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EFFECTS OF IMIPRAMINE ON THE Na^+-DEPENDENT EXCHANGE AND RETENTION OF γ-AMINOBUTYRIC ACID BY MOUSE BRAIN SUBCELLULAR PARTICLES

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Abstract—The effects of imipramine were investigated with respect to exchange and net content of γ-aminobutyric acid (GABA) in mouse brain subcellular particles in the presence of Na^+ at 2°. The effects of chlorpromazine and orphenadrine on the system were also investigated. Evidence is presented indicating an inhibition by these three drugs of the Na^+-dependent binding of GABA to sites on the membrane which mediate a transmembrane flux of GABA. In the presence of greater concentrations of these drugs a pronounced increase in a nonmediated efflux of endogenous GABA from particles was detected. Studies of the binding of tritiated imipramine demonstrated a complex relationship to drug concentration, indicative of two distinct uptake processes. This paper discusses the possible correlation between the two components of the imipramine binding and the effects of the drug on the exchange and net release of GABA by the particles.

REVIEWS on the effects of psychotropic drugs[1,2] indicate that a number of these drugs reduce membrane permeability at low drug concentrations and increase permeability at higher concentrations. Seeman[2] has suggested a model in which the membrane is viewed as porous and as going through a transition to a tight nonporous molecular configuration and then to a more porous configuration. This paper analyzes the effects of imipramine, chlorpromazine and orphenadrine on permeability in a situation where the permeating substance, γ-aminobutyric acid (GABA), has been reported to traverse the membrane by a carrier-mediated process and not by free diffusion though pores.[3]

The preparation employed in these studies consisted of subcellular particles obtained from mouse brain homogenates. These particles contain endogenous GABA which does not exchange with exogenous ^{14}C-GABA unless Na^+ is introduced into the system.[3–5] The introduction of Na^+ has previously been shown to activate GABA binding in the membranes of the particles. These Na^+ dependent sites for GABA mediate an exchange between ^{14}C-GABA and endogenous GABA, and permit a net downhill efflux of GABA from the particles. These events occur at 2° and do not require any metabolically derived energy source.[3]

Because of the extensive body of information that has accumulated on the subject of GABA permeability it was felt that a detailed analysis of the effects of psychotropic drugs on the system might be more amenable to interpretation than other studies of drug-induced changes in permeability in which little or no detailed information is available regarding the mechanisms by which the permeating molecule normally traverses the membrane.

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MATERIALS AND METHODS

Preparation of particles and binding procedure. The preparation of the particles and the assay procedures have been described.[3] Briefly, a 10 per cent (w/v) homogenate of whole mouse brain was prepared in 0·25 M sucrose at 0–2°. The particles which sedimented between 1500 *g* (10 min) and 15,000 *g* (15 min) were resuspended up to 90 per cent of the desired final volume in a buffered solution of NaCl. The appropriate drug and [^{14}C]-GABA were then introduced. The sequence and time of introduction varied and are specified in individual experiments. One ml of the final saline suspension contained the following components; 0·2 m-moles NaCl, 0·05 m-moles Tris-HCl (pH 7·3), approximately 0·75 μg of 2-[^{14}C]-GABA (sp. act. 2·71 mc/m-mole) in addition to the endogenous GABA which was distributed between particles and suspension fluid, and brain particles containing 3·0–3·2 mg protein as determined by the method of Lowry *et al.*[6] Drug concentrations are specified in the individual experiments. A temperature of 2° was maintained throughout all experiments. It has been reported that no metabolites of [^{14}C]-GABA are produced at this temperature.[4]

After appropriate times of incubation, 10-ml portions of the radioactive saline suspensions were removed. The particles were sedimented for 15 min at 15,000 *g* and the supernatant was decanted. Residual droplets of fluid were removed from the tube with a cotton swab and the pellet, which occupied 3 per cent of the sample volume, was resuspended in water to a fixed volume. The distribution of [^{14}C]-GABA and GABA between the particles and suspension fluid was then determined. Three 40-μl portions of the resuspended particles and the decanted supernatant were assayed for radioactivity in a liquid scintillation counter. The pH of the remaining material was adjusted to 4·5 with HCl and approximately 1 mg of activated charcoal (Norit) was introduced per ml of sample. Fifteen min later, the samples were placed in a boiling water bath for 10 min, centrifuged and the fluids filtered through Whatman No. 50 filter paper to remove any residual charcoal. The activated charcoal treatment was found necessary to remove the drugs from the sample and prevent them from interfering with the enzymatic assay of GABA. A comparison of the radioactive content of the fluid before and after these steps indicated that no adsorption of [^{14}C]-GABA to the Norit or filter paper had occurred. The clear filtrates were adjusted to a pH of 7–8 with NaOH and dried under an infrared lamp. Two ml of water was added to the dried sample. Any insoluble debris was removed by centrifugation and duplicate portions of the clear fluid were monitored for radioactivity[3] and assayed for GABA.[7] This enzymic assay converts GABA to succinic semialdehyde via a transamination reaction with αKG. The semialdehyde in turn is converted to succinate via a NADP-dependent oxidation. The reduction of NADP was monitored at 340 mμ. A standard curve was run with each group of samples. The concentration of GABA in the unknown samples was adjusted so that the ΔOD_{340} was 0·300 or greater. The difference between duplicates was generally less than 0·015 OD units. In the event of greater variation another set of enzymic determinations was made. In some cases internal standards were also run and good agreement with the standard curve was obtained. The radioactivity determinations served two functions. They yielded values for the specific activity of the samples and were used to monitor the mechanical losses throughout the procedure. In an individual experiment each experimental condition was duplicated and the results were not accepted unless the variation between duplicates was 5 per cent or less. The averaged values of individual experiments are

reported. All experiments were repeated at least two times to verify the general conclusions. The results are expressed as radioactivity (counts/min) and GABA content (μg) of the pellet and supernatant fluid obtained from 1 ml of the radioactive suspension. The pellet obtained from 1 ml of suspension occupied 0·03 ml. The [^{14}C]-GABA radioactivity and GABA content of 0·03 ml of the supernatant was subtracted from the pellet content thus yielding the maximum conceivable correction for fluid entrained by the pellet. It should be noted that the time intervals stated in this paper are based on the times at which the sedimentation of the particles was initiated. The suspension fluid was decanted between 20–23 min after the centrifugation step was initiated.

Method for measuring the equilibrium binding of [^{3}*H*]*-imipramine.* Equal volumes of various concentrations of [^{3}H]-imipramine (activity 3×10^5 counts/min/μmole) were mixed with portions of a saline suspension of particles in polyethylene centrifuge tubes. Each tube contained a particle content equivalent to 3·1 mg protein in a final volume of 1 ml and final concentrations of 0·16 M NaCl and 0·05 M Tris-HCl buffer, pH 7·3. Immediately after the introduction of imipramine, 30 and 60 min later triplicate portions of 40 μl were removed from the suspension (particles dispersed in the suspending fluid) for the determination of radioactivity. The observed radioactivity was in agreement with the expected concentrations. It was therefore concluded that no detectable losses of the drug from the suspension had occurred by means of adsorption to the walls of the polyethylene tubes. The remaining suspension was then centrifuged at 15,000 *g* for 15 min in a No. 40 Spinco rotor. Centrifugation of identical samples was done immediately after the introduction of imipramine, 30 and 60 min later. After centrifugation, triplicate supernatant portions of 40 μl were taken for the determination of radioactive concentrations. The radioactivity in the supernatant did not vary as a function of time. It could therefore be concluded that an equilibrium distribution of imipramine between the particles and the supernatant occurred rapidly and was maintained during the time interval of the experimental period. It was also determined that the introduction of exogenous GABA, 0·75 μg/ml of final suspension did not affect the binding of imipramine. The following general formula was used for calculating the amount bound, $X = T - S(F)$. The amount bound in 40 μl of suspension is X. T is the radioactive content of 40 μl of the suspension. S is the radioactivity in 40 μl of supernatant and F is a correction factor. Various values can be assigned to F which are dependent upon different sets of measurements and assumptions. A correction factor can be used which requires inulin space measurements. In this case all the radioactivity associated with the particles is considered as bound, and the correction factor F is the ratio of the extraparticulate fluid volume over the total volume of the suspension. If it is assumed or known that the drug can enter the particle and exist in a nonbound state within the intraparticulate fluid space at a concentration equal to the external fluid concentration, then the correction factor F should be the ratio of the intraparticulate plus extraparticulate fluid volume over the total volume of the suspension. The values obtained for F were 0·98 based on inulin space measurements and 0·995 based on the total fluid content of the system. A third procedure is to assign a value for F of 1·0 on the supposition that the contribution of the particles to the total volume is so small that it can be neglected. This last procedure will always yield a value for the amount bound which is less than the true value.

The amount bound was calculated using the three values of F of 0·98, 0·995 and 1·0.

Plots of the three sets of values revealed relatively insignificant shifts in the shape of the adsorption isotherm and the same general conclusions could be reached on the basis of any of the curves. The plots shown in the results section on imipramine binding are those obtained when F is 1·0. The use of this procedure, which slightly overcorrects, completely eliminates the possibility that any increase in binding as a function of supernatant concentration may be a methodological artifact.

RESULTS

(1) *The release by imipramine of GABA and radioactivity from the particles.* Particles were first incubated with [^{14}C]-GABA for 30 min to permit them to accumulate radioactivity in the absence of the drug. At the end of this incubation, the particles from 1 ml of suspension contained 4·1 μg of GABA and 17,250 counts/min. Imipramine was then added at various concentrations to portions of the suspension, the controls receiving equal volumes of water. The effects of the drug were assessed at various time intervals thereafter. No net changes were found to occur in the total radioactivity (36,750 counts/min/ml) and GABA (6·1 μg/ml) contents of the system (particles + fluid). Figure 1 shows the changes in the radioactivity and GABA contents of the particles as a function of time at various imipramine concentrations. In the absence of the drug the radioactivity and GABA content of the particles decreased slightly over

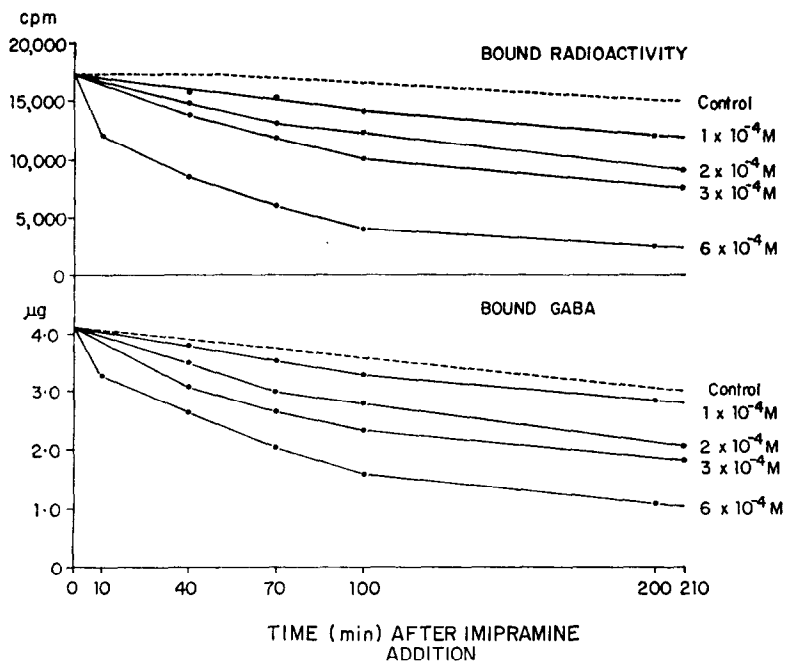

FIG. 1. The release of [^{14}C]-γ-aminobutyric acid and γ-aminobutyric acid from the particles as a function of time in the presence of various concentrations of imipramine. One ml of the buffered saline suspension (particles plus suspension fluid) contained 6·1 μg of GABA and 36,750 counts/min. Radioactivity ([^{14}C]-GABA) was allowed to accumulate in the particles for 30 min prior to the introduction of imipramine.

a period of 3–4 hr. The decrease was enhanced by the presence of imipramine. The effects were progressively greater with increasing concentrations of the drug.

Figure 2 shows the changes in the radioactivity and GABA contents of the particles as a function of imipramine concentration after 40 and 205 min of incubation after the addition of the drug. At both time intervals a low drug concentration (10^{-4} M) affected the radioactivity considerably more than the GABA content of the particles. At higher drug concentrations the radioactivity and GABA content of the particles decreased in an approximately linear fashion.

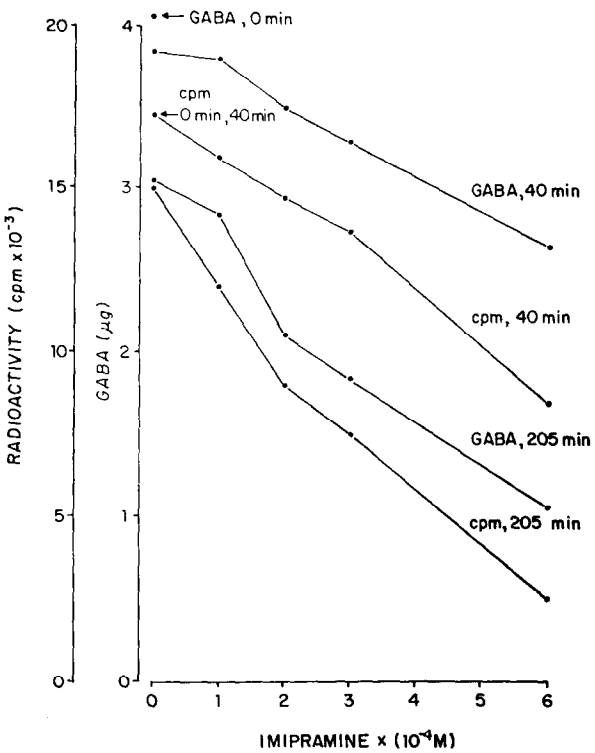

FIG. 2. The release of [^{14}C]-γ-aminobutyric acid and γ-aminobutyric acid from the particles as a function of imipramine concentration after 40 and 205 min in the presence of the drug. Imipramine was introduced 30 min after the accumulation of [^{14}C]-GABA was initiated. One ml of the suspension (particles plus suspension fluid) contained 6·1 μg of GABA and 36,750 counts/min.

(2) *The effects of 30-min preincubation in the presence of imipramine on the [^{14}C]-GABA accumulation by the particles.* Portions of the buffered saline suspension received imipramine at various final concentrations. Thirty min after the introduction of the drug, [^{14}C]-GABA was added and the suspensions were incubated for another 30 min prior to centrifugation and analysis. The results are shown in Fig. 3.

The GABA content of the particles was essentially unaffected by the imipramine at concentrations up to 6×10^{-5} M, but was progressively reduced at higher concentrations. In the range from 1 to 6×10^{-4} M, the GABA decrease in the particles was

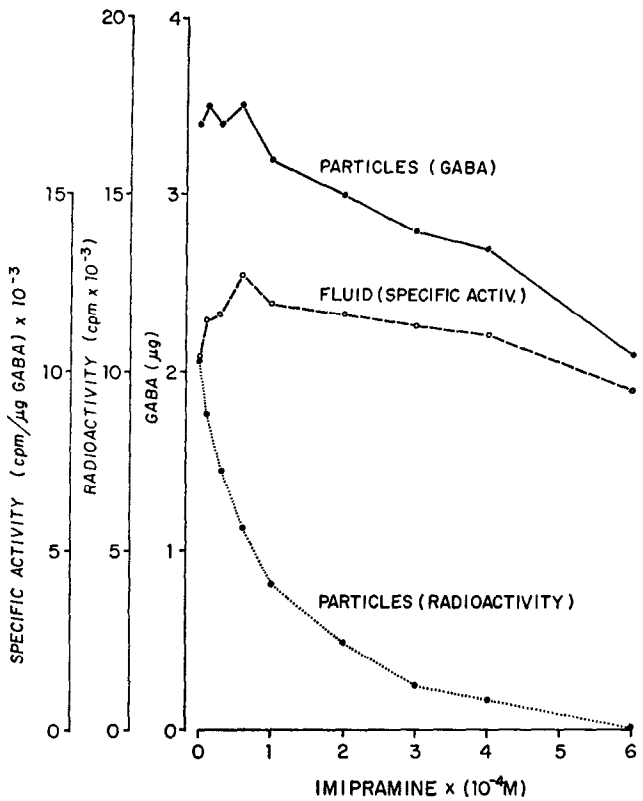

FIG. 3. The effect of a 30-min exposure to various concentrations of imipramine on the subsequent capacity of the particles to accumulate [^{14}C]-γ-aminobutyric acid within a 30-min interval. The γ-aminobutyric acid content of the particles and the specific activity (counts/min/μg) of the suspension fluid are also shown. One ml of the suspension (particles plus fluid) contained 5·9 μg GABA and a radioactivity of 36,200 counts/min.

practically a linear function of the drug concentration, in agreement with the previous experiments. Subsequent experiments showed this relationship to hold at drug concentrations, at least as high as $1{\cdot}35 \times 10^{-3}$ M. On the other hand, the ability of the particles to accumulate exogenous [^{14}C]-GABA was impaired by imipramine at even the lowest concentration tested (1×10^{-5} M) and further inhibited at progressively higher drug concentrations. The accumulation process was completely abolished with 6×10^{-4} M imipramine, a concentration at which the particles were found capable of retaining a considerable amount of preaccumulated isotope in the previous experiment (Fig. 2), even after 205 min of exposure to the drug. The decrease in isotope accumulation was a strikingly different function of imipramine concentration from that observed for isotope retention. The function was found by mathematical analysis to be a rectangular hyperbola.

Figure 3 also shows the specific activities calculated for the suspending fluid at the various drug concentrations. It has previously been shown[2] that the specific activity of the fluid declines as a function of time. The present data indicate that such a decline was progressively reduced by increasing concentrations of imipramine up to a level of

6×10^{-5} M, that is in the range where the GABA content of the particles did not appear to be affected by the drug. Further increases of the imipramine levels resulted in lower specific activity values, but only at 6×10^{-4} M did the value decrease below that of the control (no drug). Thus, it appears that the imipramine had inhibited the release of some endogenous, nonradioactive GABA from the particles. The complexity of such an effect is indicated by the fact that the specific activity rose to a maximum and then declined.

(3) *Comparison of the effects of imipramine, chlorpromazine and orphenadrine.* The previous experiments have shown that imipramine exerts two distinct effects on the exchange and net release of GABA by the particles. One is an inhibition of ability to accumulate exogenous [^{14}C]-GABA, an effect bearing a hyperbolic relationship to the concentration of the drug. The other is the decrease in GABA content of the particles which varied in an approximately linear fashion with drug concentrations above 1×10^{-4} M. Similar experiments to that described in Fig. 3 were carried out using two other psychotropic drugs, chlorpromazine and orphenadrine (Fig. 4), the latter

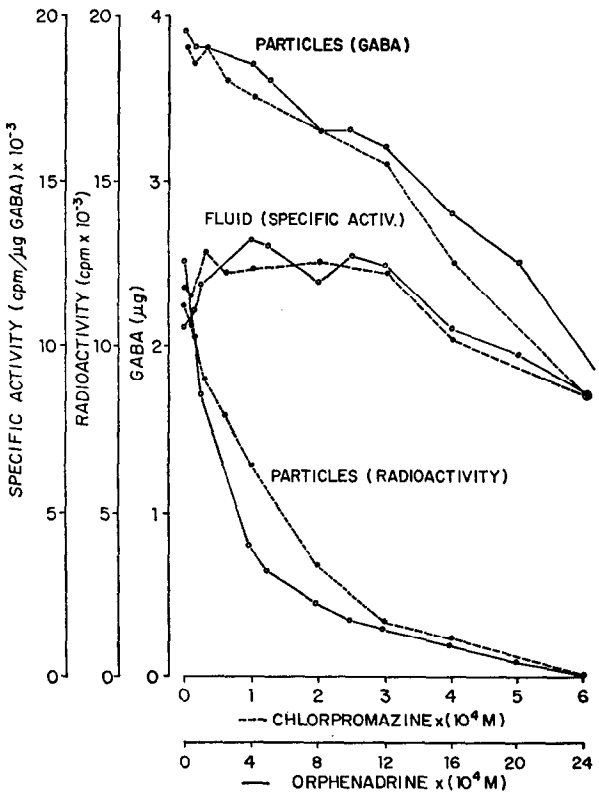

FIG. 4. The effect of a 30-min exposure to various concentrations of chlorpromazine or orphenadrine on the subsequent capacity of the particles to accumulate [^{14}C]-γ-aminobutyric acid within a 30-min interval. The γ-aminobutyric acid content of the particles and the specific activity (counts/min/μg) of the suspension fluid are also shown. In the chlorpromazine experiment, 1 ml of suspension contained 5·9 μg GABA and 35,900 counts/min. In the orphenadrine experiment there was 6·1 μg of GABA and 35,700 counts/min.

in a concentration range about 4-fold higher than that of the others. The effects of these two drugs were qualitatively similar to those of imipramine, both in terms of the hyperbolic inhibition of [^{14}C]-GABA accumulation and the approximately linear decline of particulate GABA. The specific activity of the suspending fluid again suggested some inhibitory effects of the drugs on GABA release, more conspicuous at the lower than the higher drug concentrations.

A rigorous quantitative comparison could not be made because of the differences in GABA and radioactivity levels in the control preparations from experiment to experiment. From the linear trends for endogenous GABA release one can calculate that a 50 per cent depletion of the particulate GABA would be induced, under the conditions of these experiments, at approximate concentrations of 8×10^{-4} M, 5×10^{-4} M, and $2{\cdot}4 \times 10^{-3}$ M for imipramine, chlorpromazine, and orphenadrine, respectively, showing chlorpromazine to be 1·6 times more effective than imipramine and 4·8 times more effective than orphenadrine. In contrast, a 50 per cent impairment of the ability to accumulate exogenous GABA was achieved by the three drugs at, respectively, $0{\cdot}7 \times 10^{-4}$ M, $1{\cdot}25 \times 10^{-4}$ M and $2{\cdot}4 \times 10^{-4}$ M, indicating that imipramine was 1·8 times more effective than chlorpromazine and 3·4 times more effective than orphenadrine. For each drug, this inhibitory effect was achieved at drug concentrations 4- to 11-fold lower than those required for the release effect.

(4) *The effects of* 1×10^{-4} M *imipramine on the two GABA pools of the particles.* It was previously demonstrated that the [^{14}C]-GABA which becomes associated with the particles at 2° in the presence of Na^+ is distributed between at least two pools. One pool is distinguished by the fact that its existence is Na^+-dependent and it exchanges at a relatively rapid rate with the nonbound GABA of the suspending fluid (rapidly exchanging pool, or REP), while the second pool is distinguished by a much slower rate of exchange (slowly exchanging pool, or SEP). The previously established interrelationship between these two pools[3] will be reviewed in the Discussion section. A method for estimating the size of the two pools by adding an excess of nonradioactive GABA has been previously described.[3]

[^{14}C]-GABA and imipramine (1×10^{-4} M) were introduced simultaneously into a freshly prepared saline suspension. Twenty and 150 min later, portions of the suspensions were centrifuged with and without the addition of the nonradioactive GABA for the determination of the two pools. The results of the analysis are shown in Table 1. The radioactivity and GABA content of the total suspensions (summed values from particles and suspending fluids) were in reasonable agreement among the four portions. At both time intervals the presence of 1×10^{-4} M imipramine resulted in a slight decrease of the GABA content of the particles and a marked inhibition of their ability to accumulate radioactivity. The specific activity of the suspension fluid was greater in the presence than in the absence of the drug. The radioactivity that could be rapidly displaced from the particles (REP) was considerably lower in the presence than in the absence of the drug and so was the calculated size of the REP. The size of the REP in both cases increased with time, the increase being less marked in the drug-treated sample. On the other hand, the SEP of the imipramine-treated particles contained more GABA and less radioactivity than did the control. The size of the SEP decreased as a function of time, but the SEP decline was slightly less pronounced in the presence of imipramine.

Previously, it has been shown[3] that the transfer of particles from a Na^+-containing

TABLE 1. EFFECTS OF 10^{-4} M IMIPRAMINE ON THE RAPIDLY EXCHANGING POOL (REP) AND SLOWLY EXCHANGING POOL (SEP) OF γ-AMINOBUTYRIC ACID IN THE PARTICLES

Time (min)	Drug concn. (10^{-4}M)	Total suspension (fluid + particles)		Suspension fluid			Particles					
							SEP + REP		SEP		REP	
		counts/min	GABA (μg)	counts/ min	GABA (μg)	counts/ min/μg	counts/ min	GABA (μg)	counts/ min	GABA (μg)	counts/ min	GABA (μg)
20	0·0	37,750	5·63	22,750	1·74	13,074	15,000	3·89	7250	3·30	7750	0·59
20	1·0	37,250	5·54	32,250	1·83	17,622	5000	3·71	2500	3·57	2500	0·14
150	0·0	37,000	5·45	21,500	2·46	8739	15,500	2·99	7500	2·08	8000	0·91
150	1·0	37,000	5·47	28,500	2·66	10,714	8500	2·81	5500	2·52	3000	0·29

to a Na^+-free medium inactivates the Na^+-dependent binding sites of the particles and thus abolishes their REP. Freshly prepared particles were incubated at 2° in buffered 0·2 M NaCl and [^{14}C]-GABA for 90 min to permit an accumulation of radioactivity into both pools of the particles. The particles were then sedimented, resuspended to the initial volume in buffered 0·2 M KCl and sedimented again. They now contained a radioactive SEP, but no REP. Such particles were resuspended, respectively, in 0·2 M KCl and 0·2 M KCl containing 1×10^{-4} M imipramine, and portions of these two suspensions were sedimented at various intervals so as to determine the relative rates of [^{14}C]-GABA efflux from the particles. The results are shown in Fig. 5. A considerable quantity of radioactivity was observed in the suspending fluids

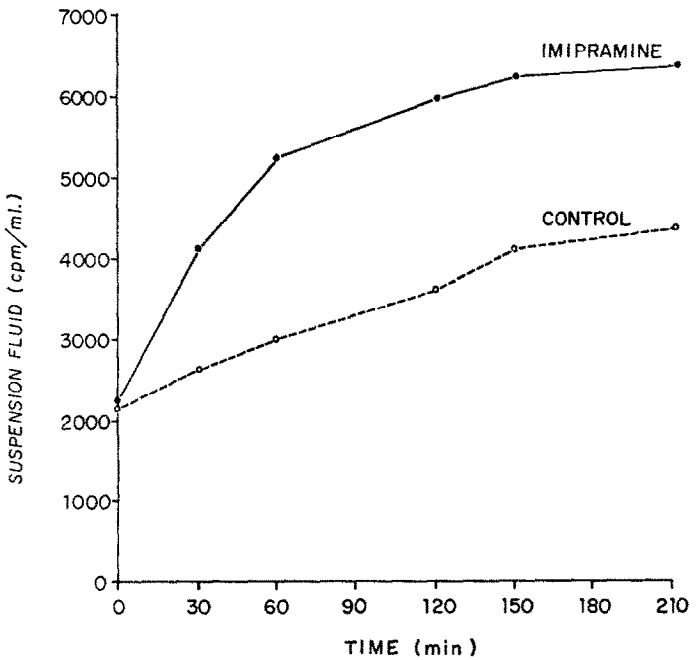

FIG. 5. The effect of 1×10^{-4} M imipramine on the release of [^{14}C]-γ-aminobutyric acid from particles suspended in 0·2 M KCl.

of both samples, even when no incubation was allowed to take place after the last resuspension. This appears to be radioactive GABA lost from the particles during the separation procedure, irrespective of the presence or absence of the drug. Additional release of [^{14}C]-GABA occurred with increasing incubation times. The presence of 10^{-4} M imipramine markedly accelerated this release. Thus, in the absence of Na^+, the drug enhanced the release of GABA from the SEP, an effect opposite to that observed in 0·2 M NaCl (Table 1), where the same amount of drug had resulted in retarding the loss of GABA SEP.

(5) *Equilibrium binding of imipramine.* The saline suspension was kept in the presence of various concentrations of [^{3}H]-imipramine at 2° for 30 min and then nonradioactive GABA was added, 0·75 μg/ml of final suspension. Thirty min later the particles were sedimented. This procedure simulated the general experimental conditions

described in section 2 of Results. The difference between the radioactivity of the suspension and of the supernatant was used to calculate the equilibrium binding of imipramine. See Methods.

Figure 6 depicts the amount of drug adsorbed as a function of the quantity of drug present in 1 ml of suspension. These values represent the distribution of the drug at equilibrium (see Methods). Up to a level of 2 μmoles of imipramine per ml of suspension there was no apparent evidence for saturation of all binding sites. On the other hand, it was previously noted that if the particles were exposed to 0·6 μmoles of

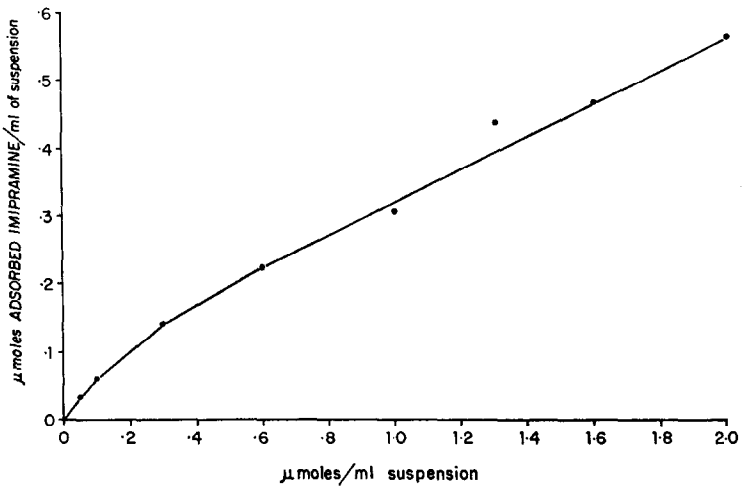

FIG. 6. Adsorption of imipramine as a function of the quantity of imipramine present in 1 ml of suspension.

imipramine per ml of suspension there was a complete inhibition in the subsequent ability of the particles to accumulate [^{14}C]-GABA, suggesting that the sites for the Na^+-dependent binding of GABA were completely inhibited. Therefore, any sites which are either directly or indirectly involved in the Na^+-dependent binding of GABA were presumably saturated by this concentration of the drug. Since imipramine binding continues to increase when the drug content was greater than 0·6 μmole per ml of suspension it would appear that there was more than one type of binding site for the drug.

The equilibrium binding of a substance is normally plotted as a function of the concentration of nonbound ligand in solution. This is shown in Fig. 7 (curve A). Since there was reason to believe that the linear slope in the high concentration range (supernatant concentrations of $3{\cdot}75 \times 10^{-4}$ M or more) was a composite made up of a class of sites that were saturated and another group which was not saturated, a commonly used procedure[8] was adopted to resolve the adsorption isotherm into its two presumed components. The observed linear slope in the high concentration range was used for the slope of a straight line which goes through the origin (Fig. 7, curve B). This is taken to represent the nonsaturating component. The difference between A and B represents the binding to the presumed group of high affinity sites which saturate and

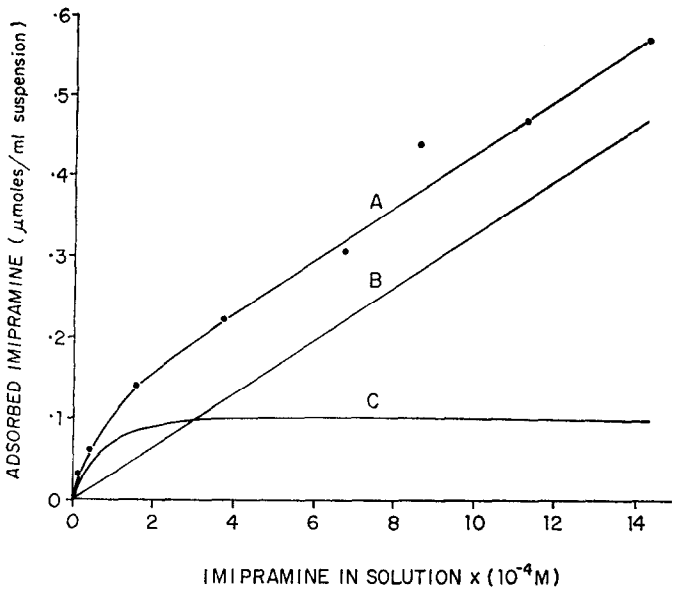

FIG. 7. Adsorption of imipramine as a function of the equilibrium concentration of nonbound imipramine (curve A) and its resolution into a linear (curve B) and nonlinear component (curve C).

is represented by curve C. This curve approximates a rectangular hyperbola. Saturation appears to be at a free drug concentration of approximately $3{\cdot}6 \times 10^{-4}$ M or about $5{\cdot}9 \times 10^{-4}$ M total drug concentration of the suspension. In this type of analysis the linear component, curve B, is assumed to represent binding to a group of low affinity sites in which only a small proportion of the total sites are occupied over the concentration range which was investigated. Under these limiting conditions the Langmuir equation for adsorption can be shown to reduce to a form in which the amount adsorbed is directly proportional to the concentration in the fluid.

It should be noted that curve A of Fig. 7 represents a slight underestimate of the amount bound. It was therefore of interest to apply a correction factor ($F = 0{\cdot}98$, see Methods) based on inulin space measurements and thus see if any significant shifts would occur in the shape of curve A and its resolution into curves B and C. It was found that the general shape was maintained but the slope increased slightly. Upon resolving this curve into its hypothetical components, curve C was identical to that shown in Fig. 7 and the linear component B had a negligibly greater slope. Thus, at the highest drug concentration which was investigated the apparent value for the amount bound in the hypothetical linear component shifted from a value of 0·49 μmoles (Fig. 7B) adsorbed to 0·51 μmole.

DISCUSSION

Previous reports[3–5] have dealt with the Na^+-dependent accumulation of [^{14}C]-GABA and release of endogenous GABA by brain subcellular particles at low temperature. It was demonstrated that membrane-bounded particles such as vesicular

microsomes, synaptosomes and mitochondria retain an appreciable amount of endogenous GABA.[5] In a sucrose or KCl medium the GABA of these particles did not exchange with [^{14}C-]GABA in the suspending fluid, indicating the existence of a permeability barrier for GABA. In the presence of Na^+ these particles accumulated [^{14}C]-GABA.[5] Further analysis[3] indicated that the [^{14}C]-GABA of the particles in the Na^+ containing medium was distributed between two pools. The two pools were apparently a constituent of each of the three types of particles. One pool of high specific activity was distinguished by the fact that its existence was Na^+-dependent and it exchanged at a relatively rapid rate with the nonbound [^{14}C]-GABA of the suspending fluid. This was designated the rapidly exchanging pool (REP). All of the remaining particulate GABA was assigned to a second pool which was referred to as the slowly exchanging pool (SEP). Changes in the size and specific activity of these two pools as a function of time as well as other types of evidence led to the following suggestions regarding the nature of these two pools and their relationship to each other: The REP apparently represented a Na^+-dependent binding of GABA to sites on the outer surface of the membranes of the particles. These sites functioned as mobile carriers which could slowly traverse the membrane in both directions and thereby mediate a slow net downhill efflux of GABA from the particles and an exchange between the [^{14}C]-GABA of the external medium and the endogenous GABA of the particles providing Na^+ was present. The SEP was viewed conceptually as including GABA-bound to Na^+-dependent sites in regions of the membrane other than the outer membrane surface of the particles plus nonbound GABA in the interior of the particles.

It is proposed that the described effects of imipramine on the GABA system may be explained on the basis of the over-lapping of two distinct actions of the drug and these effects are assumed to occur with all of the above particles. The first effect is an inibition of the Na^+-dependent binding of GABA to the membrane carrier sites. The second effect is a destruction of membrane integrity which permits a nonmediated release of GABA to occur. In the presence of low-drug concentrations, the effects on the binding of GABA and its consequences are the dominant factors to be considered. As the drug concentration is increased an increasing nonmediated release begins to exert a more significant effect. At sufficiently high drug concentrations none of the carrier sites are functional and the changes in GABA content of the particles as a function of drug concentration is solely a reflection of an increasing destruction of the general barrier properties of the membrane. The fitting of this interpretation to the results follows.

An analysis of the effects of 10^{-4} M imipramine on the REP and SEP (Table 1) illustrates the consequences of an inhibition of binding. Previous studies[3] demonstrated that the REP represents the Na^+ dependent binding sites and the present results illustrate that imipramine inhibits the binding to these sites. The expected consequences of this event are an inhibition of the mediated exchange between the [^{14}C]-GABA of the external fluid and the GABA which is located in the interior of the particle (SEP) and an inhibition of the rate at which the mediated net downhill efflux occurs from the SEP. All of these effects were observed. If there was an increased nonmediated release from the SEP because of breakdown of the membrane barrier by 10^{-4} M imipramine, this effect was presumably small and masked by the inhibition of the mediated release. To test this possibility the efflux of [^{14}C]-GABA from the SEP was

investigated after transferring the radioactive particles from NaCl to KCl (Fig. 5). Under these conditions a minimal number of Na^+ activated carriers would be involved in determining the net rate of efflux. Any differences which might then be observed should be attributed to an effect of 10^{-4} M imipramine on the nonmediated release of [^{14}C]-GABA from the SEP. The results shown in Fig 5 indicate that the drug had increased the nonmediated release. On the basis of these experiments it can be concluded that in the presence of Na^+ the 10^{-4} M imipramine apparently exerted two opposing effects on the release of GABA from the SEP, an inhibition of the mediated release and an increase in the nonmediated release. The former effect was dominant. The total GABA content of the particles was not grossly affected by this concentration of the drug because the decreased release from the SEP was offset by the smaller size of the REP. Finally the decreased size of the REP retarded the exchange as illustrated by the observation that the specific activity of the suspension fluid declined more slowly in the presence of the drug.

The effects of pretreatment of the particles with various concentrations of imipramine (see Fig. 3) can now be more readily analyzed. Preincubation with increasing concentrations of imipramine decreased the number of sites to which [^{14}C]-GABA could bind and subsequently accumulate in the interior of the particle. This phenomenon was reflected in the inhibition of the accumulation of [^{14}C]-GABA by the particles and was a hyperbolic function of the drug concentration. A complete inhibition of the Na^+-dependent binding and mediated flux occurred in the presence of 6×10^{-4} M imipramine. As the number of carrier sites diminished, the rate of exchange decreased and the specific activity of the suspension fluid increased. Beyond a drug concentration of $0{\cdot}6 \times 10^{-4}$ M this trend reversed. Presumably at this point the imipramine induced nonmediated release of GABA was sufficiently large so as to offset the effects of inhibiting the mediated exchange. The relatively stable level of GABA retention by the particles up to a drug concentration of $0{\cdot}6 \times 10^{-4}$ M imipramine is interpretated in terms of the loss of GABA binding sites being compensated for by the increased retention of GABA in the interior of the particle. Beyond this drug concentration, the nonmediated release of GABA became increasingly significant while further changes in REP size and concomitant inhibition of mediated efflux from the SEP became increasingly small. At high drug concentrations the total particulate GABA should therefore reflect with increasing accuracy the relationship between drug concentration and the degree of destruction of the membrane barrier. This effect appears to be linear with respect to drug concentration.

The effects of chlorpromazine and orphenadrine (Fig. 4) suggest that the mode of action of these drugs is analogous to that of imipramine. The finding that the relative abilities of the three drugs to prevent [^{14}C]-GABA accumulation was not in the same sequence as that observed for the net release of GABA would be compatible with the suggestion that the action of the drugs on the binding sites is distinct and separate from that on the membrane barrier.

The observation that imipramine is less effective in removing [^{14}C]-GABA from the particles than in preventing its accumulation (compare Figs. 1 and 3) may be explained in the following manner. If [^{14}C]-GABA is first permitted to accumulate in the REP and SEP it is then subject to the previously discussed factors which modify the release of GABA; it should then be found that concentrations of imipramine which completely eliminate the REP would still leave considerable quantities of radioactivity

in the SEP. On the other hand, pretreatment with a concentration of imipramine which completely destroys the mediated exchange also eliminates the mechanism by which [^{14}C]-GABA is trapped and accumulated in the interior of the particles.

The suggestion that the adsorption of radioactive imipramine to the particles may be resolved into a curvilinear component which appears to reach saturation at or slightly below the point where 6×10^{-4} M imipramine is introduced into the suspension and a linear component which did not reach saturation is of some interest. Imipramine, 6×10^{-4} M was the concentration at which complete inhibition of the Na^+-dependent binding of GABA occurred. In addition, the inhibition of the Na^+-dependent sites for GABA as well as this suggested component of the imipramine binding was described by grossly similar curvilinear functions. Thus, some but not necessarily all of the binding of the drug to this postulated group of sites would appear to be related to the Na^+-dependent GABA binding sites. The linear component of the imipramine adsorption curve would presumably include binding to sites that modify the structural integrity of the membrane. If this proposed hypothetical treatment and analysis of the drug binding data has any merit the following predictions should hold true. A comparison of the binding of chlorpromazine, orphenadrine and imipramine should all be resolvable into two components. The slope of the linear component which is assumed to be related to membrane destruction should differ for the three drugs. Chlorpromazine should have the steepest linear slope and orphenadrine the least steep slope. This prediction is based on the reported effects of these drugs on net GABA retention. A comparison of drug concentrations required for 50 per cent saturation of the postulated saturating component should fall in the following sequence of increasing drug concentrations: imipramine, chlorpromazine and orphenadrine. This predicted sequence is based on the reported different concentrations of drug required for a 50 per cent impairment in the ability of the particles to accumulate exogenous [^{14}C]-GABA.

In conclusion, it has been suggested how a group of drugs may alter the apparent permeability of the membrane to a substance in a complex manner. The interpretation presents an alternative to the model of Seeman[2] in which the membrane was viewed as going through a transition from its normal porous configuration with increasing drug concentration. Whether the interpretations presented in this paper will serve as a general model will require studies of mediated flux phenomena with other permeating molecules.

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