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EFFECT OF CHLORPROMAZINE ON L-METHIONINE TRANSPORT IN THE RAT INTESTINE

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Abstract—The inhibitory effect of chlorpromazine on L-methionine transport was studied in the everted sac preparation of the rat intestine. Chlorpromazine inhibits only the sodium-dependent active component of L-methionine transport, without affecting the sodium-independent diffusion component. Inhibition of methionine transport by chlorpromazine is time dependent and concentration dependent. Half-maximal inhibition was observed at 5.2×10^{-4} M chlorpromazine in the medium. No significant metabolism of chlorpromazine was observed during the experiment, suggesting that the unchanged chlorpromazine was the active inhibitor. Studies on a few chlorpromazine metabolites showed that demethylation in the aminopropyl side chain or ring hydroxylation at the 7-position in the chlorpromazine molecule did not affect the inhibitory activity on methionine transport, while oxidation at the sulfur atom completely abolished it.

Chlorpromazine (CPZ)* is a phenothiazine psychotropic agent given orally and chronically to psychiatric patients. The possibility that prolonged chronic oral ingestion of CPZ results in changes in the gastrointestinal tract function was studied in our laboratory [1]. Our previous report showed that 42 per cent of oral CPZ was recovered from the small intestine at 2 hr, 28 per cent at 6 hr, and 9.6 per cent at 24 hr after a dose. L-Methionine transport in intestinal segments obtained from both acutely and chronically CPZ-treated rats was significantly inhibited.

The present study describes the nature of CPZ inhibition on intestinal transport of L-methionine using the everted sac technique. Some structure–activity relationships were studied by testing the inhibitory activity of a few CPZ metabolites on the intestinal transport of L-methionine.

MATERIALS AND METHODS

Materials. CPZ HCl was supplied by Smith, Kline & French Laboratories. Nor_2-CPZ HCl, 7-OH-CPZ, and CPZ-SO-HCl were kindly provided by Dr. Albert A. Manian of the Psychopharmacology Research Branch, National Institute of Mental Health, Chevy Chase, MD. L-Methionine was obtained from Sigma Chemical Co.; ^{14}C-ring-labeled CPZ HCl from Applied Science Laboratories, Inc., State College, PA; and L-[1-^{14}C]methionine and Nuclear Chicago Solubilizer (NCS) (tissue solubilizer) from Amersham/Searle Corp. All other chemicals were of reagent-grade purity and were purchased from Fisher Scientific Co., Rochester, N.Y.

Animals. Male Sprague–Dawley rats (250–375 g), maintained on Purina Chow laboratory diet, were used in all the experiments. The animals were deprived of food for 14–18 hr before sacrifice but had free access to water.

Handling of samples containing CPZ. As CPZ is susceptible to photo-oxidation [2], samples containing CPZ were protected from light. CPZ was added to the flasks after the oxygenation and shortly before incubation with the tissues.

Assay of CPZ. Separation of unchanged CPZ from its metabolites was done by selective solvent extraction, as described earlier [1] using [^{14}C]CPZ.

General experimental procedure. All animals were killed by stunning and decapitation. The abdomen was opened by incision. The small intestine was isolated, and the first 25 cm, beginning with the pylorus, was discarded. From the next portion (the mid-jejunum) four to eight segments (each about 6 cm long) were cut and placed in individual aluminum pans containing ice-cold modified Krebs–Tris buffer (pH 7.0; 37°) gassed with 100% oxygen. The buffer contained 118 mM NaCl, 25 mM Tris–Cl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 1.2 mM KH_2PO_4. In experiments requiring sodium-free Krebs–Tris, all the NaCl was replaced by an equivalent concentration of Tris–Cl. The segments were everted as described by Wilson and Wiseman [3], filled with 0.5 ml of the same buffer as the incubation medium plus 5 mM glucose as metabolic substrate, ligated, and placed in 25 ml of the appropriate buffer (Krebs–Tris or sodium-free Krebs–Tris) in 50-ml Erlenmeyer flasks. (In a few experiments the volume of incubation medium was 5 ml and incubation was done in 25-ml Erlenmeyer flasks). No glucose was added to the incubation medium. Labeled substrates and other drugs were added to the mucosal solution only. After all the additions, the pH of the incubation medium was readjusted to pH 7.0 with 1 N NaOH or 1 N HCl. Incubations were carried out at 37° in a Dubnoff metabolic incubator shaking at 100 oscillations/min. At the end of the incubation period, the sacs were quickly removed and rinsed for 5 sec in 5–10 ml of ice-cold modified Krebs–Tris buffer. Each

* The abbreviations used in the text are: CPZ, chlorpromazine; nor_2-CPZ, didesmethylchlorpromazine; 7-OH-CPZ, 7-hydroxychlorpromazine; and CPZ-SO, chlorpromazine sulfoxide.

sac was cut open and the serosal fluid was drained and collected. In the early experiments, the serosal volumes measured 0.43 to 0.48 ml, with no significant differences between the treated and control groups. Hence, in later experiments, a 0.5-ml serosal volume was assumed for the calculations. After removal of the serosal fluid, each sac was placed in a preweighed scintillation vial and dried in the oven at 105° overnight. The vial with the dry tissue was then weighed, and the difference in weight of the vial with and without tissue gave the dry tissue weight. The tissues were then solubilized in 1.5 ml of NCS tissue solubilizer.

No extracellular volume measurements or intracellular concentration measurements were attempted in the study because of the uncertainties associated with these measurements [4]. Incubation periods of 5, 15 and 30 min were used in most of the time-course studies.

Determination of radioactivity. Radioactivity from aliquots of serosal fluid (0.2 ml) and incubation medium (1 ml) and whole solubilized tissue, added to 10 ml of Triton X-100 toluene scintillation liquid [5], was assayed in a Packard Tricarb scintillation spectrometer. Counting efficiencies ranged from 75 to 78 per cent for the aqueous samples and from 50 to 60 per cent for the solubilized tissue samples. All values were corrected for quenching and background.

Expression of the data. The results are expressed in the following form:

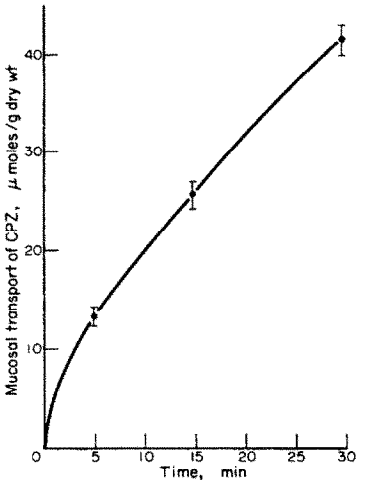

Fig. 1. Mucosal transport of CPZ and its effect on medium CPZ concentration. Everted sacs were incubated in 25 ml of standard Krebs–Tris incubation medium containing 1 mM CPZ (including 0.4 μCi [^{14}C]CPZ). Incubations were terminated at indicated times and values of the mucosal transport were determined. The preparation of sacs, conditions of incubation, assay methods, and the method of calculation and expression of data are described in Materials and Methods. Each point represents the mean ±S.E. for three sacs.

(a) Mucosal transport:

$$\frac{\text{amount of substance in tissue + amount of substance in serosal compartment}}{\text{g of dry tissue}}$$

(b) Serosal transfer:

$$\frac{\text{amount of substance in serosal compartment}}{\text{g of dry tissue}}$$

(c) Tissue content:

$$\frac{\text{amount of substance in tissue}}{\text{g of dry tissue}}$$

(d) Serosal/tissue ratio:

$$\frac{\text{serosal transfer}}{\text{tissue content}}$$

(e) Per cent control value:

$$\frac{\text{mean mucosal transport for treated at time } t}{\text{mean mucosal transport for control at time } t} \times 100$$

(f) $\Delta_{t_2-t_1}$ Per cent control value:

$$\frac{(\text{mean mucosal transport for treated at time } t_2) - (\text{mean mucosal transport for treated at time } t_1)}{(\text{mean mucosal transport for control at time } t_2) - (\text{mean mucosal transport for control at time } t_1)} \times 100.$$

RESULTS

CPZ mucosal transport and medium concentration. Our early studies indicated that some of the characteristics of CPZ transport in the intestine needed to be considered before studies of the effect of CPZ on L-methionine or any other transport substrate were made. Figure 1 shows some of these characteristics. From a 1-mM CPZ medium concentration, the mucosal transport was 13.3 μmoles/g of dry tissue at 5 min and 41.4 μmoles/g of dry tissue at 30 min. Ninety-seven per cent of the mucosal uptake was observed

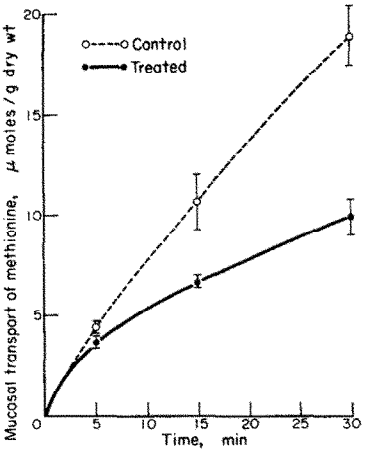

Fig. 2. Time course of effect of CPZ on L-methionine mucosal transport. Everted sacs were incubated in 25 ml of standard Krebs–Tris incubation medium containing 1 mM L-methionine (including 0.4 μCi L-[^{14}C]methionine) with (treated) or without (control) 1 mM CPZ. Incubations were terminated at indicated times and values of the mucosal transport were determined in the two groups. The preparation of sacs, conditions of incubation, assay methods, and the method of calculation and expression of data are described in Materials and Methods. Each point represents the mean ±S. E. of values from eight sacs.

to be in the tissue and very little was in the serosal fluid. This could be due to the high tissue-binding characteristics of CPZ [2, 6]. To test what portion of the observed CPZ tissue content at 5 min represented trapped or adsorbed CPZ, shorter incubation periods (10 sec–5 min) were used. The tissue CPZ content was found to increase gradually with time in this period. The 10-sec tissue content (which would represent mostly trapped or adsorbed CPZ) was only 12 per cent of the 5-min tissue CPZ content, suggesting that most of the 5-min tissue CPZ content represented mucosal transport as opposed to trapping.

The large mucosal transport of CPZ can affect the incubation medium concentration of CPZ. Using 5 ml of incubation medium (the volume often used in everted sac studies), CPZ concentration decreased by 15 per cent from initial concentration in 5 min, and 40 per cent with 30 min of incubation. When the incubation volume was 25 ml, there was only a 10 per cent drop in the medium concentration of CPZ at the end of a 30-min incubation. To maintain a steady concentration of CPZ, 25 ml of incubation medium was used in the rest of the experiments in this study.

Table 1. Effect of CPZ on the serosal transfer, tissue content and serosal/tissue ratio of L-methionine*

Group	Serosal transfer (μmoles/g dry wt)	Tissue content (μmoles/g dry wt)	Serosal/tissue ratio
Treated	3.4 ± 0.5†	6.4 ± 0.5†	0.5 ± 0.1
Control	6.9 ± 0.7	11.9 ± 0.9	0.6 ± 0.0

* The values of serosal transfer, tissue content and serosal/tissue ratio were calculated for the 30-min time points in the experiments illustrated in Fig. 2, as described in Materials and Methods. Values are means ± S. E. for eight sacs.

† Significantly different from control ($P < 0.01$).

Table 2. Effect of CPZ on sodium-independent component of L-methionine mucosal transport*

Group	Sodium-independent L-methionine mucosal transport (μmoles/g dry wt) 5 min	15 min	30 min
Treated	2.1 ± 0.1	3.8 ± 0.2	5.8 ± 0.2
Control	2.3 ± 0.0	4.1 ± 0.2	5.9 ± 0.2

* Everted sacs were incubated in 25 ml of sodium-free Krebs–Tris buffer containing 1 mM L-methionine (including 0.4 μCi L-[^{14}C]methionine) with (treated) or without (control) 1 mM CPZ. Other details are similar to those described in Fig. 2. Values are means ± S. E. for three sacs.

CPZ effect on L-methionine transport. L-Methionine was rapidly taken up and transported by the intestinal tissue (Fig. 2). The transport was faster in the first 5 min, a finding similar to that of Schultz *et al.* [7] with the transport of L-alanine in rabbit intestinal mucosal strips. A CPZ concentration of 1 mM in the incubation medium inhibited the mucosal transport of L-methionine significantly by 15 and 30 min when compared to the controls ($P < 0.05$ and 0.001 respectively).

CPZ inhibition of L-methionine mucosal transport increased with time. The treated group transported 81 per cent of the control in the 0- to 5-min period, 48 per cent in the 5- to 15-min period, and only about 39 per cent in the 15- to 30-min period.

The decreased mucosal transport in the CPZ-treated group could be due to decreased serosal transfer or decreased tissue content, or both. Table 1 shows that both the serosal transfer and the tissue content of methionine were significantly less in the treated group. The serosal/tissue ratios, indicative of tissue release of methionine to the serosal side, were not different between the treated and control groups,

Table 3. Effect of CPZ on sodium-dependent component of L-methionine mucosal transport*

Group	Na^+-dependent L-methionine mucosal transport (μmoles/g dry wt) 5 min	15 min	30 min
Treated	1.5	2.8	4.0
Control	2.1	6.5	12.9
Per cent control	71.4	43.1	31.0
	Δ_{5-0} per cent control = 71.4		
	Δ_{15-5} per cent control = 29.5		
	Δ_{30-15} per cent control = 18.8		

* The values of the sodium-dependent component were obtained by subtraction of the mean values of sodium-independent mucosal transport at the time points shown in Table 2 from the corresponding mean values of total mucosal transport shown in Fig. 2. The per cent control and $\Delta_{t_2-t_1}$ per cent control values were calculated as described in Materials and Methods.

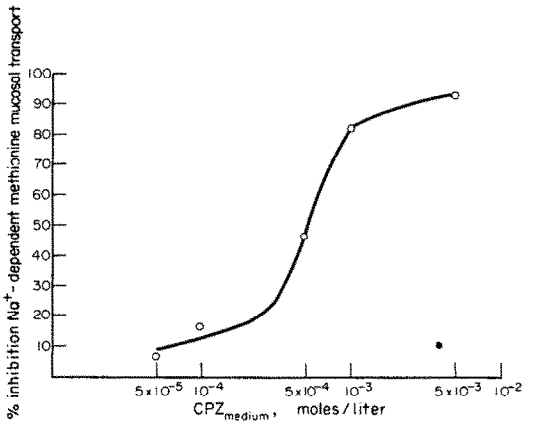

Fig. 3. Log concentration–effect curve for CPZ inhibition of the sodium-dependent component of L-methionine mucosal transport. Everted sacs were incubated in 25 ml of standard Krebs–Tris incubation medium containing 1 mM L-methionine (including 0.4 μCi L-[^{14}C]methionine). The medium also contained CPZ at different concentrations (control had no CPZ). Incubations were terminated after 30 min and the values of total mucosal transport in the various groups were determined as described in Materials and Methods. From these values, the value of the 30-min sodium-independent component of L-methionine mucosal transport (taken as the mean of 30-min values for control and treated groups in Table 2) was subtracted to obtain the values of the 30-min sodium-dependent component of L-methionine mucosal transport for the control and the treated (different CPZ concentration) groups. The treated values were expressed as per cent of control and the per cent inhibition values (100 per cent, control value) were calculated. Results at each concentration are values from two sacs.

suggesting that the CPZ effect was at the level of mucosal uptake.

CPZ effect on sodium-independent and sodium-dependent components of L-methionine transport. The total transport of L-methionine in intestine, like that of other neutral amino acids, has a sodium-dependent and a sodium-independent component [8].

The effect of 1 mM CPZ concentration on the transport of 1 mM L-methionine by the everted sac in a sodium-free Krebs–Tris medium is shown in Table 2. CPZ had no significant inhibitory effect on the sodium-independent component of L-methionine mucosal transport. Tissue CPZ concentration monitored on the same day after a 15-min incubation in normal and sodium-free Krebs–Tris medium showed no significant difference (30.2 ± 2.0 and 33.9 ± 0.9 μmoles/g of dry tissue respectively), suggesting that the negative effect of CPZ on the sodium-independent transport of L-methionine was not due to low tissue CPZ in sodium-free buffer. The effect of CPZ on the sodium-dependent component of L-methionine transport is shown in Table 3. The values for the sodium-dependent mucosal transport were obtained by subtracting the observed sodium-independent mucosal transport shown in Table 2 from values obtained with the regular Krebs–Tris buffer shown in Fig. 2. The Δ_{30-15} per cent control value of 18.8 per cent shows that about 81 per cent of the sodium-dependent transport of L-methionine is blocked after exposure of intestinal tissue to 1 mM medium concentration of CPZ for over 15 min.

Log concentration–effect curve. A semilogarithmic plot of the concentration–effect (Fig. 3) gave the classical sigmoid curve. Half-maximal inhibition occurred at a CPZ medium concentration of 5.2×10^{-4} M.

CPZ metabolism under the experimental conditions. There is some disagreement among investigators about the metabolism of CPZ in the gut tissue. Some workers [9] reported CPZ metabolism in the gut, whereas others [10, 11] found insignificant metabolism. We investigated CPZ metabolism occurring in the everted sacs under our experimental conditions in order to determine whether CPZ, CPZ metabolites or both could be responsible for inhibition of L-methionine transport. Everted sacs were incubated for 30 min in 5×10^{-4} M [^{14}C]CPZ. After incubation, unchanged CPZ was 100.1 ± 1.3 per cent and 99.8 ± 0.4 per cent in the tissue and incubation medium, respectively, suggesting that the inhibition of L-methionine transport was due to unchanged CPZ.

Effect of CPZ metabolites on L-methionine mucosal transport. The effect of three metabolites of CPZ on L-methionine transport was studied. The structures of these metabolites, as well as of CPZ, are shown in Fig. 4. CPZ and nor$_2$-CPZ were about equipotent in their inhibitory effect, 7-OH-CPZ was slightly less potent than CPZ and nor$_2$-CPZ, and CPZ-SO, in contrast, had an insignificant effect on L-methionine mucosal transport (Table 4).

DISCUSSION

In the present work, the characteristics of CPZ inhibition of L-methionine intestinal transport were studied using the everted sac technique. This is the simplest technique *in vitro* available for the study of intestinal transport wherein the polar characteristics of the intestine are preserved and transcellular transport can be monitored. It has been used extensively

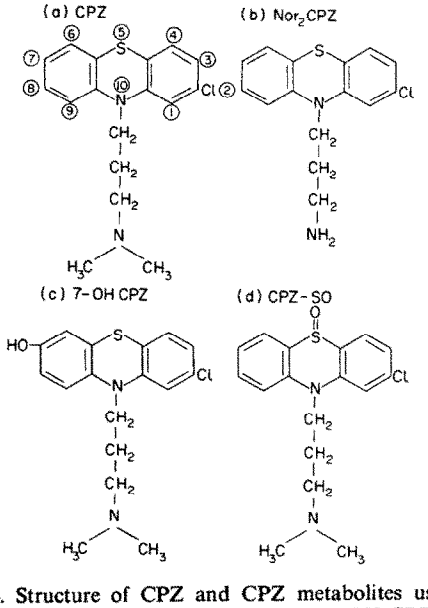

Fig. 4. Structure of CPZ and CPZ metabolites used in the study: (A) CPZ; (B) nor$_2$-CPZ; (C) 7-OH-CPZ; and (D) CPZ-SO.

Table 4. Effect of CPZ and CPZ metabolites on L-methionine mucosal transport*

Additions to mucosal solution	Mucosal transport (μmoles/g dry wt)	Per cent of control
Control	24.7 ± 0.5	100
CPZ (1 mM)	9.1 ± 1.2	36.8
Nor_2-CPZ (1 mM)	8.9 ± 0.8	36.0
7-OH-CPZ (1 mM)	12.7 ± 0.9	51.4
CPZ-SO (1 mM)	22.8 ± 1.8	92.3

* Everted sacs were incubated in 25 ml of standard Krebs–Tris incubation medium containing 1 mM L-methionine (including 0.4 μCi L-[^{14}C]methionine). The medium in addition contained the indicated concentrations of CPZ or CPZ metabolites ("control" had nothing in addition). Incubations were terminated after 30 min, and the values of mucosal transport in the different groups were determined as described in Materials and Methods. Results are the means ± S. E. for three sacs.

in the last 20 years and is generally thought to be reliable [4]. Although some workers have questioned the validity of the everted sac preparation on histological grounds [12], other workers have clearly shown its reliability using both histologic [13] and functional [14] parameters. L-Methionine was chosen as the transport substrate because (a) it is actively transported in the rat intestine via the neutral amino acid pathway [15], (b) it is negligibly metabolized by the rat intestine [16], and (c) the intestinal tissue levels of the amino acid are negligible [17].

In the present study, the inhibition of L-methionine transport in the intestine by various concentrations of CPZ occurred as early as at 5 min and increased with time. The observed time-course of CPZ action could be due to metabolites of CPZ rather than to CPZ itself, but the negligible CPZ metabolism observed under the experimental conditions makes this possibility unlikely. Time-dependent inhibition would also be expected to occur if CPZ uptake by the tissue was important for the inhibitory effect. We have found that the tissue CPZ levels generally correlate with percentage inhibition of L-methionine transport. In recent studies,* we have characterized the time dependence of the CPZ effect in more detail.

Several characteristics of the action of CPZ—its ability to produce a graded, concentration-related biological response, the classical sigmoid concentration–effect curve, and the structure–activity relationship observed (even with the limited number of congeners tried)—suggest the existence of a specific site of action ("receptor"). However, the potency is not quite in keeping with what would be expected of a specific "drug–receptor" interaction. The high concentrations of CPZ required to produce a significant effect on the intestinal transport of L-methionine in the present study may be related to the fact that intact tissues were used in the experiments. CPZ binds avidly to tissue proteins [2]; hence a lot of CPZ in the preparation might be bound nonspecifically. Differential potency of CPZ effects in the presence and absence of protein (with comparatively less effect in the presence of protein for the same CPZ concentration) is well documented [18].

The structure–activity relationship of a few congeners showed that demethylation in the aminopropyl side-chain (as in nor_2-CPZ) or even introduction of an oxygen atom in the ring at the 7-position to form a hydroxyl group (as in 7-OH-CPZ) does not greatly alter the inhibitory activity on L-methionine transport. But the attachment of oxygen to the sulfur in the ring results in loss of the inhibitory activity. The lack of inhibitory effect of CPZ-SO could not be due to poor tissue uptake because of its greater polarity, since 7-OH-CPZ is more polar than CPZ-SO [19]; yet it has inhibitory activity. Further studies with other congeners would help to clarify the structure–activity relationship.

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* P. R. Sundaresan and L. Rivera-Calimlim, unpublished observations.