Table 1. Distribution of loperamide in the wall of the guinea pig ileum

Tissue	Recovered loperamide, expressed in ng/g tissue (means ± S.E.M.; $n = 5$–8) I	II	III
Longitudinal muscle + myenteric plexus	10.09* ± 1.66	59.29† ± 12.35	196.84‡ ± 41.54
Total ileum segment	2.70 ± 0.80‖	6.59 ± 1.22‖	20.78 ± 2.80‖
Ileum segment without mucosa	4.62 ± 1.25§	21.70 ± 4.93‖	34.02 ± 5.46‖
Circular muscle + mucosa	2.73 ± 0.77‖	5.82 ± 0.72‖	16.55 ± 4.01‖
Circular muscle	6.50 ± 1.74	7.37 ± 1.01¶	16.57 ± 6.35‖
Mucosa	0.86 ± 0.24‖	0.79 ± 0.30‖	1.08 ± 0.39‖

The ileum was incubated with loperamide at 1.25 (I), 5 (II) and 20 (III) ng/ml. The significance of the differences of values *, † and ‡ from the other values of the corresponding concentrations, is calculated by the Mann–Whitney U-test two-tailed; §$P \leqslant 0.01$; ‖$P \leqslant 0.005$; ¶$P \leqslant 0.001$.

nal muscle–myenteric plexus of the ileum.

It has been suggested that the local inhibitory action of loperamide on intestinal motility may be due to its effect on nervous structures [4]. The present findings that the drug is found preferentially in the longitudinal muscle–myenteric plexus, rather than in either the circular muscle or the mucosa, indicate that loperamide acts on the intramural ganglia (myenteric plexus) and on nerve endings within the longitudinal muscle layer.

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Carbonic anhydrase activity of brush border and plasma membranes prepared from rat kidney cortex

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The distribution of carbonic anhydrase in the mammalian proximal tubule cell is of great physiological interest because of its role in bicarbonate reabsorption. The enzyme has been found in the microsomal membranes of rat, dog and human cortical cells as well as the cytoplasm of the cell [1, 2]. During the course of present work, carbonic anhydrase was found in the brush border and basal–lateral membranes of rat renal cortical cells [3]. Inhibition studies with these membranes indicated an enzyme 2–6 times less sensitive to the action of sulfanilamide, ethoxzolamide and CL 13475 than the cytoplasmically located enzyme [3]. If such differences were real, they point to a low activity form of the enzyme different than that found in the cytoplasm and perhaps an adjustment in dose or concentration of the drugs in physiological and clinical work. The present investigation was undertaken to reaffirm the localization of the enzyme within the plasma membranes of the rat renal cortical cell and to ascertain if the sensitivity of the membrane bound enzyme to the inhibitors methazolamide and acetazolamide is truly different from that of the cytoplasmic enzyme. The observation that isolated membranes contained carbonic anhydrase was confirmed but no difference in inhibitory sensitivity between the membrane-associated and cytoplasmic enzyme could be found.

Preparation of the kidney membranes. Male Sprague–Dawley rats, weighing 300–600 g, were killed by decapitation, the kidneys removed and perfused through the renal artery with cold sucrose–Tris buffer (0.25 M sucrose–0.01 M Tris–OH, pH 7.6 with HCl) to flush out contaminating red blood cells and thus their carbonic anhydrase. Cortical slices were obtained and a 10 per cent (w/v) renal homogenate was prepared in the sucrose–Tris buffer. Each experiment is from the kidneys of six rats. Plasma membranes were isolated by the method of Fitzpatrick *et al.* [4]. Brush border membranes were prepared by the method of Booth and Kenny [5] using 10 mM $CaCl_2$. The techniques are such that the preparation of the plasma membranes included both the brush border and basal–lateral membranes, but the brush border membrane preparation excludes basal–lateral membranes.

Enzyme assays. Prior to measuring the enzyme activities,

all membrane preparations were freeze-dried at −20° overnight and stored at −20° until analysis. Freeze drying releases maximal ATPase activity from the homogenates and membrane fractions [6]. We have confirmed this finding for the other enzymes determined in this study. Also, tissue treated in this manner can be stored at −20° for at least three months with no loss of enzyme activity. The freeze-dried membrane preparations were resuspended in 0.25 M sucrose–5.0 mM EDTA to give a protein concentration of around 10 mg/ml. All enzyme assays except carbonic anhydrase were run at 37°.

Alkaline phosphatase, a brush border membrane marker, was assayed by the method of Russell *et al.* [7]. Na, K-ATPase activity, a basal–lateral membrane enzyme was determined as described by Kinne *et al.* [8]. Succinic dehydrogenase activity was determined by the method of Gibbs and Reimer [9]. The determination of lactic dehydrogenase was carried out according to Caboud *et al.* [10].

Carbonic anhydrase activity was measured by the method of Maren [11] but modified using barbital buffer (4 mM) at pH 7.9. Enzyme units for carbonic anhydrase activity are defined as (uncatalyzed time–catalyzed time)/catalyzed time per gram wet weight of tissue and refer specifically to the reaction in which the volume is 1 ml, temperature 1° and CO_2 gas is 100 per cent. The activity of the enzyme is given in enzyme units as well as CO_2 hydrated per mg protein per hr. In the inhibition studies, the drugs acetazolamide and methazolamide, in four different concentrations, were added to the reaction mixture 2 min before the start of the assay so that the equilibrium between the drug and enzyme was assured.

In order to ensure that the calcium chloride used in the brush border isolation did not inhibit the carbonic anhydrase activity, 10 mM $CaCl_2$ was added to hemolyzed blood and subsequently assayed for enzyme activity or the calcium was added directly to the assay chamber. Neither procedure affected carbonic anhydrase activity.

Protein determinations were carried out according to Lowry *et al.* [12] using bovine serum albumin as a standard.

Table 1 shows the specific activities and relative enrichments of the marker enzymes for the basal–lateral membranes (Na, K-ATPase) and brush border membranes (alkaline phosphatase) of the plasma membrane and brush border membrane fractions. Alkaline phosphatase and Na, K-ATPase were enriched in the plasma membrane fractions 8.3 and 5.4 fold, respectively. The brush border membrane preparation showed a high enrichment, 31 times, of alkaline phosphatase and a correspondingly low enrichment, 0.8 times, of Na, K-ATPase. Mitochondrial and cytosolic contamination, as indicated by the activities of succinic dehydrogenase and lactic dehydrogenase respectively, were very low in both membrane preparations.

Carbonic anhydrase activity was found in both the brush border membrane and in the plasma membrane fractions of the renal cortical cell (Table 1), the enrichments being 0.33 and 0.21 respectively. The plasma membrane fraction contained significantly more carbonic anhydrase activity than expected from the amount of brush border membranes found in this fraction. Assuming that the brush border membrane fraction is highly purified, a ratio of carbonic anhydrase/alkaline phosphatase activity of 36 can be calculated versus a ratio in the plasma membrane fraction of 208. Thus it appears that carbonic anhydrase is present both in the luminal and contraluminal faces of the cell membrane.

Table 2 shows the inhibition by methazolamide and acetazolamide for the brush border and plasma membranes and for the homogenate. The enzyme activity in all fractions was fully inhibited at 80×10^{-8} M acetazolamide and 50 per cent inhibited at 2×10^{-8} M. With methazolamide, the activity in the plasma membranes fractions (brush border and basal–lateral membranes) was completely inhibited at 80×10^{-8} M and approximately 50 per cent at 2×10^{-8} M. At the latter concentration the enzyme in the homogenate was inhibited 50 per cent. A further study was undertaken with brush border and basal–lateral membranes isolated by free-flow electrophoresis from Wister rats [13] and the present method for assessment of inhibition [11]. The I_{50} (or K_i, see footnote Table 2) for acetazolamide and methazolamide against both the basal–lateral and brush border membranes was again 2×10^{-8} M. Note that these data agree with those of Table 2 and differ from those reported [3].

These data agree with those found previously for less pure membrane fractions [1]. A K_I (or I_{50}) of 2×10^{-8} M generally implies an enzyme with a high turnover number, the prototype exemplified by human red cell carbonic anhydrase C and characteristic of secretory carbonic anhydrases. It is important to note that essentially complete inhibition of the enzyme activity in the homogenate, which represents mainly cytosolic enzyme, and of the enzyme activity in the membranes was achieved at approximately 10^{-6} M, a concentration readily achieved *in vivo* following ordinary doses of these drugs [14].

Table 1. Activity of enzymes in rat kidney cortical plasma membranes and brush border membranes

	Specific activity*			Enrichment†	
	Homogenate ($n = 8$)	Plasma membrane ($n = 4$)	Brush border ($n = 4$)	Plasma membrane	Brush border
Alkaline phosphatase	4.5 ±0.8	38 ±6	142 ±40	8.3	31
Na, K-ATPase	2.6 ±0.4	14 ±0.6	2 ±1	5.4	0.8
Carbonic anhydrase	23900 ± 2650 (4550)	7900 ±490 (1504)	5100 ± 950 (971)	0.33	0.21
Succinic dehydrogenase‡	0.2 ±0.07	n.d.	0.004 ±0.001	0	0.02
Lactic dehydrogenase§	4669 ± 668	267.8 ± 93.3	445.6 ±143.4	0.09	0.06

* Specific activity is defined as μmoles substrate converted/mg protein · hr at 37°. Values are means ± standard error. n.d. = not detectable.

† Enrichment is the ratio of specific activities of enzymes in these membrane fractions to that of the homogenate.

‡ Values at 0°. Enzyme units for carbonic anhydrase activity per mg wet tissue shown in parenthesis. On this scale of unitage, rat red cells are about 3000 units/ml.

§ Lactic dehydrogenase (LDH) activity is expressed as LDH units/mg protein.

Table 2. Per cent inhibition of carbonic anhydrase in kidney fractions by acetazolamide and methazolamide*

	Acetazolamide		Methazolamide	
	80×10^{-8} M	2×10^{-8} M	80×10^{-8} M	2×10^{-8} M
Homogenate	100	52	100	47
	(3)	(3)	(4)	(5)
Plasma membrane	100	48	100	47
	(2)	(2)	(3)	(4)
Brush border	100	56	—	—
	(4)	(4)		

* The mean per cent inhibition is given. 100% inhibition indicates that the reaction rate is indistinguishable from that of the uncatalyzed rate. The number of experiments is given in parenthesis and (—) means the inhibition was not determined. In these tests the concentration of enzyme (E_0) was $1 - 2 \times 10^{-9}$ M. Since this is less than the I_{50}, $K_i = I_{50}$.

Although carbonic anhydrase has classically been thought of as a cytoplasmic enzyme, the present results, as well as those of other studies [1, 3], demonstrate its presence in the membranes of the proximal tubule. The question may be raised as to whether the enzyme found in the membrane is an artifact due to absorption of cytoplasmic enzyme on the plasma membrane or owing to enclosure of the cytoplasm into membrane vesicles. Two lines of evidence speak against these possibilities. One, lactic dehydrogenase, a cytoplasmic enzyme, showed considerably less enrichment than did carbonic anhydrase (in the brush border membranes, 0.06 vs 0.21) and it might be expected that absorption of cytoplasm would give similar enrichments for carbonic anhydrase and lactic dehydrogenase. Furthermore, Wistrand and Kinne [3] have actually shown that cytoplasmic carbonic anhydrase was not absorbed to isolated membrane fractions and the specific activity of the carbonic anhydrase of their membrane fractions are the same as those observed in the present study.

Inhibition studies with acetazolamide and methazolamide clearly show that in experiments where doses of these drugs which generate plasma and tissue concentrations of 10^{-4} M (10–20 mg/kg) are used [14], essentially all the carbonic anhydrase present in the renal cell, be it membrane bound or cytoplasmic, is abolished.

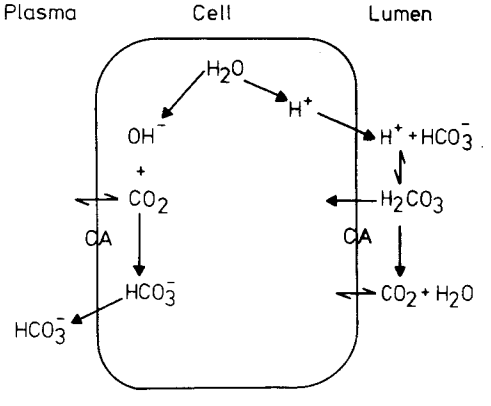

Fig. 1. A model of the role of membrane bound carbonic anhydrase (CA) in the renal acidification process.

Water is split resulting in the accumulation of protons luminally and hydroxyl ions basal–laterally. The brush border membrane CA dehydrates luminal H_2CO_3 which is formed from proton secretion. The basal–lateral enzyme catalyzes the formation of HCO_3^- from CO_2 and OH^-.

The role of the membrane bound carbonic anhydrase is speculative but can be conceived as shown in the model presented in Fig. 1 [15]. In this model, water is split intracellularly and an asymmetric release of the products H^+ and OH^- leads to an accumulation of protons on the luminal side of the cell and hydroxyl ions on the plasma side. After proton secretion into the lumen, the brush border carbonic anhydrase could then function in the rapid dehydration of the H_2CO_3. However, H_2CO_3 is a highly diffusive substance and thus would have access to the high concentrations of carbonic anhydrase in the cytoplasm. Generation of a "disequilibrium pH" proximally by carbonic anhydrase inhibitors is thus consistent with the drug action at either the brush border or the cytoplasm [16]. The basal–laterally located carbonic anhydrase would catalyze the buffering of OH^- by CO_2 thus sustaining the separation of charge. This model is supported by the evidence that acidification of the urine is always accompanied by alkalinization of the peritubular fluid [17].

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Tolerance to barbiturate and chlorpromazine-induced central nervous system sedation—Involvement of calcium-mediated stimulus-secretion coupling

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It has been well documented that calcium ions are essential for neurotransmitter secretion in the central nervous system [1–3]. Subsequent to depolarization of the nerve end, calcium ions flow down a concentration gradient and couple cytoplasmic events which result in neurotransmitter release. This process is known as "stimulus–secretion coupling". "Stimulus–secretion coupling" has become of interest recently as a potential site of action for drugs which produce sedation. For example, recent work has shown that barbiturates depress depolarization-induced calcium influx across synaptosomal membranes, and subsequent transmitter release, in concentrations which are consistent with *in vivo* sedative doses [4, 5]. To implicate further the involvement of "stimulus–secretion coupling" in the production of central nervous system sedation, recent work in our laboratories has shown that chronic *in vivo* barbiturate administration results in the development of tolerance to depolarization-induced synaptosomal calcium accumulation in parallel with the development of behavioral signs of tolerance [6]. A similar correlation between behavioral tolerance and tolerance to synaptosomal membrane calcium movement also has been reported recently for morphine [7, 8].

In the present report, we have used chlorpromazine to characterize further the involvement of "stimulus–secretion coupling" in the production of sedation. Chlorpromazine was chosen for this study because it is a drug known to produce marked sedation followed by tolerance [9], but on the other hand it produces an antipsychotic effect to which tolerance does not develop or develops quite slowly. Our results show, as we have found with barbiturates, that behavioral indices of sedation and tolerance with chlorpromazine agree quite well with depression of depolarization-induced synaptosomal calcium influx and the development of tolerance at the membrane level to this effect.

Ten- to twelve-week-old male DBA/2J mice, 20–25 g, were housed individually for at least 5 days prior to experimentation and kept on a 12/12 hr light–dark cycle with food and water *ad lib.* Mice were then divided randomly into three experimental groups: control, acute and tolerant. Control mice were maintained on a milled Purina Lab Chow diet *ad lib.* Acute animals received a single dose of chlorpromazine hydrochloride, 15 mg/kg, i.p., and then were killed 30 min later during the peak sedative effect (after loss of righting reflex). Animals in the tolerant group received a food cup containing a milled diet (Purina Lab Chow) thoroughly mixed with 1.0 mg chlorpromazine/g diet as their sole food source. This procedure for dietary drug administration is described more completely elsewhere [10]. Animals in the tolerant group were maintained on the chlorpromazine diet for 7 days prior to being killed. During the 7 days, the animals showed no appreciable loss in weight (average weight day 0 = 23.86 ± 2.65 g; average weight day 6 = 22.28 ± 2.10), and food and chlorpromazine consumption increased markedly (Fig. 1).

Grid test scores (a measure of neuromuscular impairment) were used to determine the degree of intoxication and tolerance development for animals in the tolerant group. This procedure is described in detail elsewhere [10], but briefly involves a determination of the number of times that a mouse's foot slips through a wire grid floor (number of errors) over a 12 cm distance. A reduction in grid test scores over a period of time represents a reduction in intoxication, which can be used as an index of fuctional tolerance. It has been established that tolerance to chlorpromazine-induced sedation is a functional or tissue tolerance and not metabolic, although it has been suggested that chlorpromazine may induce its own metabolism [9]. Figure 1 shows that there was a significant reduction in grid test scores over the 7-day diet period, while at the same time there was a dramatic increase in food and chlorpromazine consumption. Behaviorally, the mice showed marked signs of intoxication after day 1 of the chlorpromazine diet, while the mice on day 6 appeared much more alert. These data provide evidence for substantial tolerance development by day 6.

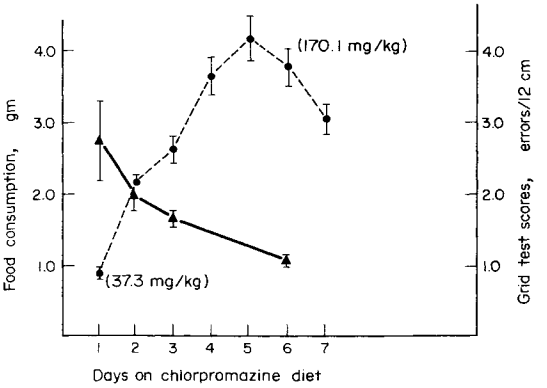

Fig. 1. Grid test scores and chlorpromazine diet consumption over a 7-day period. Each point represents the mean ± S.E.M. of five observations. Each mouse was tested only once to avoid learning effects. The grid tests were performed as described by Belknap *et al.* [10]. The numbers in parentheses represent the dietary consumption of chlorpromazine on day 1 (37.3 mg/kg) and day 6 (170.1 mg/kg). Statistical analysis (Student's *t*-test) showed that grid test scores on day 1 vs day 6 were significantly different ($P < 0.05$) and that chlorpromazine consumption on day 6 was significantly greater than that for day 1 ($P < 0.001$).