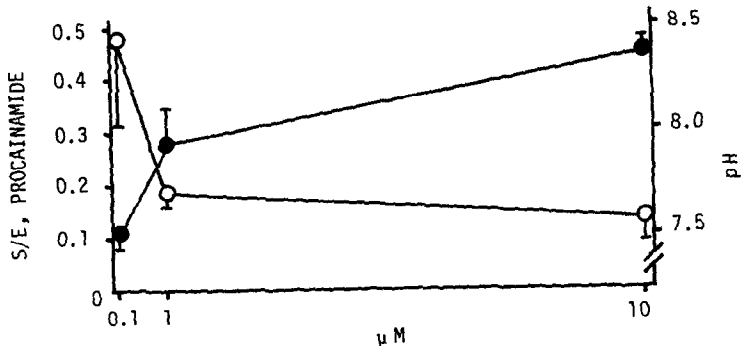

Fig. 1. Effect of ACh dose on the S/E ratio of procainamide (○) and salivary pH (●) when the rat mandibular gland was perfused with 10 μg/ml of the drug. Krebs–Ringer bicarbonate buffer solution (pH 7.4) containing 5% PVP, oxygenated with 95% O_2–5% CO_2 and kept at 37°, was used as the perfusion medium which was pumped at 1.5 ml/min. Salivary pH was measured by a previously described method [6]. Each point is the mean ± SD of three to four rats (15–20 data points).

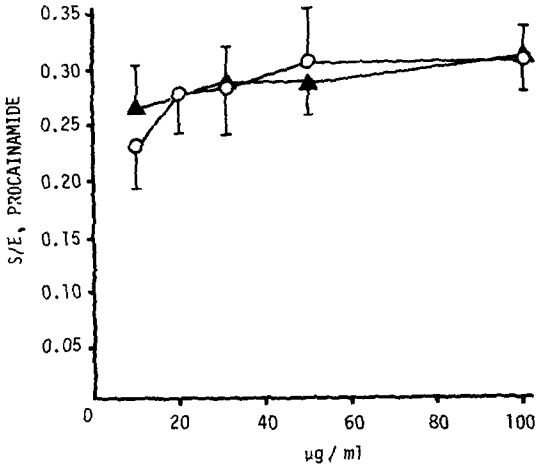

Fig. 2. Effect of the perfusate procainamide level on its S/E ratio in the perfused rat mandibular gland which was stimulated with 1 μM ACh (○) or 10 μM pilocarpine (●). Other perfusion conditions were the same as described in the legend of Fig. 1. Each point is the mean ± SD of three rats. There was a significant difference between 10 and 100 μg/ml under ACh stimulation ($P < 0.05$).

of the two stimulation conditions (e.g. at 30 μg/ml PA, 0.284 ± 0.04 and 0.285 ± 0.04 with 1 μM ACh and 10 μM pilocarpine respectively) despite a marked difference in the salivary pH (e.g. 7.46 ± 0.07 and 6.78 ± 0.26 with ACh and pilocarpine, respectively).

Comparison of the observed S/E ratio with that calculated by Matin's equation or Bozelleca's model. In the preliminary experiments, the binding of PA to PVP was measured in the perfusion buffer and the control (blank) venous effluent. At 10–30 μg/ml of PA, the mean (± SD) free fraction in the venous effluent (f_E) was 0.942 (±0.035) which was identical with that in the perfusate. The fraction in the saliva (f_S) was assumed to be 1.0, since the salivary protein level (0.368 ± 0.114 mg/ml with ACh and 0.540 ± 0.124 mg/ml with pilocarpine) was lower than the level *in vivo* and was almost negligible compared with the plasma protein level [6]. The pK_a value for PA has been reported to be 9.4 [12]. The S/E (i.e. C_S/C_E) ratio was then calculated by Matin's equation (Eq. 2) where C_p, f_p and pH_p were substituted by C_E, f_E and pH_E (venous effluent pH which

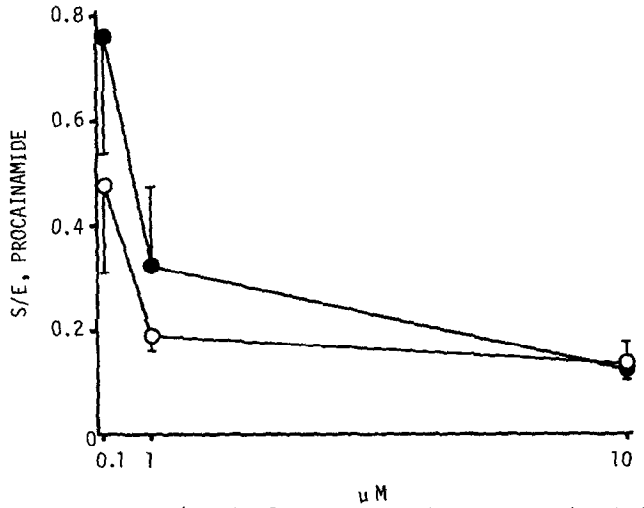

Fig. 3. Comparison of the observed S/E ratio (○) for procainamide with the ratio calculated according to Matin's equation (●) in the perfused rat mandibular gland which was stimulated with 0.1 to 10 μM ACh. The perfusate procainamide level was fixed at 10 μg/ml. Other perfusion conditions were the same as described in the legend of Fig. 1. Each point is the mean ± SD of three rats.

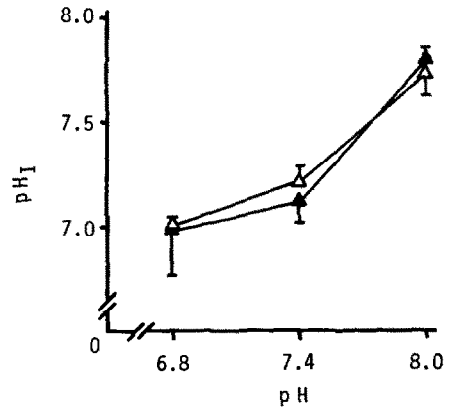

Fig. 4. Intracellular pH (pH_I) of the perfused rat mandibular glandular cells stimulated with 1 μM ACh (△) or 10 μM pilocarpine (▲). Perfusate pH was varied from 6.8 to 8.0. The pH_I was determined by estimating intracellular spaces for 5,5-dimethyl-2,4-oxazolidinedione and inulin [10, 11]. Each point is the mean ± SD of three to four rats.

is equal to the perfusate pH)

$$\frac{S}{E} = \frac{C_S}{C_E} = \frac{1 + 10^{pK_a - pH_S}}{1 + 10^{pK_a - pH_E}} \cdot \frac{f_E}{f_S} \qquad (2)$$

respectively. Figure 3 compares the observed S/E ratios with the calculated values when the gland was perfused with 10 μg/ml of PA at pH 7.4 and stimulated with ACh (0.1 to 10 μM). The calculated ratio showed the same tendency as the observed ratio in repsonse to ACh of maintaining a relatively high value except at 10 μg/ml. Amost the same discrepancy between the observed S/E ratio and the value calculated by Eq. 2 was found in other experimental conditions (see below in Fig. 5).

Figure 4 shows the intracellular pH (pH_I) value of the perfused mandibular gland against the effluent pH (pH_E) which was modified at 6.8 to 8.0 in the perfusate, during stimulation with ACh or pilocarpine. When the perfusate pH was equal to the physiological pH (i.e. pH 7.4) or higher, the intracellular pH was lower than the perfusate pH. According to the model of Borzelleca and Putney [2], the unbound fraction of PA in the glandular cells (f_I) was defined as $f_I = f_E/(C_I/C_E)$, where C_I/C_E was the intracellular to venous-effluent drug concentration ratio. Therefore, Eq. 2 may be modified by incorporating the above relationship as follows (Eq. 3),

$$\frac{S}{E} = \frac{C_S}{C_E} = \frac{C_S}{C_I} \cdot \frac{C_I}{C_E} = \frac{1 + 10^{pK_a - pH_S}}{1 + 10^{pK_a - pH_I}} \cdot \frac{f_E}{f_S} \qquad (3)$$

Figure 5 compares the observed S/E ratio of PA when perfused at 30 μg/ml with those calculated based on Equations 2 and 3 under both stimulation conditions at three different perfusate pH levels. During stimulation with ACh (1.0 μM), at pH 7.4, the calculated ratio based on Eq. 3 (0.365 ± 0.055) was closer to the observed ratio (0.284 ± 0.040) than that calculated by Eq. 2 (0.551 ± 0.083). The same tendency was obtained at pH 8.0, whereas an opposite trend was found at pH 6.8. In the case of stimulation with pilocarpine (10 μM), almost the same results were obtained (Fig. 5).

DISCUSSION

In vivo salivary Na^+ and K^+ levels in rats at a

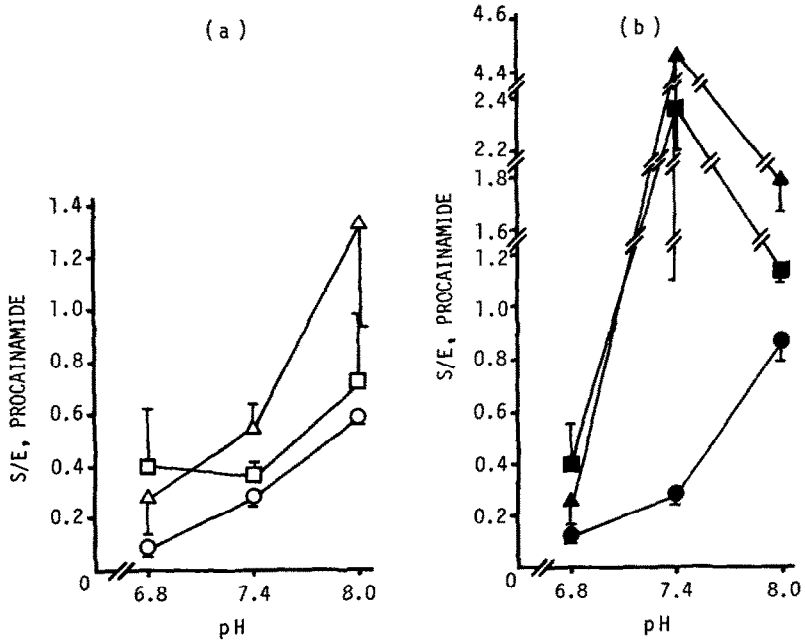

Fig. 5. Comparison of the S/E ratio for procainamide calculated by Matin's equation using venous effluent pH values (△, ▲) or intracellular pH values (□, ■) with the observed ratio (○, ●) in the perfused rat mandibular gland which was stimulated with 1 μM ACh (a) or 10 μM pilocarpine (b). The perfusate procainamide level was fixed at 30 μg/ml, but the perfusate pH was varied from 6.8 to 8.0. Each point is the mean ± SD of three to four rats.

relatively low flow rate (11 μl/min/g gland) when stimulated with ACh (i.a. infusion at 0.83 to 18 nmol/min) have been reported as 7.9 ± 5.8 (N = 42) and 42.0 ± 6.5 (N = 5) mM respectively [13]. Those levels at a salivary flow of 20–40 μl/min/g gland when stimulated with pilocarpine (i.p. injection at 0.2 to 2.0 ng/kg) have also been reported as 4.7 ± 1.1 (N = 5) and 42.1 ± 4.8 (N = 5) mM respectively [14]. The present *in situ* perfusion condition, in which the doses of both stimulants (ACh and pilocarpine differed from those *in vivo* [13, 14] yielded higher (1.7 to 2 times) and lower (80%) salivary levels of Na^+ and K^+, respectively, than the *in vivo* levels. On the other hand, the salivary flow rate during the stimulation with ACh and pilocarpine in our present experiments was much higher than the value reported *in vivo* [13, 14]. It has been reported that the rat mandibular Na^+ level increases whereas the K^+ level decreases with salivary flow in a certain flow rate region, due to the active reabsorption and secretion of Na^+ and K^+, respectively, in the mandibular striated duct [14]. Taking into account the difference of the salivary flow rate in the *in vivo* study from that in the present (*in situ*) experiment, the present salivary Na^+ and K^+ levels were regarded as almost normal.

The present perfusion medium including 5% PVP but no exogeneous protein constituent yielded far lower salivary protein levels than those (1.3 to 3.0 mg/ml) reported in the *in vivo* experiments [6, 14]. The relatively low protein level in the mandibular saliva was favored to neglect the contribution of salivary protein binding of the drug to the S/E ratio in Equations 2 and 3. After 15 min perfusion, the salivary flow rate, pH, and levels of protein, Na^+ and K^+ were almost stabilized over another 45 min. Therefore, the present *in situ* perfusion method was considered valid for the investigation of salivary drug excretion.

The effect of the dose of stimulant on the S/E ratio of PA was examined with ACh. A higher ACh dose yielded a higher salivary pH and a lower S/E ratio of the drug (Fig. 1), suggesting that the effect of an increased dose of ACh may be mediated by a rise in the salivary pH. It has also been reported in human subjects that the S/P ratio of PA is reduced with increasing salivary pH [3]. There was no difference in the S/E ratio at a given PA level (20 or 30 to 100 μg/ml) in the perfusate between either stimulation condition, with 1 μM ACh or 10 μM pilocarpine (Fig. 2), despite a significant difference in the salivary pH (i.e. pH 7.64 and pH 6.78 with ACh and pilocarpine respectively). This suggests that Matin's equation cannot explain the S/E ratio of PA when the mandidular gland was perfused with this drug, and some factors other than the perfusate (or venous effluent) pH may determine the S/E (or S/P) ratio of the drug.

In fact, Matin's equation failed to predict any value close to the observed S/E ratio for PA except that with 10 μM ACh (Fig. 3). When the perfusate PA level was fixed at 30 μg/ml, the discrepancy between the observed ratio and the ratio calculated by Matin's equation was maximum under both stimulation conditions (1 μM ACh and 10 μM pilocarpine). Koup *et al.* [3] have suggested that the reason why the S/P ratio cannot be explained by Matin's equation may be a significant difference in the salivary pH measured after collection and that of the primary saliva in the acinus. On the other hand, Borzelleca and Putney [2] have proposed a model describing the importance of intracellular pH of the glandular epithelial cells in determining salivary drug excretion, i.e. the S/P ratio. Their model includes a lipid barrier only at the luminal side of the glandular epithelial cell membrane and assumes that the arterial (serosal) and interstitial pH values are identical. There were no studies that had predicted the S/P or S/E ratio by using intracellular pH values that were measured directly. With the DMO method, the rat mandibular intracellular pH *in vivo* was 7.17 [15] and that of the sliced gland was 7.24 [11]. When the mandibular gland was perfused at pH 7.4 and 8.0, intracellular pH was lower than the perfusate pH (Fig. 4), and the present calculation using these intracellular pH values yielded S/E ratios relatively close to the observed ratios when compared with those calculated by Matin's equation which employed the venous effluent pH (Fig. 5).

In conclusion, the present results suggest that the intracellular pH of the salivary glandular cells may be an important factor in determining the saliva-to-plasma concentration ratio of some drugs in the study of the kinetics and mechanisms salivary excretion.

REFERENCES

1. S. B. Matin, S. H. Wan and J. H. Karam, *Clin Pharmac. Ther.* **16**, 1052 (1974).
2. J. F. Borzelleca and J. W. Putney, *J. Pharmac. exp. Ther.* **174**, 527 (1970).
3. J. R. Koup, W. J. Jusko and A. L. Goldfarb, *J. pharm. Sci.* **64**, 2008 (1975).
4. R. L. Galeazzi, L. Z. Benet and L. B. Sheiner, *Clin. Pharmac. Ther.* **20**, 278 (1976).
5. M. Pirovino, G. Karlaganis and R. L. Galeazzi, *Schweiz. med. Wschr.* **110**, 1415 (1980).
6. J. Watanabe, I. Koyama, K. Iwamoto and S. Ozeki, *J. Pharm. Pharmac.*, **39**, 912 (1987).
7. T. C. Butler, *J. Pharmac. exp. Ther.* **108**, 11 (1953).
8. N. Iwamitsu, K. Kuzuno, A. Irie, H. Hisashiro, H. Sakakibara and J. Kodama, *Seitai Shiryo Bunseki* **2**, 17 (1979).
9. W. J. Waddel and T. C. Butler, *J. clin. Invest.* **38**, 720 (1959).
10. J. W. Putney and J. F. Borzelleca, *J. Pharmac. exp. Ther.* **177**, 263 (1971).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
12. S. H. Weily and E. Genton, *Arch. intern. Med.* **130**, 366 (1972).
13. M. T. Coroneo, A. R. Denniss and J. A. Young, *Pflügers Archs* **381**, 223 (1979).
14. J. A. Young and E. Schogel, *Pflügers Arch. ges. Physiol.* **291**, 85 (1966).
15. A. K. Cho, S. H. Curry and S. Jacobsen. *Biochem. Pharmac.* **18**, 2323 (1969).