Br. J. Pharmac. (1969), **35**, 304–312.

An *in vitro* study of the intestinal absorption of pyridinium aldoximes

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1. The transfer rates of three pyridinium aldoximes, a non-quaternary pyridine aldoxime and choline across the wall of sacs from the jejunum of rats were measured *in vitro*.

2. The transfer rates observed for any one of the quaternary compounds could be inversely correlated with the transmural potential of the particular sac studied, but there was no correlation with the rates of water or glucose transfer.

3. 2-hydroxyiminomethyl-N-methylpyridinium iodide (PAM) had a transfer rate seven times less than that of its non-quaternary analogue, 2-hydroxyiminomethyl pyridine.

4. Neither neostigmine nor EDTA affected the transfer rate of PAM in the conditions used.

5. It was concluded that the transfer of the quaternary compounds could be explained by diffusion through aqueous pores.

The search for nucleophilic agents capable of reactivating cholinesterase inhibited by organophosphorus compounds has led to the use of various salts of 2-hydroxyiminomethyl-N-methylpyridinium (PAM) for this purpose. Many studies have been made of the absorption, distribution and metabolism of this compound when administered parenterally or by mouth (Ellin & Wills, 1964). Levine & Steinberg (1966) have examined the absorption of PAM and related compounds using the *in vivo* loop technique, but so far no study of the intestinal absorption using an *in vitro* preparation has been reported.

Results obtained from the study of PAM absorption should be of general interest, since it is a quaternary compound and the mode of intestinal absorption of such compounds has been a subject of speculation for some time (Schanker, 1961).

This paper describes a study of the absorption of PAM and related compounds in the small intestine of rats by the everted sac technique. The other compounds studied were: TMB-4 (1,3-di(4-hydroxyiminomethylpyridinium) propane dibromide); PAA (2-hydroxyiminomethyl-N-amylpyridinium iodide); 2-hydroxyiminomethyl pyridine and choline. The iodide salt of PAM was used.

Methods

A modification of the everted sac preparation described by Crane & Wilson (1958) was used to measure the transfer of oxime from the mucosal side to the

serosal side of the sac (serosal transfer). Male rats of the Wistar strain weighing from 250 to 320 g were denied food for 18 hr before the experiments. Each rat was stunned and killed by exsanguination. The abdomen was opened and a piece of the small intestine about 50 cm long was removed, starting from the pylorus. This was placed in Krebs-Henseleit Ringer bicarbonate (bicarbonate-saline) solution at 10° C through which 95% oxygen and 5% carbon dioxide was bubbled continuously. A 30 cm length of the intestine was everted, and a 5 cm length of this was removed and attached to a glass cannula. The free end of the gut was ligated to form a sac, and a glass bead (0.7 g) was attached to the end. Two types of sac were used, one from the upper jejunum and the other from the lower jejunum. When the intestine was cut into short pieces, the gut initially contracted considerably. The two types of sac were therefore defined by reference to the original length of intestine. Sacs from the upper jejunum were obtained 15–20 cm from the pylorus, and those from the lower jejunum at 45–50 cm. The prepared sac was transferred to the incubation tube kept at 37° C and containing aerated bicarbonate-saline. It was then left there for a 15 min period to allow equilibrium and relaxation to take place ; during this time it was filled through the glass cannula with bicarbonate-saline (0.5–0.6 ml.) to 1 cm above the outer fluid level.

To start the incubation, the sac was transferred to a second tube containing bicarbonate-saline and the compound to be studied, normally at a concentration of 5 mM. Samples of 0.05 ml. were removed from the serosal fluid at 10, 20, 30, 45 and 60 min. At 25 min the potential difference across the sac wall was measured, using bridges of 1 M KCl in 3% agar in polythene tubing connected to calomel half-cells. The voltage (serosal side always positive) was read on a Radiometer pH meter model 25SE. After 60 min the sac was removed from the incubation tube, the outside was lightly blotted with paper tissue and then the end was cut so that the internal fluid could be collected in a weighed tube. The empty sac was then dried at 90° C for 4 hr and weighed. The mean dry weight of the sac was 67 mg, and averaged 16.2% of the net weight. The serosal fluid was stored at −20° C until glucose estimations were performed.

In a second series of experiments the serosal transfer of PAM was measured for each sac first with and then without glucose in the mucosal medium (the medium bathing the mucosa). After the normal equilibration period of 15 min, an initial sample of 0.05 ml. was taken from the serosal fluid and the mucosal medium was changed to one containing both glucose and 5 mM PAM. The serosal fluid was again sampled 20 min later and the PAM transfer rate in the presence of glucose was measured. The mucosal medium was then changed to one without glucose or PAM. After 10 min equilibration, PAM alone was added and its transfer measured over a further period of 20 min in the absence of glucose in the mucosal medium. This sequence was reversed in other experiments so that the PAM transfer rate was measured first without and then with glucose in the mucosal medium.

The parameters measured in these experiments were the rates of accumulation of oxime, of glucose and of water on the serosal side of the sacs. The term serosal transfer is used for these movements, without intending to imply the operation of any particular transfer mechanism.

The composition of the Krebs-Henseleit Ringer (bicarbonate-saline) solution used was (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 28. No adjustment of composition was made when other compounds were

added. The fluid was continually gassed with 95% oxygen and 5% carbon dioxide. The pH before and after the experiments was between 7.2 and 7.4.

Estimation of the pyridine derivatives was performed spectrophotometrically. For the pyridinium compounds, the 0.05 ml. samples were mixed with 2.8 ml. of water, and 0.2 ml. of 5N NaOH was added just before measuring the optical density. PAM and PAA were read at 336 mμ, and TMB-4 at 344 mμ. For 2-hydroxyiminomethyl pyridine, the samples were added to phosphate buffer at pH 6.5 and readings were taken at 305 mμ. Then 0.2 ml. of 5N NaOH was added and the optical density was measured again at the same wavelength. The change in optical density was used as a measure of the compound present. These estimations were calibrated using standard solutions of the pure compounds. The possible presence of interfering compounds was checked by performing blank assays on serosal fluid during the equilibrium period, and by recording the absorption spectra of representative samples from 200 to 450 mμ in a Unicam S.P.800 spectrophotometer.

For the experiments with choline, a solution of known specific radioactivity [Me-^{14}C]-choline chloride (Radiochemical Centre, Amersham) was mixed with a standard solution of choline. This solution was then used to prepare the incubation medium. Radioactivity in the samples after incubation was measured in a Beckman LS 100 liquid scintillation counter using a dioxan-based scintillant.

Glucose was assayed by the colorimetric method of Nelson (1944).

Results

Entry of PAM into the tissues of the jejunal wall

The rate at which PAM entered the actual wall of the jejunal sacs was measured as a preliminary to the measurement of entry into the fluid bathing the serosal sur-

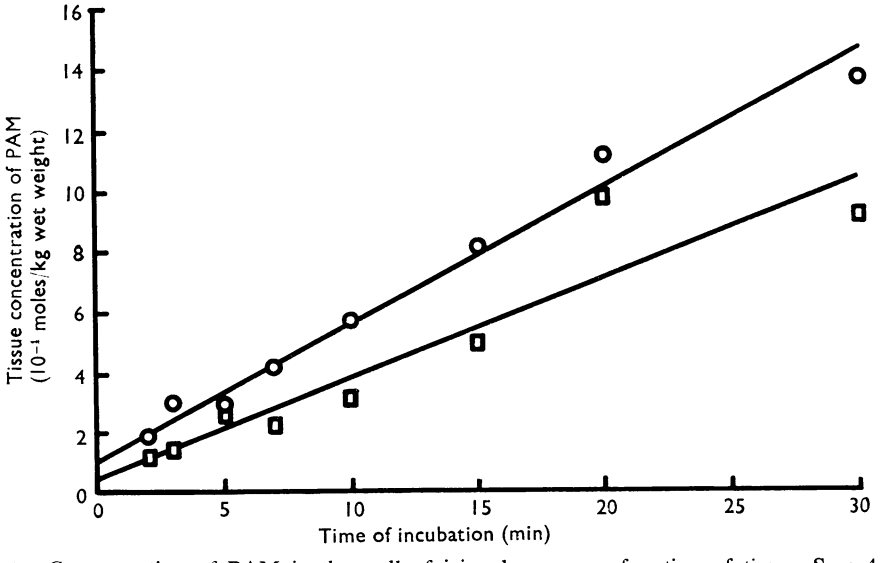

FIG. 1. Concentration of PAM in the wall of jejunal sacs as a function of time. Sacs 4 cm long, from the region 15 to 30 cm from the pylorus, were filled with 0.25 ml. bicarbonate-saline (not containing PAM) and tied at each end. After incubation in bicarbonate-saline with 5 mM PAM at 37° C, the sacs were cut open, drained and blotted. The concentration of PAM in the tissue was then estimated by the method of Creasey & Green (1959). □——□, Sacs everted (mucosa outermost); ○——○, sacs not everted (serosal surface outermost). The regression lines were calculated by the method of least squares.

face (serosal transfer). The concentration of PAM in the wall rose proportionally with time for 30 min (Fig. 1), but in other experiments it was found that the rate of rise diminished around 60 min. The tissue concentration rose more slowly when PAM penetrated from the mucosal side (everted sacs) rather than from the serosal side, which shows that the mucosal surface presented a slightly greater bar to PAM movement than did the serosal surface. After 30 min the tissue concentration of PAM (expressed as moles/kg wet weight) was 3 to 4 times that in the fluid inside the sac, but this ratio fell to 1.5 after 60 min.

Serosal transfer rates of oximes

For the quaternary oximes, the quantity of the compound in the fluid bathing the serosa increased in direct proportion to time up to 60 min, but a reduction of the rate of transfer occurred after 30 min with 2-hydroxyiminomethyl pyridine and for choline. The rates expressed here for the upper jejunum (Table 1) are calculated from the slopes of lines fitted by eye to the early points of the graphs, so that they represent initial rates. The differences between the rates for various compounds shown in Table 1 are all significant at the 5% or lower level, except that between the rates for PAA and TMB-4. The rate for PAM is seven times lower than that for its non-quaternary analogue 2-hydroxyiminomethyl pyridine. When the PAM concentration is lowered to 1 mM, the rate of transfer falls proportionately, indicating a direct relationship over this concentration range. The variability of the results for the quaternary compounds is quite large, which suggests that there is at least one uncontrolled factor in the experiments.

For each of the incubations shown in Table 1 the serosal transfer rates of water and of glucose and the transmural potential were also measured. There was a good correlation between glucose and water transfers ($P=0.1\%$). Glucose transfer rates (effected against a concentration gradient) ranged from 3 to 55×10^{-8} moles/hr/per mg dry weight and water transfer rates from 0.2 to 12 μl./hr/per mg. A graphical plot gives a straight line passing through the origin, with a slope of 4.8×10^{-8} moles glucose/1 μl. water; this is equivalent to movement of a 48 mM glucose solution, as opposed to the 28 mM glucose in the incubation medium. The correlation between transmural potential difference and both water and glucose transfer is significant at the 5% level. The transfer rate of quaternary oximes can be correlated with the transmural potential difference only ($P=5\%$). As shown in Fig. 2 for PAM, this is an inverse relationship, with the highest potential being accompanied by the lowest oxime transfer rate. The other quaternary oximes PAA and TMB-4 behaved like PAM in this respect.

TABLE 1. *Rates of serosal transfer of five compounds through everted sacs of the upper jejunum*

Compound	Serosal transfer rate
PAM	$10\cdot7\pm4\cdot45\times10^{-9}$ (12)
PAM [1 mM]	$1\cdot8\pm0\cdot55\times10^{-9}$ (6)
PAA	$6\cdot5\pm3\cdot12\times10^{-9}$ (6)
TMB-4	$6\cdot1\pm5\cdot09\times10^{-9}$ (8)
Choline	$2\cdot1\pm0\cdot63\times10^{-8}$ (13)
2-hydroxyiminomethyl pyridine	$7\cdot6\pm1\cdot54\times10^{-8}$ (6)

Units are moles/hr per mg dry weight; Results are given as means $\pm$ S.D. Number of determinations in brackets. The concentration of the compounds at the mucosal surface was 5 mM, except for one series of results obtained with 1 mM PAM.

Further study of transmural potential difference effects

In order to study more fully the effects of the transmural potential on the serosal transfer rate of PAM modifications to the previous experiments were made. First sacs from the lower jejunum were used because these are known to have higher transmural potentials in the presence of glucose (Barry, Dikstein, Mathews, Smyth & Wright, 1964), and second the presence or absence of glucose in the mucosal medium was used to increase or decrease the transmural potential differences. The effect of glucose on the transmural potential of the preparation is shown in Fig. 3. When prepared in the presence of glucose, the sac had a transmural potential which remained steady at about 10 mV. The withdrawal of glucose from the mucosal medium resulted in an immediate fall of the potential by 4–6 mV followed by a slower decline to below 2 mV. Restoration of glucose to the mucosal side caused an increase of the potential by 6 mV to a new steady value but the initial potential was never regained. Figure 3 also shows that glucose produced the converse effects of a sac equilibrated initially in the absence of this substance.

The results of experiments in which the rate of PAM transfer was measured for the same sac in conditions of high and low transmural potential are illustrated in Fig. 4. Here the ratio of the rate of transfer at low potential to that at high potential is plotted against the change in average potential between the two periods. It is clear that an alteration in potential will markedly affect the PAM transfer rate, up to four-fold in some cases. A change of at least 5 mV seems necessary, however, for a demonstrable variation in PAM transfer rate. These results may also be expressed as PAM transfer rates against absolute potential, but this treatment destroys the advantages of pairing the results for one sac, and consequently the variability is increased; in this case a graph similar in form to that shown in Fig. 2 is obtained, in which the correlation is significant at the 1% level.

The slope of the regression line for results obtained in the sacs from the lower intestine is different from that obtained for the upper jejunum. The two regions of

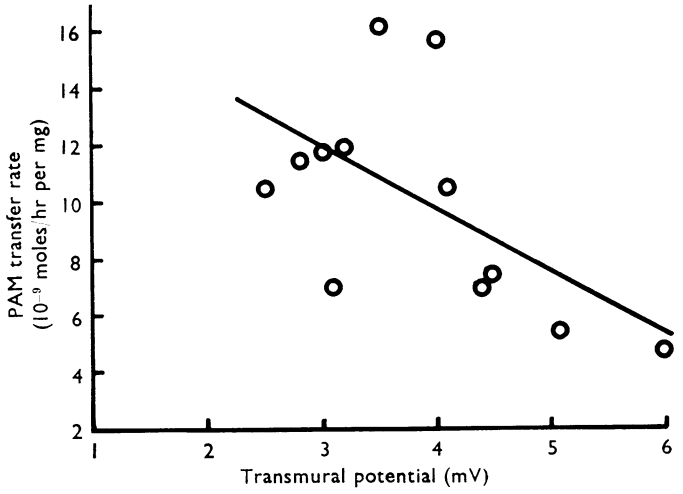

FIG. 2. Relationship between serosal transfer rate of PAM and transmural potential for sacs of the upper jejunum. The regression line was calculated by the method of least squares ($P=5\%$).

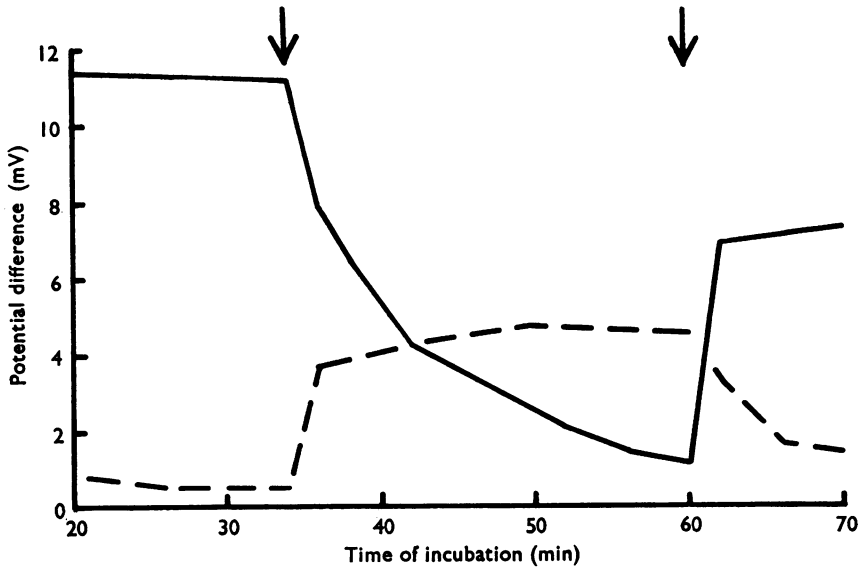

FIG. 3. Effect of glucose in the mucosal medium on the transmural potential of sacs from the lower jejunum. The continuous line records the potential for a sac prepared in the presence of 28 mM glucose, the interrupted line that of a sac prepared in the absence of glucose. At the first arrow glucose was removed from the mucosal medium (continuous line) or added (interrupted line). At the second arrow the reverse changes were effected. Glucose was present in the serosal fluid throughout the experiments. The potential difference was recorded at 2 min intervals.

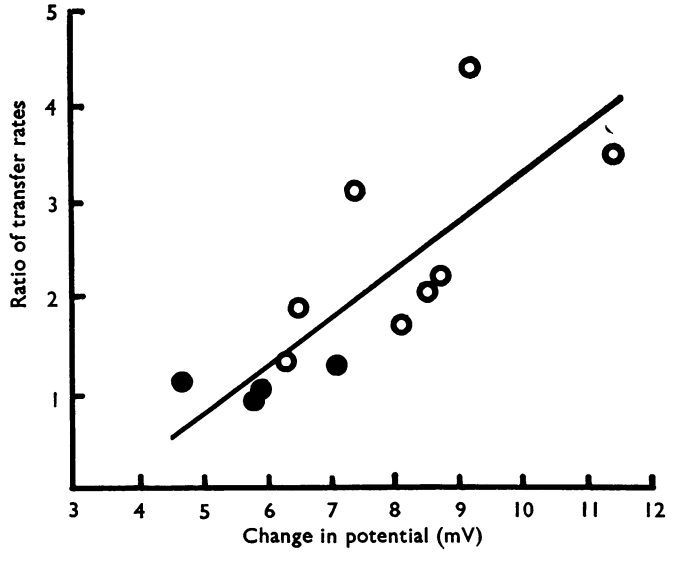

FIG. 4. PAM serosal transfer rates for individual sacs at low and high transmural potential, as a function of the change in potential. The ordinate represents the transfer rate at low potential divided by that at high potential. The abscissa represents the change in potential. The regression line was calculated by the method of least squares. ○——○, Sacs incubated with 28 mM glucose initially present in mucosal fluid; ●——●, sacs incubated with glucose absent at start.

the intestine are compared in Table 2, from which it can be seen that the lower jejunum shows a slower transfer rate and a smaller effect of the potential difference on this rate than does the upper jejunum.

Effect of PAM on the transmural potential difference

The effect of PAM on the transmural potential difference was examined in order to test whether this compound affected the properties of the sacs directly.

Sacs were set up and equilibrated for 15 min as before. Then the mucosal medium was changed to bicarbonate-saline containing 5 mM PAM, and the transmural potential was monitored for 15 min. The fall of potential over this period was compared with that of sacs incubated in bicarbonate-saline with no additions. There was no significant difference between two sets of eight results, so it was concluded that PAM did not markedly affect the transmural potential.

Effects of other compounds on PAM serosal transfer

Neostigmine has been reported by Green (1965) to increase the absorption of sulphisoxazole from guinea-pig intestine and ethylenediamine tetraacetic acid (EDTA) was shown by Schanker & Johnson (1961) to increase the intestinal absorption of decamethonium, among other compounds.

Neostigmine, when present in the mucosal fluid at concentrations of 2×10^{-7}M or 2×10^{-5}M, did not affect the serosal transfer rate of PAM. Neither was there any effect when 2.5 mM EDTA was present, nor when the calcium concentration in the bicarbonate-saline was lowered from 2.5 mM to 1 mM. Changes in the transfer rate greater than two-fold would have been detected with certainty.

Chloroform-water partition ratios of the oximes

When 5 mM solutions of the oximes at pH 7.4, buffered with Tris or with phosphate, were shaken with chloroform at 22° C and the relative concentrations in the two layers measured, PAM and TMB-4 were found to have chloroform to water concentration ratios less than 2×10^{-4}. PAA had a ratio of 3.7×10^{-3}. The non-quaternary compound 2-hydroxyiminomethyl pyridine had a much higher chloroform solubility, the ratio being 5.4×10^{-1}. This compound had a ratio of 4×10^{-3} at pH 1 and 8×10^{-3} at pH 10.

When PAM was present in the aqueous layer in a concentration of 50 mM, partition ratios of 4 to 9×10^{-5} were obtained at pH values corresponding to degrees of ionization of the oxime group from zero to 80%. There was an indication that increased ionization of the oxime group might yield a slightly higher concentration in chloroform. This would be in accord with the formation of a resonance hybrid carrying a zero net charge (Larsson & Wallerberg, 1962); however, even at 100% ionization of the oxime group the partition ratio was still less than 2×10^{-4}.

TABLE 2. *Comparison of the serosal transfer rates of PAM in the upper and lower jejunum*

	Upper jejunum	Lower jejunum
Rate of PAM serosal transfer (moles 10^{-9}/hr per mg)	10·7 ± 4·45 (12)	3·8 ± 2·5 (12)
Transmural potential in mV	4·2 ± 1·0 (6)	8·8 ± 2·2 (12)
Variation of PAM transfer rate with potential (slope of calculated regression line in moles $\times 10^{-9}$/hr/mg per mV)	−2·2	−0·38

Results are given as means ± S.D. Number of determinations in brackets.

Discussion

These results show that the movement of the quaternary oximes across the wall of intestinal sacs is dependent on their concentration and the transmural potential difference, but is independent of glucose or water transport. The concentration range studied for PAM is limited and the possibility remains that a saturable system of facilitated diffusion or active transport could exist at lower concentrations.

The effect of the transmural potential difference on transfer rate is to be expected in the present situation in which the positively charged ion moves against the potential gradient. The position, however, must be more complex, for the observed potential is the resultant of at least three components. These are the potential difference across the luminal surface of the mucosal cells, that across the serosal surface and potential differences created by diffusion in the submucosal tissues of the preparation (Wright, 1966). Irrespective of the means by which the potential exerts its effect, however, it can account for much of the variation in results presented in Table 1. It is relevant that the greatest variation in the rate of transfer was observed for the *bis*-quaternary oxime (TMB-4) and the least variation for the non-quaternary compound (2-hydroxyiminomethyl pyridine).

The fact that the transfer rate of PAM is independent of the active glucose transport is some indirect evidence that PAM is not moved by an active transport system, because the efficiency of an individual sac in performing one energy-dependent task would be expected to be reflected in the performance of similar tasks with other compounds. Similarly, the lack of correlation with water movement tends to eliminate any solvent drag effect such as that described by Fisher (1955) for small neutral molecules. It should also be noted that the final PAM serosal concentrations (0.3 to 1.7 mM) do not correlate with the observed rates of PAM entry. This independence from water movement indicates that the phenomenon described by Munck (1968) does not influence the present results. Munck found that the apparent effect of glucose on proline and valine transfer was partly an indirect result of water transport, as this latter factor varied the volume of the fluid into which the amino-acids were diffusing.

The rate of intestinal absorption of unionized or weakly ionized drugs can be explained in terms of lipid solubility (Schanker, 1961). The quaternary oximes studied here, however, are obligatory cations and consequently have very low lipid solubility. Also, although the oximate species or zwitterionic form of PAM is reported to have a chloroform solubility of about 2.5% (Larsson & Wallerberg, 1962), ionization of the oxime group (pK 7.7) did not affect the chloroform-water partition ratio greatly. In our experiments the rate of transfer of the quaternary oximes did not seem to bear any relation to the partition ratio. Thus PAA had a ratio more than ten times greater than PAM, yet it had a serosal transfer rate half that of PAM. The higher rate of 2-hydroxyiminomethyl pyridine transfer compared with that of PAM is a reflection of the fact that this compound is unionized at pH 7.4 and hence has a high partition ratio and no hindering charge.

This investigation therefore leads us to conclude that quaternary oximes cross the mucosal membrane by diffusion through the aqueous pores, the existence of which is postulated on the basis of other data (Smyth & Whittam, 1967). The rate of transfer would then be dependent chiefly on the charge on the oxime and the transmural potential, as shown here, and on the size of the ion. Because this latter

factor is complicated by hydration we cannot readily link the relative sizes of the oxime cations to the rate of entry. When hydration is neglected, however, the PAM cation can be fitted into a sphere of 4.3 Å radius (*anti* form) or 4.8 Å (*syn* form), according to measurements made on Courtald atomic models. These values compare with hydrated ionic radii of 2.0 Å and 2.6 Å for potassium and sodium respectively (Gorin, 1939). The average radius of the pores in the intestinal wall is estimated as 4 Å (Smyth & Whittam, 1967), so that it is reasonable to suppose that the PAM cation can diffuse through a proportion of the pores the radii of which are slightly larger than the mean value. This would not apply to larger organic cations such as benzomethamine, studied by Levine & Pelikan (1961), for which the mechanism of entry could be quite different.

Our value for the serosal transfer rate of PAM agrees well with the value reported for the absorption of various salts of PAM by Levine & Steinberg (1966), who used the *in vivo* loop technique. Similarly the explanation of variations in absorption rates advanced in the present paper, namely their dependence on ionic size and charge, fits with the values presented by Levine and Steinberg for various oximes.

The authors are grateful to Mr. E. M. Smith for technical assistance.

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(*Received August* 26, 1968)