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EFFECTS OF PHENOTHIAZINES ON AMINO ACID TRANSPORT AND PROTEIN SYNTHESIS IN ISOLATED NERVE ENDINGS

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Abstract—The uptake of ^{14}C-labeled leucine, phenylalanine, aspartic acid and proline by isolated synaptosomal particles was strongly inhibited by low concentrations of promazine and chlorpromazine. Chlorpromazine was consistently the more inhibitory of the two drugs. The inhibition of leucine uptake by phenothiazines was of the competitive type. Hill plots of inhibition of leucine and phenylalanine uptake suggested that the inhibition occurs at a single rate-limiting step. Analysis of the inhibition of proline and aspartic acid by chlorpromazine and promazine suggested a basic difference in the modes of action of these two compounds. The incorporation of [^{14}C]leucine into protein by intact synaptosomal systems was also inhibited by promazine and chlorpromazine. The inhibition was competitive in nature. Evidence is presented to indicate that phenothiazines may exert independent inhibitory effects on synaptosomal amino acid transport and on amino acid incorporation into protein.

SYNAPTOSOMAL fractions isolated from cerebral tissues are capable of incorporation of labeled amino acids into protein[1,2] by a process which is presumably dependent on endogenous sources of energy. This incorporation is not enhanced by addition of ATP or other energy-yielding substrates.[3] Synaptosomal incorporation of labeled amino acids into protein probably involves the initial active transport of the amino acid across the intact synaptosomal membrane,[4] and we have recently obtained evidence (unpublished) which indicates the presence of several distinct types of transport systems for amino acids in isolated nerve endings.

Phenothiazine compounds are known to affect a number of processes which depend on membrane permeability and the production of respiratory energy. Such a basic effect on membrane permeability accounts for the effects of phenothiazines on many biochemical reactions. A report from this laboratory,[5] however, described inhibitory effects of promazine and chlorpromazine on amino acid incorporation into protein by brain ribosomal systems. This incorporation process did not depend on the transport of the substrates (amino acids and aminoacyl-tRNA) across an intact membrane, and therefore the inhibition was in this case direct rather than secondary to changes in the characteristics of an interceding membrane.

In the present investigation, the possibilities were considered that phenothiazines may inhibit the active transport of amino acid by synaptosomes (thereby indirectly interfering with protein synthesis), may have a direct inhibitory effect on the mechanism of synaptosomal protein synthesis, or may affect both of these processes.

MATERIALS AND METHODS

Materials

^{14}C-amino acids. Uniformly labeled ^{14}C-amino acids were purchased from New England Nuclear Corp. and had the following specific activities (in millicuries per millimole): [^{14}C]leucine, 248; [^{14}C]phenylalanine, 390; [^{14}C]proline, 210; [^{14}C]-aspartic acid, 150. Promazine (Sparine) was kindly provided by Dr. H. H. Koepke of Wyeth Laboratories, Philadelphia, Pa. Chlorpromazine was a gift from Smith, Kline & French Laboratories, Philadelphia, Pa.

Methods

Synaptosomal fractions were isolated from brain cortices of young adult rats (30–60 days of post-natal age) on Ficoll density gradients, essentially as described by Kurokawa *et al.*[6] The synaptosomal pellets were suspended in a medium[7] containing: tris–HCl buffer, 10 mM (pH 7·4); NaCl, 125 mM; KCl, 25 mM; and $MgCl_2$, 15 mM.* To measure incorporation of amino acids into protein, portions of the fractions containing 1 mg of synaptosomal protein were incubated in a final volume of 1 ml with 0·5 μc ^{14}C-amino acid under conditions described in the figures and tables. The incubations were terminated by addition of 10 per cent trichloroacetic acid (TCA) containing unlabeled amino acid. The TCA-insoluble material was washed twice with 5% TCA, then suspended in TCA solution and heated for 15 min at 90° to solubilize RNA. The insoluble protein was washed two additional times with 5% TCA and then dissolved in formic acid and assayed for ^{14}C by liquid scintillation spectroscopy. For measurement of amino acid uptake by synaptosomal particles, unless otherwise specified in the tables and figures, 0·25 mg of synaptosomal protein was incubated with 0·1–0·25 μc ^{14}C-amino acid for 3 min. The incubations were terminated by the addition of 4 vol. of ice-cold medium. The synaptosomal particles were then collected on millipore filters (0·85 μ) under gentle pressure, and washed on the filters with a total of 40 ml of cold medium. The filters were dissolved in counting solution of the composition described by Herberg[8] and assayed for ^{14}C.

To establish the identity of ^{14}C-amino acids taken up by the synaptosomes, the synaptosomal particles were incubated with 1 μc of the ^{14}C-amino acids for 3 min under conditions described above. The washed filters containing the radioactivity were eluted with water and the eluates were subjected to descending paper chromatography in the solvent systems *n*-butanol–acetic acid–water (4:1:1, by vol.) and phenol–water (4:1 by vol.). Scans of the resulting chromatograms indicated that over 90 per cent of the radioactivity was present as the added amino acid. Protein was estimated by the method of Lowry *et al.*[9]

RESULTS

Effects of phenothiazines on synaptosomal amino acid uptake

Uptake of labeled leucine. Synaptosomal fractions rapidly accumulated labeled leucine during the first 3–5 min of incubation. After this period of rapid influx, there was a net efflux of the amino acid through the next 40–60 min of incubation. Promazine inhibited the early influx of the amino acid into synaptosomal particles.

* In experiments on the uptake of [^{14}C]aspartic acid and [^{14}C]proline, the incubation medium contained NaCl (150 mM) but no KCl, since 25 mM K^+ was found to inhibit the accumulation of these two amino acids.

At a concentration of 250 μM, promazine inhibited the influx of [^{14}C]leucine by 60 per cent (Fig. 1). Similar results were obtained in experiments using chlorpromazine instead of promazine.

Experiments were carried out to determine the nature of the phenothiazine inhibition of amino acid uptake.

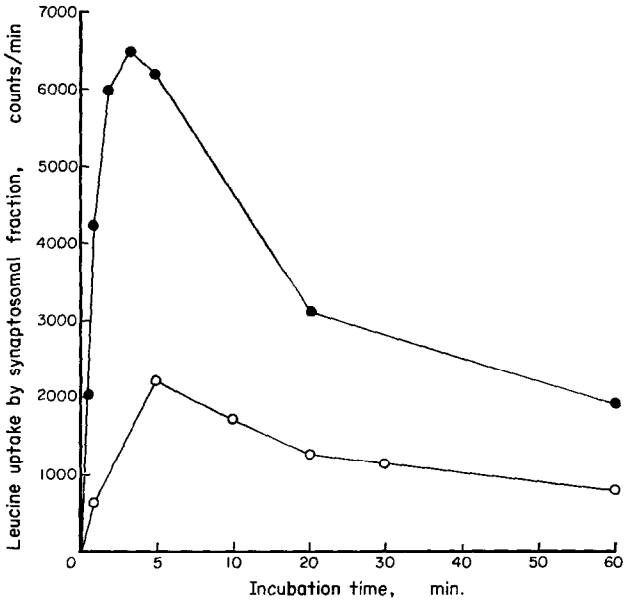

FIG. 1. Recovery of [^{14}C]leucine from synaptosomal fractions as a function of incubation time. Synaptosomal fractions were incubated with and without promazine for varying time intervals under the conditions described in the text. ●—●, Control; ○—○, 250 μM promazine.

In Fig. 2, the rate of [^{14}C]leucine influx has been plotted as a function of synaptosomal protein concentration. In the presence of both promazine and chlorpromazine, straight lines passing through the origin were obtained, but the slopes of these lines were less than that of the control (absence of inhibitor). This indicates that phenothiazine inhibition of [^{14}C]leucine uptake by synaptosomes is of the reversible type (see reference 10). A Dixon–Webb plot of the effect of promazine on [^{14}C]leucine uptake (Fig. 3) indicates that the inhibition was of the competitive type. The K_i value, derived from the plot was 160 μM, and the K_m value for leucine uptake was 14·6 μM. The competitive nature of the inhibition and the values of the constants were verified by Lineweaver–Burk plots derived from data of independent experiments.

The inhibition of [^{14}C]leucine uptake by promazine and chlorpromazine was also examined using a Hill-type plot, as described by Loftfield and Eigner.[11] At concentrations up to 500 μM, the plots of the velocity function against the log of inhibitor concentration yielded straight lines with a slope of approximately 1 (Fig. 4). An interpretation of this result would be that the inhibitor interfered with a single rate-limiting reaction, and that the enzyme–inhibitor complex involved one molecule of the inhibitory compound.

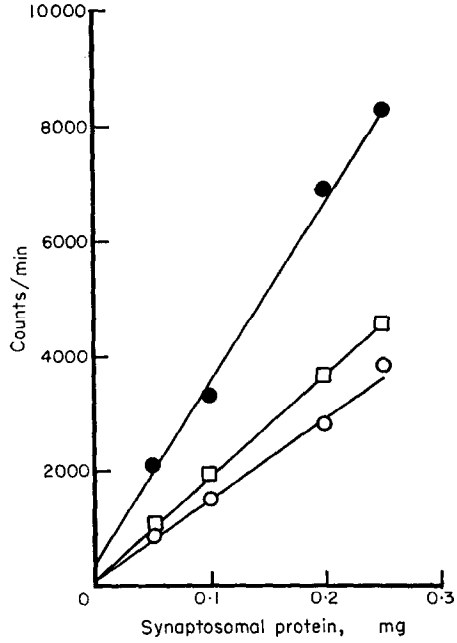

FIG. 2. Recovery of [^{14}C]leucine from synaptosomal fractions as a function of synaptosomal protein concentration. Synaptosomes were incubated with [^{14}C]leucine for 3 min in the presence and absence of the inhibitors. Other conditions were as described in the text. ●—●, Control; □—□, 100 μM promazine; ○—○, 100 μM chlorpromazine.

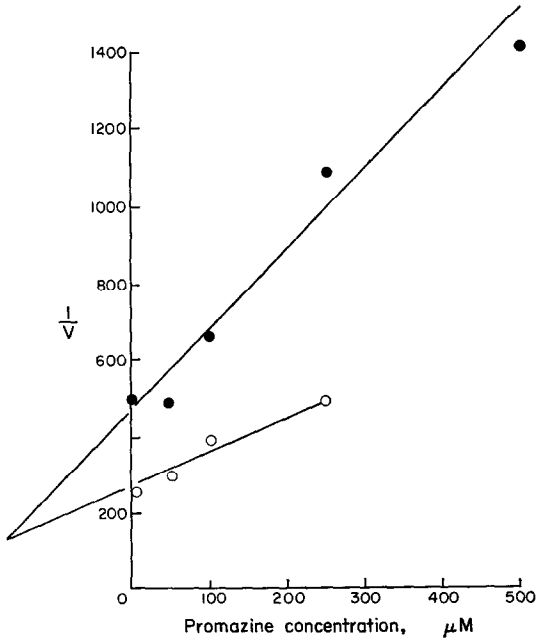

FIG. 3. Dixon–Webb plot of promazine inhibition of [^{14}C]leucine uptake by synaptosomal fractions. Synaptosomal fractions were incubated for 3 min with various concentrations of promazine and with leucine concentrations of 7 μM (●—●) and 17 μM (○—○). V is 10^3 × micro-moles of leucine taken up by synaptosomes (0·25 mg protein) during the 3-min incubation period.

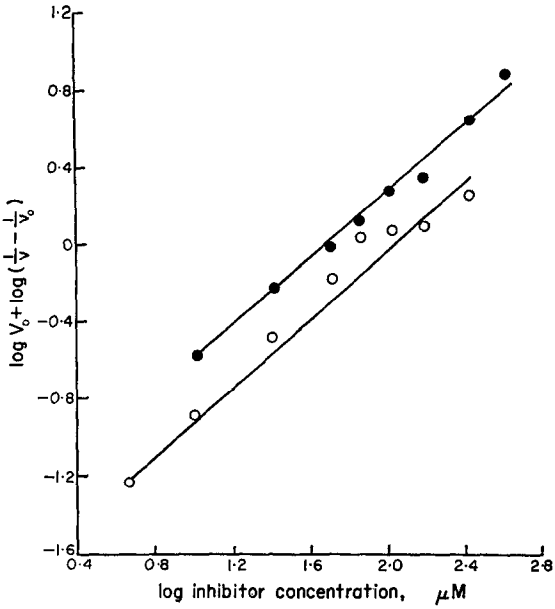

FIG. 4. Inhibition of [^{14}C]leucine influx into synaptosomal fractions by promazine (○—○) and chlorpromazine (●—●). Synaptosomal fractions were incubated for 3 min with [^{14}C]leucine and various concentrations of the inhibitors. Slope value for: (a) promazine inhibition, 0·85; (b) chlorpromazine inhibition, 0·95. V_o and V are the uninhibited and inhibited uptake rates respectively.

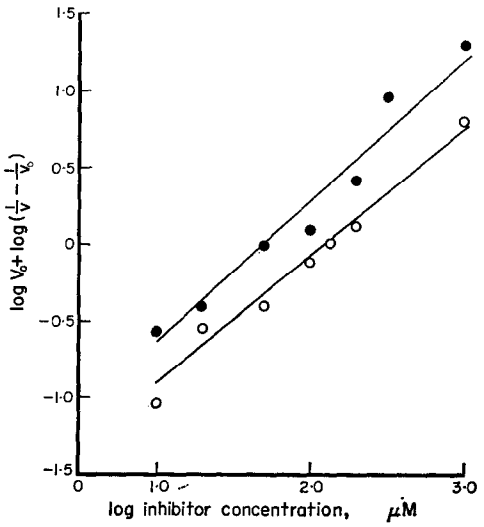

FIG. 5. Inhibition of [^{14}C]phenylalanine uptake into synaptosomal fractions by promazine (○—○) and chlorpromazine (●—●). Conditions of incubation were as in Fig. 4. Slope value for: (a) promazine inhibition, 0·86; (b) chlorpromazine inhibition, 0·94. V_o and V are the uninhibited and inhibited uptake rates respectively.

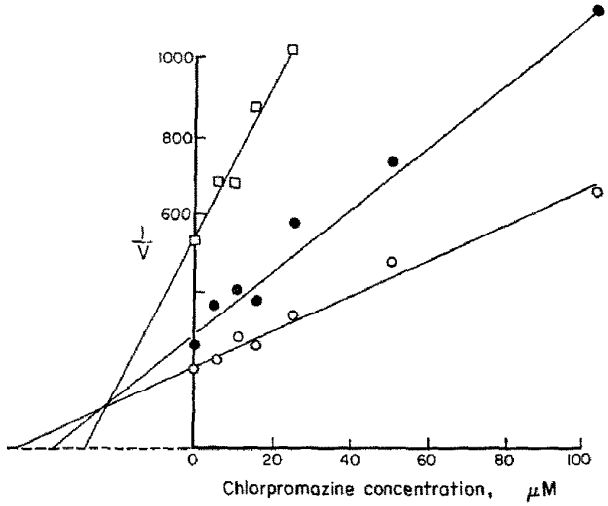

FIG. 6. Dixon–Webb plot of chlorpromazine inhibition of [^{14}C]proline uptake by synaptosomal fractions. □—□, 2 μM proline; ●—●, 5 μM proline; ○—○, 7 μM proline. V is 10^{-3} × micromoles of proline taken up by synaptosomes (0·25 mg protein) during the 3-min incubation.

Uptake of other amino acids. The effects of phenothiazines on the synaptosomal uptake of three other amino acids, namely phenylalanine, proline and aspartic acid, were examined. The inhibitory effects of chlorpromazine and promazine on phenylalanine uptake were similar to their effects on leucine uptake. Hill plots of the inhibition (Fig. 5) were linear with slope values of unity, and chlorpromazine was more inhibitory than promazine.

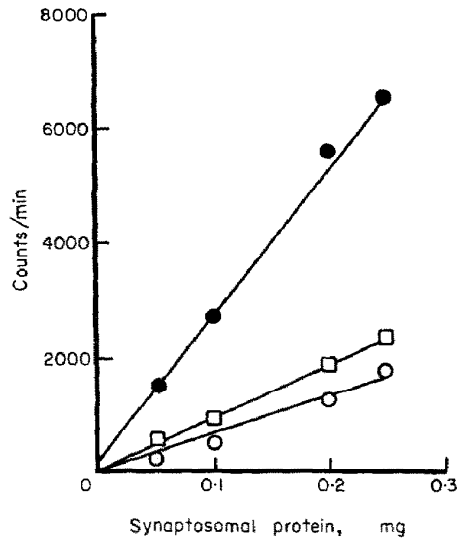

FIG. 7. Recovery of [^{14}C]proline from synaptosomal fractions as a function of synaptosomal protein concentration. Conditions of incubation were as in Fig. 2. ●—●, Control; △—△, 100 μM promazine; ○—○, 100 μM chlorpromazine.

Both promazine and chlorpromazine inhibited the accumulation of proline to a significantly greater extent than the accumulation of leucine and phenylalanine. The Dixon–Webb plot of chlorpromazine inhibition of proline uptake (Fig. 6) yielded a K_I value of 22 μM, approximately one-eighth of that derived for the inhibition of leucine uptake. As with leucine, the inhibition of proline by both compounds was reversible (Fig. 7), and was not dependent on the concentration of synaptosomal protein. The Hill plots (Fig. 8) for the inhibition of proline uptake by promazine and chlorpromazine suggest a basic difference in the mode of action of these two compounds. Thus, the plot for promazine inhibition was linear up to a concentration of 500 μM with a slope value of approximately 1. In contrast, the plot for chlorpromazine inhibition of proline uptake was characterized by two distinct segments. At lower chlorpromazine concentrations ($<$ 250 μM) the plot was linear with a slope value which approximated unity, whereas at concentrations above 250 μM the slope of the plot sharply increased to a value between two and three.

The Hill plot for promazine inhibition of aspartic acid uptake (Fig. 9) was linear, but with a slope value of 2·0, suggesting an interaction of two molecules of promazine

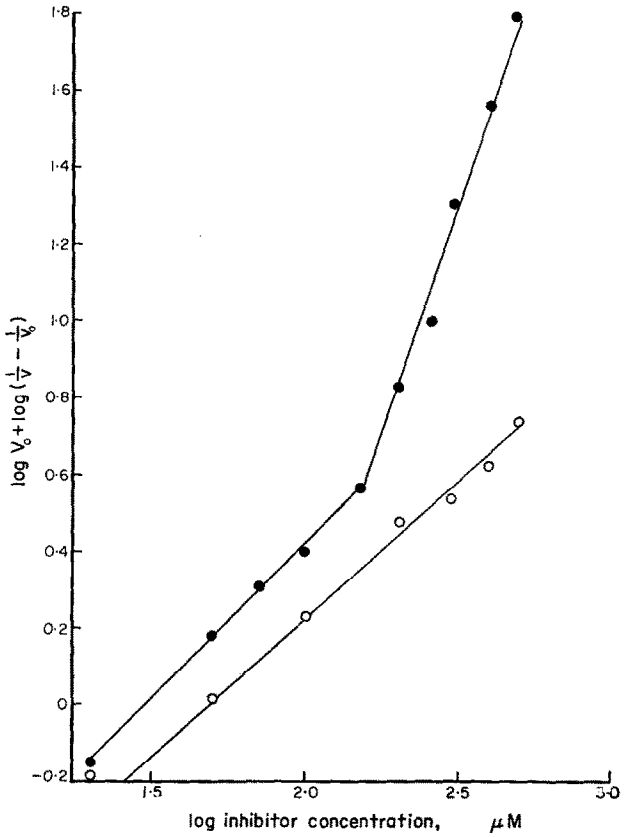

FIG. 8. Inhibition of [^{14}C]proline influx into synaptosomal fractions by promazine (○—○) and chlorpromazine (●—●). Synaptosomal fractions were incubated for 3 min with [^{14}C]proline and various concentrations of the inhibitors. V_0 and V are the uninhibited and inhibited uptake rates respectively.

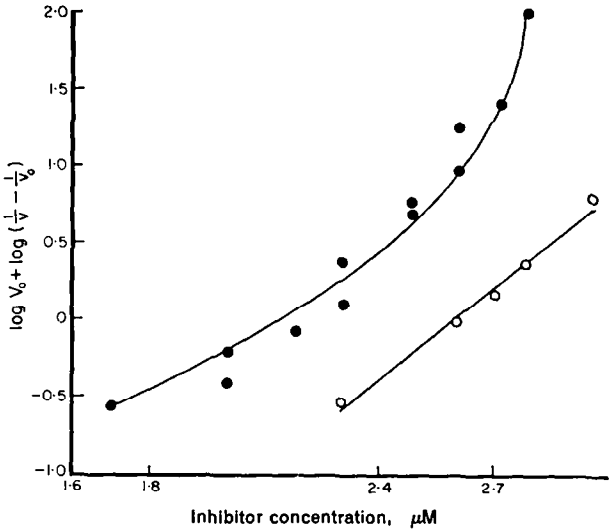

FIG. 9. Inhibition of [^{14}C]aspartic acid influx into synaptosomal fractions by promazine (○—○) and chlorpromazine (●—●). Synaptosomal fractions were incubated with [^{14}C]aspartic acid and various concentrations of inhibitors. Incubation time, 3 min. Slope value for promazine inhibition was 2·0. V_0 and V are the uninhibited and inhibited uptake rates respectively.

with a single step in the aspartic acid transport system. Aspartic acid uptake, furthermore, was much less sensitive to promazine than to chlorpromazine, as indicated by the displacement of the curve to the right (50 per cent inhibition occurred with a promazine concentration of approximately 400 μM). The Hill plot for chlorpromazine inhibition of aspartic acid uptake is nonlinear with a gradually increasing slope, suggesting a more complex interaction between chlorpromazine and transport of this amino acid.

Effects of phenothiazines on incorporation of [^{14}C]*leucine into protein by isolated synaptosomes*

The time course for the incorporation of [^{14}C]leucine into synaptosomal protein was linear over a 40-min incubation period (Fig. 10). The inhibitory effect of chlorpromazine was essentially not dependent on incubation time. Inhibition of [^{14}C]-leucine incorporation by promazine was competitive with a K_i value of 120 μM (Fig. 11). With both the compounds, the Hill plots were linear within the concentration range studied (50–1000 μM; Fig. 12). Although the slopes of the Hill plots for inhibition of amino acid incorporation were close to unity, the data from a number of experiments of the type shown in Fig. 12 yielded slope values which were in fact significantly greater than 1. Thus, in six different experiments on promazine inhibition of leucine incorporation, the average value of the slope was 1·28 $\pm$ 0·08, and in three experiments on inhibition by chlorpromazine the average value was 1·12 $\pm$ 0·04. In no case was the slope value less than 1·08. This would imply that the inhibition of amino acid incorporation into protein involved an enzyme–inhibitor complex with a second molecule of the phenothiazine compound which had an incomplete interaction.

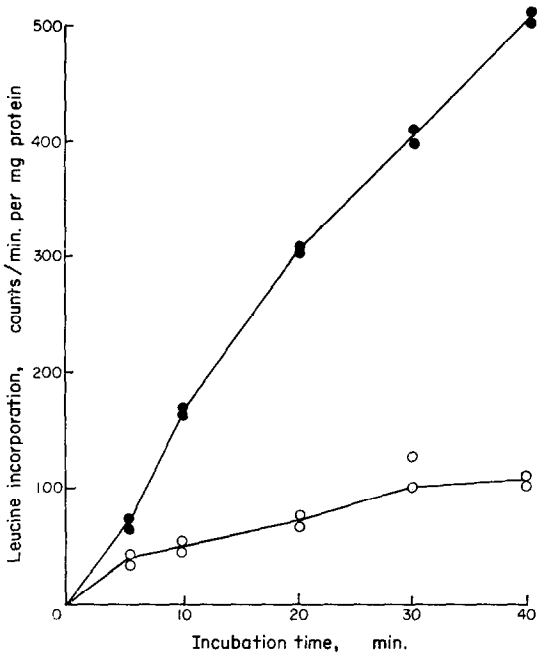

FIG. 10. Chlorpromazine inhibition of [^{14}C]leucine incorporation into synaptosomal protein. Synaptosomal fractions (1 mg protein) were incubated with [^{14}C]leucine for various time intervals under the conditions described in the text. Reactions were terminated by addition of TCA and the proteins were processed for assay of ^{14}C content. ●—●, Control; ○—○, 500 μM promazine.

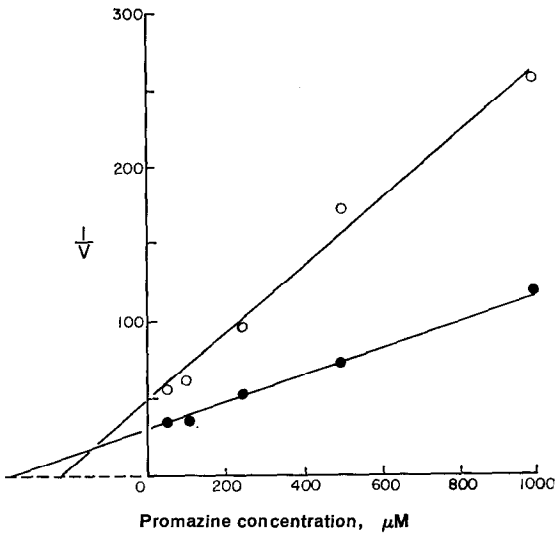

FIG. 11. Dixon–Webb plot of promazine inhibition of [^{14}C]leucine incorporation into protein by synaptosomal fractions. Fractions were incubated for 30 min with final leucine concentrations of 17 μM (●—●) and 7 μM (○—○). V is 10^5 × micromoles of leucine incorporated per milligram of protein per 30 min.

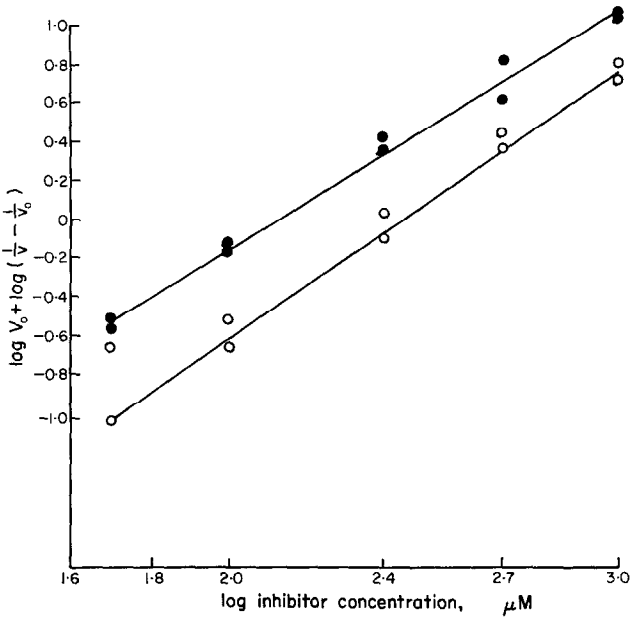

FIG. 12. Inhibition of [^{14}C]leucine incorporation into synaptosomal proteins by promazine (●—●) and chlorpromazine (○—○). The fractions were incubated for 30 min with [^{14}C]leucine and various concentrations of the inhibitors. Slope value for: (a) promazine inhibition, 1·38; and (b) chlorpromazine inhibition, 1·10. V_0 and V are the uninhibited and inhibited uptake rates respectively.

An attempt was made to determine if chlorpromazine inhibited the synaptosomal amino acid-incorporating mechanism independent of the transport of amino acid into the synaptosomal particle. Synaptosomal fractions were disrupted by osmotic shock by the following procedure. Synaptosomal pellets were suspended in distilled water (15–20 mg protein/ml of water), homogenized gently and allowed to stand at 0° for 30 min. Portions of the suspension were then incubated under conditions described in Table 1. In the absence of ATP and an ATP-regenerating system, the lysed fractions incorporated 90 counts/min/mg of protein. The addition of ATP–creatine phosphate–phosphokinase system to the incubation mixtures increased this incorporation by approximately 4-fold. This energy-dependent incorporation exhibited other properties that are characteristic of a cytoplasmic cell-free protein-synthesizing system. Thus, the incorporation was inhibited by ribonuclease and by puromycin. Chlorpromazine at concentrations of 250 and 500 μM had little or no effect on the incorporation of [^{14}C]leucine into protein by the lysed system. At higher concentrations (1 mM), chlorpromazine inhibited this incorporation significantly.

DISCUSSION

In a previous study,[5] we had shown that phenothiazines inhibit the incorporation of ^{14}C-amino acids into protein by rat brain ribosomal systems, but not by liver systems. We further suggested that this inhibition results from an interaction between the drugs and the soluble brain transferases. The present investigation deals with the effects of promazine and chlorpromazine on the transport of amino acids across the

TABLE 1. EFFECTS OF CHLORPROMAZINE ON THE INCORPORATION OF [^{14}C]LEUCINE INTO PROTEIN BY OSMOTICALLY SHOCKED SYNAPTOSOMAL SYSTEM*

Addition	[^{14}C]leucine incorporated (counts/min/mg protein)
None	90
ATP, creatine phosphate, creatine phosphokinase†	355
Puromycin (50 μg/ml)	85
Ribonuclease (100 μg/ml)	198
Chlorpromazine (0·25 mM)	340
(0·5 mM)	372
(1·0 mM)	154

* Lysed synaptosomal particles (1 mg protein) were incubated in a medium containing: tris–HCl buffer, 10 mM (pH 7·4); NaCl, 125 mM; KCl, 25 mM; and $MgCl_2$, 15 mM. Final volume, 1 ml; incubation time, 30 min.

† Concentrations of these compounds were as given in ref. 5.

synaptosomal membrane and on the incorporation of amino acids into protein by isolated synaptosomal fractions.

Synaptosomal particles rapidly accumulated labeled leucine, phenylalanine, aspartic acid and proline during 3 min of incubation at 37°. The uptake of each of the amino acids was strongly inhibited by low concentrations of promazine and chlorpromazine. Chlorpromazine was consistently the more inhibitory of the two compounds. Leucine and proline accumulation was inhibited competitively by both compounds, and the inhibition was reversible. Analysis of the Hill plots of the inhibition of leucine and phenylalanine influx showed that the inhibition involves an interaction of a single molecule of the drug with a rate-limiting step in the transport process. Apart from the greater inhibitory effect of chlorpromazine, there appeared to be no essential differences in the nature of the effects of the two compounds on leucine and phenylalanine accumulation. The inhibition of proline and aspartic acid by the compounds, however, presented a different picture. The plot for inhibition of proline uptake by promazine was linear with a slope of 1, while that for chlorpromazine inhibition showed an abrupt increase in the slope value at an inhibitor concentration of about 250 μM, indicating that an additional step in the transport became inhibited at the higher chlorpromazine concentrations. Aspartic acid uptake was less sensitive to promazine than the other amino acids tested; 50 per cent inhibition occurred only at promazine concentrations of 400 μM. The Hill plot (Fig. 9) for the inhibition was linear, but with a slope value of 2, suggesting that this inhibition was second order with respect to promazine concentration. Furthermore, the plot for chlorpromazine inhibition of aspartic acid was nonlinear with an increasing slope over the entire concentration range studied, indicating a more complex type of inhibitory mechanism.

In view of these differences between the effects of chlorpromazine and promazine on the transport of proline and aspartic acid, the possibility was entertained that the two compounds exerted their inhibitory effects at dissimilar reactive sites in the transport of leucine also. Mixed inhibition experiments were carried out with promazine and chlorpromazine in which the rate of leucine influx was measured in the

presence of a mixture of promazine and chlorpromazine at various concentrations. The experimentally measured influx rates were then compared with hypothetical rates (V_{p+c}) calculated using the following relationships:[12]

$$\frac{V_{p+c}}{V_0} = \frac{1}{1+K_p[p]+K_c[c]} \tag{1}$$

$$\frac{V_{p+c}}{V_0} = \frac{1}{1+K_p[p]+K_c[c]+K_p[p]K_c[c]} \tag{2}$$

in which $[p]$ and $[c]$ are the concentrations of promazine and chlorpromazine, respectively, and V_0 is the uninhibited influx rate. Values for K_p and K_c were derived from the data which yielded Hill plots. Equation (1) applies if the inhibitory compounds share a common reaction site; equation (2) applies if the compounds exert their inhibition at independent nonreacting sites. The experimental values for V_{p+c}/V_0 were in considerably closer agreement to values calculated from equation (1) than to those calculated from equation (2). A statistical assessment was made by expressing the experimental V_{p+c}/V_0 values as a ratio of the values calculated from each equation and then computing the probability that the average ratio deviated from unity. Based on this assessment, it was established that a common reactive site for the two compounds is roughly 80 times more likely than independent sites.

In a recent communication,[13] we have described the characteristics of the accumulation of several amino acids by isolated nerve endings. These experiments have established that the accumulation of proline and aspartic acid by synaptosomal fractions, unlike that of several other amino acids tested, was totally dependent on the presence of Na^+ in the incubation mixture, and that the Na^+-dependent transport was stoichiometrically inhibited by K^+. The uptake of the other two amino acids reported in this present study, phenylalanine and leucine, showed little if any dependency on added Na^+. Although the exact mechanisms for the transport of various amino acids across the synaptosomal membrane are not known, it seems possible that transport of proline and aspartic acid may derive energy directly from a transmembrane Na^+ gradient and follow the downhill movement of Na^+ into the particle. While the effect of phenothiazines on leucine and phenylalanine transport may be by interference with an energy-dependent transport enzyme system, the chlorpromazine inhibition of aspartic acid and proline transport may, in addition, involve alterations in membrane permeability which in turn affect Na^+ influx.

It is of interest to compare the effects of phenothiazines on amino acid incorporation into protein by isolated intact synaptosomes and by brain ribosomal systems. The synaptosomal system was much more sensitive to phenothiazines than the cell-free system reported by us earlier.[5] Thus, the incorporation of [^{14}C]leucine into protein by synaptosomes was inhibited by 50 per cent at chlorpromazine concentrations of 0·12 mM, whereas in the ribosomal system a similar inhibition of ^{14}C-amino acid incorporation was achieved only at concentrations of over 1·0 mM.

The evidence presented in this study further suggests that the inhibitory effects on amino acid incorporation into protein by intact synaptosomes may not be due entirely to inhibition of transport processes. Morgan and Austin[14] have demonstrated that osmotically shocked synaptosomal fractions incorporate amino acid into protein only in the presence of added ATP, and they have provided evidence to suggest that such

incorporation represents the activity of a eukaryotic ribosomal system. In such a system, chlorpromazine had little or no effect on amino acid incorporation into protein at concentrations up to 0·5 mM, but markedly inhibited the incorporation at 1 mM concentration. Additional studies on the effects of phenothiazines on the soluble synaptosomal system are warranted, particularly in view of our earlier demonstration that these compounds inhibited the incorporation of amino acids into protein by brain ribosomal systems but not by liver systems. These studies might also prove valuable in understanding the mechanism of protein synthesis in synaptosomal fractions.

The Hill plots for phenothiazine inhibition of uptake of leucine and its incorporation into protein lend additional support to the view that these drugs may exert independent inhibitory effects on these two processes. Whereas the slope for inhibition of [^{14}C]leucine influx never exceeded unity, that for inhibition of incorporation of this amino acid into protein was consistently greater than one, thus implying different mechanisms[11] for the inhibition. However, the possibility that phenothiazines also interfere with intrasynaptosomal mitochondrial processes (transport of amino acid or mitochondrial protein synthesis or both) cannot be ruled out.

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