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COMMUNICATIONS

The binding of chlorpromazine to some fractions of homogenized rat brain

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Investigations of the binding of drugs to proteins have been carried out for a variety of reasons. Specific binding of drug to protein may be used to elucidate the nature and number of binding receptors (Pert et al 1974). Binding of drugs to serum albumin, the most widely investigated of generalized drug-protein interactions, has been studied both for the light it throws on the forces controlling such interactions (see e.g. Peters & Sjoholm 1978) and for the information it gives as to drug disposition in the body (Jusko & Gretsch 1976). We have been interested in factors which might influence the disposition of drugs in the brain, and report here preliminary findings on the binding of chlorpromazine to some fractions obtained from rat brain.

Adult male rats (Anglia Livestock, Wister CF HB) were killed by cervical dislocation, the brain rapidly removed and homogenized in isotonic phosphate buffer pH 7·4 at a ratio of buffer volume to brain weight of 2. The homogenates were centrifuged at 10 000 *g* for 30 min and the supernatants then dialysed against three changes of 1 litre isotonic phosphate buffer overnight. The temperature of the brain tissue was kept at 0–4°C. The supernatant remaining after dialysis against isotonic buffer is SSI (Table 1). 4 ml of a saturated ammonium sulphate solution was added, with stirring, to SSI, giving a final solution 32% saturated in ammonium sulphate. The precipitate formed (SP2) was separated from the supernatant (SS2) by centrifugation. A portion of the supernatant SS2 was made 66% saturated in ammonium sulphate by addition of further saturated ammonium sulphate solution. After centrifugation to remove the precipitate (SP3) a portion of the supernatant (SS3) was brought to 90% saturation by careful addition of solid ammonium sulphate and again centrifuged to give a trace of precipitate (SP4) and supernatant (SS4). The three precipitates obtained by this procedure were each resolubilized in isotonic phosphate buffer and the original dialysed supernatant and the six fractions assayed for protein content (Lowry et al 1951) and for their ability to bind chlorpromazine.

After homogenization and dialysis as above, acetone was added to the dialysed supernatant (SP1) to give a solution 20% w/v in acetone. The precipitate which formed (AP2) was collected by centrifugation while the acetone in the supernatant was evaporated by warming at 37°C. The resulting solution (AS2) was then divided into two portions, one of which was chilled before the addition of 59·3% w/v acetone, and the above procedure repeated to give a precipitate fraction (AP3) and a supernatant (AS3). Finally acetone sufficient to produce a 78·4% w/v solution was added and precipitate (AP4) and supernatant (AS4) separated. The three precipitates were each resolubilized in isotonic buffer. The original dialysed supernatant and the six fractions, free of acetone, were each assayed for protein content and for ability to bind chlorpromazine.

The binding assays were carried out by centrifugal filtration using CF20 centriflow cones (Amicon Ltd). The cones were charged with 3 ml of the appropriate fraction together with 3 ml of 2×10^{-5} M chlorpromazine (May & Baker) solution, the whole buffered at pH 7·4. The cones were centrifuged at 1500 rev min^{-1} for 15 min at 4°C. The solution passing through the cones was analysed spectrophotometrically for chlorpromazine, after checking that no protein had leaked

Table 1. Effect of ammonium sulphate fractionation on the binding of chlorpromazine to water soluble protein from rat brain. S = supernatant and P = precipitated fractions.

Fraction	$(NH_4)_2SO_4$ % saturation	Total protein (mg)	Prot. concn (mg ml^{-1}) for binding assay	$10^5 \times$ bound chlorpromazine (mol mg^{-1} protein)
SS 1	0	359·04	13·2	15·64
SS 2	32	106·62	3·15	73·92
SP 2		170·21	7·30	22·22
SS 3	66	60·82	0·84	98·19
SP 3		30·03	1·95	39·24
SS 4	90	10·56	0·10	34·01
SP 4		0·44	0·09	—

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through. A correction was made for cone-bound chlorpromazine. The cones vary widely in their ability to bind chlorpromazine. Those used were selected as having a low but reproducible binding ability.

The total protein content, protein concentration and amount of protein bound chlorpromazine following ammonium sulphate fractionation are shown in Table 1. Similar results for fractions prepared by acetone precipitation are shown in Table 2.

The values for chlorpromazine bound per mg protein shown in Table 2 indicate that as the acetone concentration increases the binding ability of the protein in solution decreases, while that of the precipitated protein is increased. Ammonium sulphate fractionation shows the opposite behaviour (Table 1); there is an increase in binding ability of the solute protein compared with the precipitates as the ammonium sulphate concentration is increased. It might be thought that the differences in amount bound per mg portion are caused mainly by the differences in protein concentration of the test solutions used. That this is not so is shown clearly by Table 3 where a hypothetical association constant K has been calculated on the assumption that all fractions are composed of the same protein of molecular weight 100 000 with one chlorpromazine binding site.

A number of authors have inferred, from the results of studies of the binding of phenothiazines to albumin, that the lipophilic nature of the bulk of the molecule is of importance in determining the degree of binding (Kriegelstein et al 1972; Perrin & Hulshoff 1976). The results from both methods of fractionation used in the present study imply that chlorpromazine is better bound to protein which is overall more hydrophilic; though such proteins may well have lipophilic regions within them which are essential to the binding process. It is always possible, of course, that fractionation is removing lipid which inhibits the binding.

Although the nature of the various fractions obtained from rat brain is not known it is certain that some albumin will be present. It would be expected that this would precipitate in fractions SP 3 and SP 4 (Cohn et al 1940) but the results suggest that albumin binding does not account for most of the chlorpromazine bound to rat brain protein.

We define the binding capacity of a fraction as the product of the third and fifth columns of Tables 1 and 2; that is, it is nominally the total number of moles of chlorpromazine that can be bound to protein within a given fraction. Bearing in mind the protein loss involved in each fractionation step, most of the stages in both ammonium sulphate and acetone fractionations show an overall conservation of binding capacity. However, the initial ammonium sulphate fractionation of the supernatant SS1 causes a three fold rise in binding capacity which suggests the removal of an inhibitor of chlorpromazine binding at this stage.

Specific binding of drug to protein would be expected to show a very low concentration of drug very highly bound. Thus, for the opiate drugs specific binding of the order of 0·025 p mol mg^{-1} of brain protein is observed (Simon 1979). Binding of chlorpromazine reported here is three to four orders of magnitude greater than this, while the association constants calculated (Table 3) do not show a very tight binding. Thus the interaction between chlorpromazine and the various brain fractions would appear to be non-specific.

The data presented in Tables 1–3 indicates that, despite the favourable lipophilicity of the drug, chlorpromazine binding to brain lipid and lipoprotein is less favourable than its binding to more hydrophilic species. Penetration of drug molecules from the general circulation to the brain is subject to the blood-brain barrier. Crone & Thompson (1970) have pointed out that general capillary cells allow diffusion of drug molecules in and out via clefts in the cell membrane. However, brain capillary cells have no such clefts, so that drug must enter the cell membrane in order to reach the brain. It is often assumed that only lipophilic drugs can enter the brain, presumably because the ready solubility of such drugs in the cell membrane will help drug transport across it. However any binding which occurs within the brain will itself facilitate transport across the barrier

Table 2. Effect of acetone fractionation on the binding of chlorpromazine to water soluble protein from rat brain. S = supernatant and P = precipitated fractions.

Fraction	% Acetone (v/v)	Total protein (mg)	Protein concn (mg ml^{-1}) used	10^{7} × chlorpromazine bound (mol mg^{-1} protein)
AS 1	0	112·56	13·4	15·64
AS 2	20	91·38	10·75	11·715
AP 2		4·73	0·43	7
AS 3	59·3	35·22	2·38	1·0
AP 3		22·95	2·125	56·11
AS 4	78·4	23·09	1·8	14·53
AP 4		11·34	1·4	22·38

Table 3. Hypothetical association constants for binding of chlorpromazine to protein in fractions of rat brain. Calculated on the assumption that all the fractions contain the same single protein of mol. wt 10^5 and with one binding site.

Fraction	K × 10^{-3} litre mol^{-1}	Fraction	K × 10^{-4} litre mol^{-1}
SS 1	1·10	AS 1	1·10
SS 2	8·57	AS 2	0·79
SS 3	14·70	AS 3	0·06
SS 4	2·56	AS 4	0·96
SP 2	1·62	AP 2	3·95
SP 3	3·15	AP 3	5·26
SP 4	—	AP 4	1·57

membranes by increasing the concentration gradient. The results reported here show that non-specific binding of chlorpromazine to brain constituents can occur, and will therefore account to some extent for the distribution of drug to the brain.

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Clozapine's anti-acetylcholine property modulates its antistereotypic action in the mesolimbic sytem

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Clozapine is a unique neuroleptic agent. Compared with the classical neuroleptics such as haloperidol, it possesses strong anti-acetylcholine properties (Miller & Hiley 1974; Snyder et al 1974) and lacks antistereotypic and cataleptic actions in animals (Stille et al 1971) as well as extrapyramidal side effects in man (Simpson 1974). In an attempt to explain this, it has been suggested that the neuroleptic has a selective antidopaminergic effect on the mesolimbic system (Andén & Stock 1973; Zivkovic et al 1975; Bartholini 1976). Since the latter system seems also to be associated with (+)-amphetamine-induced stereotypies (Pijnenburg et al 1975; Costall et al 1977), we have studied the action of clozapine alone or in combination with various acetylcholine-like and anti-acetylcholine agents given bilaterally into the nucleus accumbens septi on (+)-amphetamine-induced stereotyped behaviour. The action of central injections of low doses of haloperidol alone into the same region on (+)-amphetamine stereotypies has also been investigated.

Male Wistar rats, 200–250 g, were used in groups of 4–6, anaesthetized with halothane and implanted bilaterally with stainless steel cannulae into the nucleus accumbens septi (A = 9, V = 2, L = ± 1·5) (De Groot coordinates). 1–2 weeks after surgery the animals were challenged with a submaximal stereotypic dose of (+)-amphetamine sulphate (SKF) (3 mg kg^{-1} i.p.). After 20 min of (+)-amphetamine treatment, rats were restrained manually and given an intracerebral injection of either 0·9% NaCl (saline) or vehicle (control groups), haloperidol (Searle), clozapine (Sandoz) or a combined injection of clozapine together with oxotremorine sesquifumarate (Aldrich), physostigmine salicylate (BDH), scopolamine HBr (BW) or atropine sulphate (BDH). A 10 μl Hamilton syringe was used to deliver 2μl bilaterally at a rate of 1μl min^{-1}. All drugs were dissolved in saline except haloperidol (Serenace ampoules), and clozapine which was dissolved in saline acidified with 0·1 M HCl, and adjusted to pH 3·5 with 0·1 M NaOH.

Stereotypies were scored in individual animals at 15 min intervals for 90 min after the intracerebral injection according to the following rating scale: 1 = periodic mild sniffing, 2 = continuous sniffing, 3 = intermittent licking, gnawing or biting, 4 = continuous licking, gnawing or biting. Animals were used once and the site of injection was confirmed histologically. Statistical analysis was by the Mann-Whitney U-test.

Bilateral application of haloperidol, 2·5–5 μg into the nucleus accumbens septi (NAS) effectively antagonized (+)-amphetamine stereotypies (Fig. 1a). The antagonism appeared most pronounced at 45–90 min during which time very little stereotypic activity was observed. The application of 10 μg of clozapine into the same site produced no significant change in the stereotyped behaviour, while increasing the dose to 25 and 50 μg produced a potentiation (Fig. 1b), at both 75 and 90 min after injection.

The combination of 25 μg of clozapine together with the cholinergic agents oxotremorine (5 μg) or physostigmine (10 μg) effectively antagonized the stereotypies (Fig. 2a). The antagonism was rapid and lasted throughout the scoring period during which the animals appeared similar to those pre-treated with haloperidol. On the other hand, the combination of a subthreshold

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