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BINDING OF [^{3}H]PHENYCYCLIDINE TO RAT AND HUMAN BLOOD CONSTITUENTS

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Abstract—The binding of [^{3}H]phencyclidine (PCP) to rat serum and human plasma was studied using equilibrium dialysis. [^{3}H]PCP bound with a relatively low affinity to both rat serum ($K_D = 1.5 \times 10^{-5}$ M) and human plasma ($K_D = 6.2 \times 10^{-6}$ M). However, the binding capacity was quite large for rat serum (5.7 nmoles/ml) and human plasma (5.6 nmoles/ml). Binding was readily reversible as shown by the efflux of [^{3}H]PCP from a dialysis bag containing the rat serum–drug complex. In addition, the [^{3}H]PCP–human serum complex appeared to dissociate completely when analyzed by Sephadex gel filtration chromatography. The low affinity of PCP for serum appeared to account in large part for the high tissue-to-plasma ratios that are observed in animals and humans injected with this drug. *In vitro* equilibration of [^{3}H]PCP between rat serum and tissue homogenates resulted in at least a 10-fold accumulation of [^{3}H]PCP in the homogenates. [^{3}H]PCP was found to bind weakly to the major protein components of human serum (macroglobulins, immunoglobulins and albumins). The weak nature of the binding to serum proteins coupled with the relatively high capacity of binding probably account for the failure of other drugs to compete for PCP binding.

Phencyclidine (PCP) is a dissociative anesthetic that is commonly abused because of the psychological effects it produces. The prevalent misuse of PCP has created the problem of treating individuals suffering from untoward side effects or overdose [1]. The diagnosis of PCP intoxication is complicated by several factors, chief among which is the symptomatology itself. PCP produces multiple symptoms that not only vary with intensity but are frequently difficult to discern from some abnormal mental states [2, 3]. PCP is often co-abused with other drugs [4] which may contribute to some of its pathological effects. Additionally, the diagnosis of PCP intoxication has been complicated by the fact that blood and plasma levels of PCP span a broad range in individuals that are seemingly equally intoxicated [4–6]. Human postmortem tissues reveal that tissue levels far exceed blood levels of PCP and that tissue-to-blood ratios vary widely [7]. Probably, human plasma levels of PCP are not always a reflection of brain levels, particularly at extended periods of time after administration. A good correlation between brain and plasma levels exists for PCP in mice after i.v. administration but not after i.p. or p.o. administration [8]. The reason for high tissue-to-plasma ratios for PCP in laboratory animals and humans has not been established. It may be that PCP has a higher affinity for tissue than for plasma. Conversely, PCP may have a very low affinity for plasma proteins thereby making it readily available for tissue penetration. Low affinity for plasma proteins would suggest that the alterations in the concentration of plasma proteins in various pathological conditions or competition by other drugs for protein binding would not affect the biodisposition of PCP to a major extent. The studies reported herein were undertaken in order to measure the binding affinity of PCP to plasma and serum proteins, to examine the partitioning of PCP between sera and tissue homogenates *in vitro*, and to identify plasma constituents to which PCP binds.

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MATERIALS AND METHODS

Drugs. [^{3}H]PCP, tritiated in carbon 3 of the benzene ring (sp. act. 5.8 Ci/mmole) was obtained in toluene–ethanol (9:1) from the Research Triangle Institute (Research Triangle Park, NC, U.S.A.) as authorized by the National Institute on Drug Abuse. The compound was over 95% pure as determined by radioscan after thin-layer chromatography. [^{3}H]PCP was diluted with PCP to provide the amount of radioactivity per sample as indicated below. Δ^9-Tetrahydrocannabinol, morphine, cocaine, 4-phenylpiperidinocyclohexanol (C-4-OH-PCP), 1-(1-phenylcyclohexyl)-4-hydroxypiperidine (P-4-OH-PCP) and PCP were also supplied by the National Institute on Drug Abuse. 1-Phenyl-1-cyclohexene-3-ol (PC-3-ol), 1-phenyl-1-cyclohexene-3-one (PC-3-one) and 1,2-dihydroxy-1-phenylcyclohexene (PC-1,2-diol) were synthesized as described previously [9].

Materials. Sephadex G-200 (40–120 μm, 30–40 ml bed vol./g dry gel) was obtained from Pharmacia (Uppsala, Sweden). Gravity flow columns (5.0 × 100 cm) were used for gel filtration chromatography (Glenco Scientific, Houston, TX, U.S.A.). The dialysis tubing used was 16 mm Spectrapor membrane tubing (Arthur H. Thomas Co., Philadelphia, PA, U.S.A.).

Methods. The gel filtration chromatography was carried out at a room temperature of 20 ± 3°. Samples (2 ml) of human serum previously incubated for 2 hr at room temperature with [^{3}H]PCP (10^{-6} M) were applied to a Sephadex G-200 chromatographic column. Absorbancies were detected with an ISCO UA-5 absorbance monitor and recorder (Instrumentation Specialties Co., Lincoln, NE, U.S.A.). The eluant was counted in tubes in an automatic fraction collector. Radioactivity was conducted in a Beckman liquid scintillation counter (Beckman Instruments, Palo Alto, CA, U.S.A.), and quench was corrected by external standardization.

Human blood was collected in vacutainers, either with or without sodium heparin (Becton-Dickinson, Rutherford, NJ, U.S.A.), from non-fasting, apparently healthy individuals with no current or past history of drug abuse. When rat serum was desired, blood was collected via decapitation, in the absence of anticoagulants, allowed to clot at room temperature, and centrifuged immediately thereafter.

Equilibrium dialysis was used to determine percent binding of [^{3}H]PCP to protein. One procedure allowed for serial sampling whereas the other technique did not. The basic dialysis apparatus for serial sampling has been described before [10] and consisted of a magnetic stirrer, a stirring bar, dialysis bag, a short glass tube (inserted in a rubber stopper) to which the dialysis bag was fastened with surgical silk, and a 250-ml graduated cylinder to hold the dialyzing solution. The opening made by the glass tube allowed the serial removal of serum aliquots at various time intervals. The dialysis tubing used in these experiments was placed in a 1% EDTA solution in water, heated for 1 hr to 80°, and kept soaked in a 0.1% EDTA solution until used. Dialysis was carried out at 20 ± 3° by adding 20 ml of the phosphate-buffered saline (150 mM NaCl, 1 mM KH_2PO_4, 5 mM K_2HOP_4) to the graduated cylinders along with the appropriate concentration of [^{3}H]PCP. Two milliliters of sample was pipetted inside the bag and equilibration was allowed to proceed. Serial sampling in duplicate of both buffer (1.0 ml) and sample (50 μl) provided a time course for protein binding. The amount of [^{3}H]PCP bound to serum at equilibrium was determined by subtracting the free concentration (buffer) from the concentration in the dialysis tubing (bound plus free). The other technique was designed for sampling at only one time point, e.g. under equilibrium conditions. Human plasma (0.5 ml) was added to a dialysis bag which was sealed and placed in a test tube containing 45 ml of phosphate-buffered saline. [^{3}H]PCP (0.1 μCi) was added to the buffer, and the tube was sealed with a Teflon screw cap. The test tubes were placed on a platform rocker (Searle Buchler Instruments, Fort Lee, NJ, U.S.A.) and allowed to shake for 24 hr at 20 ± 3°. Duplicate samples of plasma (50 μl) and buffer (0.1 ml) were removed for scintillation counting.

The latter method for equilibrium dialysis was altered slightly in order to investigate the partitioning of [^{3}H]PCP between rat serum proteins and tissue homogenates. Sera were dialyzed against four different tissue homogenates (liver, lung, brain and adipose tissue). Ten milliliters of tissue homogenate (100 mg protein for liver, lung and brain, and 25 mg for adipose tissue) was placed in 15 ml test tubes. Serum containing an equivalent amount of protein was added to each dialysis tubing along with [^{3}H]PCP. The test tubes were sealed with teflon screw caps, placed on the platform rocker, and allowed to shake for 24 hr at 10°. Duplicate samples of serum (100 μl) were counted directly for radioactivity, whereas the tissue homogenates were solubilized (NCS reagent, Amersham Searle, Arlington Heights, IL, U.S.A.) prior to scintillation counting.

The distribution of [^{3}H]PCP between red blood cells (RBC) and plasma was studied by incubating 2 ml of whole human blood at 20 ± 3° with the desired amount of drug (1.0 μCi/2 ml blood) for 1 hr. At the end of the incubation, an aliquot of whole blood was taken; the tubes were centrifuged and an aliquot of plasma was removed for determination of radioactivity. The amount of [^{3}H]PCP in RBC was calculated using the following relationship: dpm/ml RBC = (dpm/ml blood − dpm/ml plasma × [1-hematocrit]) ÷ hematocrit.

Hematocrits were determined from a sample of blood from each donor. The blood was drawn into a capillary tube and spun for 5 min in an International Microcapillary centrifuge, model M.B. (International Equipment Co., Needham Heights, MA, U.S.A.), and the hematocrit was determined.

For the partial characterization of the PCP-binding fraction of plasma, chromatographic columns (Sephadex G-200, Pharmacia, 5 × 100 cm) were used to fractionate 2 ml of human serum that had been incubated with [^{3}H]PCP for 2 hr at 37°. Proteins were eluted from the column with phosphate-buffered saline. Fractions (2 ml) were collected and subjected to liquid scintillation counting. Proteins of known molecular weights (Sigma Chemical Co., St. Louis, MO, U.S.A.), were used as standards in the calibration of the chromatographic columns.

Quantitative protein determinations were made by Sutherland's [11] modification of the Folin reaction. Spectrophotometric measurements were done in a model 635 Varian UV-Vis spectrophotometer (Varian Associates, Palo Alto, CA, U.S.A.).

RESULTS

Equilibrium dialysis of rat serum. The purpose of the first experiment was to establish the time required to reach equilibrium between rat serum and phosphate-buffered saline. [^{3}H]PCP was added to the buffer to make a 10^{-9} M solution. One-half of the cylinders also received PCP (10^{-4} M). The time required to reach equilibrium was determined by removing aliquots from the dialysis bag and dialyzing buffer at various times. The plateau for total [^{3}H]PCP binding to rat serum occurred at 12 hr as shown in Fig. 1 and remained unchanged at 24 hr. In addition,

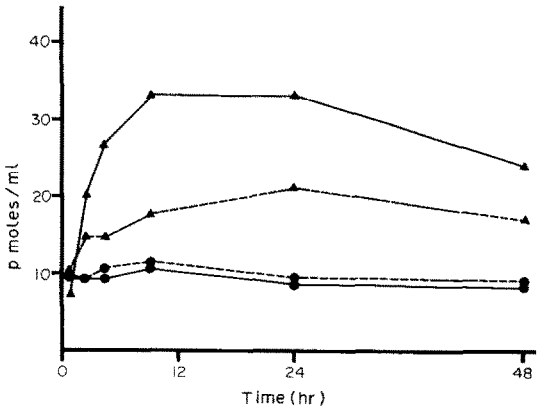

Fig. 1. Time course for the saturation of [3H]PCP (10^{-8} M) with serum proteins in the presence (- - -) and absence (——) of PCP (10^{-4} M). [3H]PCP and PCP were added to the buffer, and aliquots of serum (▲) and buffer (●) were removed at the indicated times. Averages of triplicate samples are presented.

a large excess of unlabeled PCP reduced [3H]PCP binding by approximately one-third at 24 hr. The experiment was repeated with various concentrations of [3H]PCP. The time course of binding was similar to that shown in Fig. 1, regardless of the [3H]PCP concentration. However, the amount bound varied as the [3H]PCP concentration was changed. The amount bound at 24 hr for all concentrations was analyzed by Scatchard plots, and the results are presented in Fig. 2. [3H]PCP apparently has a rather low dissociation constant ($K_D = 1.5 \times 10^{-5}$ M) for serum proteins; however, the capacity for binding is quite large (5.7 nmoles/ml).

Equilibrium dialysis was also performed by adding [3H]PCP to the serum, rather than to the dialyzing buffer, in order to verify the method as well as the time required to reach equilibrium. The results in Fig. 3 show that [3H]PCP readily dissociated from serum proteins with approximately 80% appearing in the buffer within 4 hr. As shown before, equilibrium was established at 24 hr and the ratio of bound to free drug was similar to that in Fig. 1.

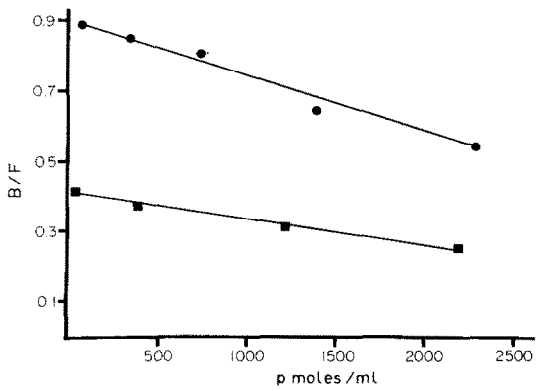

Fig. 2. Scatchard plots for the binding of [3H]PCP to rat serum (■) or human plasma (●). [3H]PCP (10^{-9} to 10^{-6} M) was dialyzed with the sample in the presence and absence of 10^{-4} M PCP. Binding in the presence of 10^{-4} M PCP was subtracted from total binding. The amount bound was determined after 24 hr of dialysis.

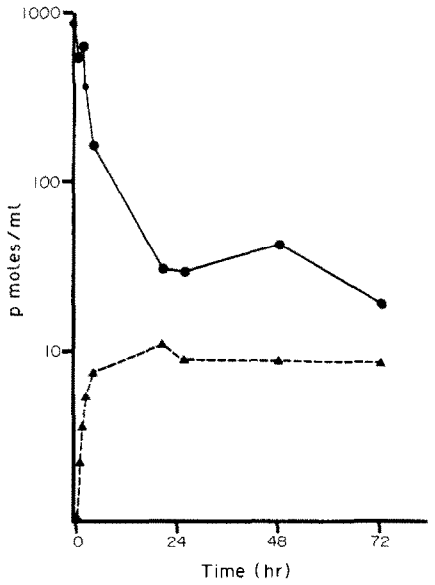

Fig. 3. Time course of the equilibrium dialysis of [3H]PCP bound to serum proteins. [3H]PCP (2 nmoles) was added to 2 ml serum in the dialysis tubing and dialyzed against 200 ml buffer. Aliquots of serum (●—●) and buffer (▲- - -▲) were removed at the indicated times. Averages of triplicate samples are presented.

To examine the partitioning of [3H]PCP between serum and tissue, dialysis was carried out with serum (inside the dialysis tubing) against tissue homogenates. The ratio of tissue-to-serum radioactivity (Table 1) demonstrates that the binding of [3H]PCP to all tissues was greater than that to serum. Not too surprisingly, adipose tissue exhibited the greatest degree of binding. Similar results were obtained when these experiments were carried out by placing the tissue homogenate inside the dialysis bag and the serum (diluted with buffer) outside the bag.

Several drugs were investigated for their abilities to compete with [3H]PCP for binding to rat serum proteins (see Table 2). PCP at 10^{-4} M reduced the binding of [3H]PCP (10^{-8} M) by 73%, whereas 10^{-6} M PCP reduced it by 24%. Two metabolites of PCP (C-4-OH-PCP and P-4-OH-PCP) were almost as effective as PCP in competing with [3H]PCP for binding. Metabolites of phenylcyclohexene (PC-3-ol, PC-3-one, and PC-1,2-diol) were not effective in reducing [3H]PCP binding. Ketamine, at 10^{-4} M, displaced [3H]PCP binding by 24% but had almost no effect when the concentration was lowered to 10^{-6} M. Several other drugs of abuse were also investigated for potential competition with [3H]PCP binding. Nicotine, diazepam, cocaine and amphetamine at a concentration of 10^{-4} M competed with [3H]PCP to a modest degree but lower concentrations (10^{-6} M) of these agents did not reduce binding.

Binding to human serum and plasma. The results presented in Table 3 indicate that [3H]PCP is not evenly distributed throughout whole blood. The levels in plasma were almost twice as high as those in red blood cells for all six subjects. The ability of human plasma to bind [3H]PCP was evaluated by

Table 1. Partitioning of [^{3}H]PCP between serum and tissue homogenates*

Tissue homogenate	Tissue homogenate-to-serum ratios	
	In vitro	*In vivo*
Liver	11.7 ± 0.1	18
Lung	11.1 ± 0.4	16
Brain	11.9 ± 0.3	6
Adipose tissue	50.7 ± 1.1	25

* Tissue homogenate-to-serum ratios were determined *in vitro* from the total radioactivity (free plus bound) per ml of serum and tissue homogenate at equilibrium. Means ± S.E.M. of four experiments are presented. Each experiment was carried out in triplicate. The tissue-to-serum ratios were determined *in vivo* 30 min after i.v. administration of [^{3}H]PCP (10 mg/kg).

Scatchard analysis as described above for binding to rat serum. The K_D was estimated to be 6.2×10^{-6} M and the maximum capacity of binding was 5.6 nmoles/ml (Fig. 2). The nature of the [^{3}H]PCP binding to human serum is illustrated by the results in Fig. 4 (a representative plot of six experiments). Four peaks were apparent from the gel chromatogram. These have been identified in their order of elution as follows: macroglobulins and lipoproteins, 7S globulins and immunoglobulins, albumins and globulins of similar size, and lastly peptides and amino acids. Greater than 90% of the radioactivity was recovered as unbound [^{3}H]PCP which shows that binding to proteins is weak and highly reversible.

Failure to identify a significant protein–[^{3}H]PCP complex by gel filtration led us to alter the conditions in order to attenuate dissociation of [^{3}H]PCP binding. In this instance, Sephadex G-200 columns were equilibrated with phosphate-buffered saline containing [^{3}H]PCP prior to the addition of the [^{3}H]PCP–serum sample (Fig. 5). The sample was eluted with buffer containing [^{3}H]PCP. Of the total radioactivity recovered, 2.3 ± 0.3% (mean ± S.E.) corresponded to the first peak (macroglobulins and lipoproteins), 3.7 ± 0.2% corresponded to the second peak (7S globulins and immunoglobulins) and 8.7 ± 0.3% eluted with the third peak (albumins). The remaining radioactivity was recovered as unbound PCP. These values for bound radioactivity in each individual peak were the average values for six different gel chromatographic experiments.

DISCUSSION

Equilibrium dialysis was used to demonstrate PCP binding to constituents of plasma and serum. Scatchard analysis of binding to rat serum revealed that PCP had very low affinity for these proteins but that there was a large binding capacity. The weak nature of the PCP binding to the serum proteins is consistent

Table 2. Competition between [^{3}H]PCP (10^{-8} M) and other drugs for binding to rat serum proteins*

Drugs	Concn (M)	% Displacement
PCP	10^{-4}	73
PCP	10^{-6}	24
C-4-OH-PCP	10^{-4}	65
P-4-OH-PCP	10^{-4}	46
Phenylcyclohexene carbonitrile	10^{-4}	0
Phenylcyclohexene	10^{-4}	0
PC-3-ol	10^{-5}	0
PC-3-one	10^{-5}	9
PC-1,2-diol	10^{-5}	22
Ketamine	10^{-4}	24
Ketamine	10^{-6}	6
Diazepam	10^{-4}	33
Diazepam	10^{-6}	0
Nicotine	10^{-4}	30
Nicotine	10^{-6}	0
Morphine	10^{-4}	15
Morphine	10^{-6}	5
Pentobarbital	10^{-4}	0
Δ^9-Tetrahydrocannabinol	10^{-4}	9
Cocaine	10^{-4}	19
Cocaine	10^{-6}	0
Amphetamine	10^{-4}	17
Amphetamine	10^{-6}	10

* Experiments were carried out at least twice and average percent displacement is presented.

Table 3. Distribution of [^{3}H]PCP between red blood cells (RBC) and plasma in human subjects

Subject	Hematocrit	Blood	Plasma	RBC	RBC/Plasma
1	47	176	106	70	0.66
2	45	183	114	69	0.61
3	47	167	105	62	0.59
4	49	182	111	71	0.64
5	44	167	97	71	0.73
6	48	180	106	74	0.69

with the high tissue-to-plasma ratios found in mice and rats *in vivo* [8, 12, 13]. For example, the brain-to-plasma ratio of PCP is approximately 4 within minutes after i.v. administration of PCP [8]. PCP also readily dissociates from rat serum proteins as demonstrated by the efflux of [^{3}H]PCP from the dialysis bag containing rat serum. Although it appears that the high tissue-to-plasma ratio of PCP resulted in large part from low affinity for plasma proteins, it is possible that an active transport system could be involved in the rapid transfer of PCP from plasma to tissues *in vivo*. However, when [^{3}H]PCP was partitioned between rat serum and rat tissue homogenates *in vitro*, [^{3}H]PCP accumulated in the homogenates of all tissues which suggested that an active transport system was not responsible for high tissue-to-plasma concentrations of PCP. The extent to which PCP accumulated in adipose tissue was to be expected in light of the high lipid : water partition ratio of PCP and its low affinity for plasma proteins.

There is always concern that other agents can alter plasma binding of a drug and thereby alter the pharmacokinetics of the drug. Two major metabolites of PCP are C-4-OH-PCP and P-4-OH-PCP [14]. It has also been reported that a major portion of PCP is converted to phenylcyclohexene (PC) during smoking [15] and that this latter compound is metabolized to form several products [9]. Moreover, PCP is frequently abused with other drugs [4, 6]. The above-mentioned agents were tested for their abilities to compete with PCP for serum binding. The metabolites of PCP were effective in competing for binding but high concentrations were required before a significant amount of displacement occurred. This latter observation supports the finding that there was a large capacity for PCP binding in serum. The metabolites probably have little effect on PCP binding *in vivo* since their plasma concentrations are usually quite low [8]. Phenylcyclohexene carbonitrile (PCC), a frequent contaminant of PCP [16], appeared to not interfere with PCP binding. Its inability to compete with PCP binding may have been due to its lability in aqueous solutions [16]. Although the pyrolysis product, PC, is relatively stable in an aqueous medium, it also had little effect on PCP binding. These data suggest that, in spite of the fact that PC and PCP blood levels may be comparable after smoking [17], plasma bound PCP was unaffected by either PC or its metabolites. The drugs that are sometimes abused with PCP were practically ineffective in displacing PCP. Their lack of interaction with PCP binding was most likely due to the low affinity of PCP for sites that are present in abundant quantities.

In the evaluation of protein–drug interactions, protein–drug affinities, fractional binding of the drug and the size of the protein to which the drug is bound are all important considerations. Analysis of the data on PCP binding to human plasma by the method of Scatchard [18] gave results similar to those found using rat serum, i.e. PCP had low affinity to sites with high binding capacity. As with binding to rat serum, low affinity for plasma proteins coupled with its high lipophilicity could account for the rapid entry and sequestration of PCP in tissue. The fractional binding to human plasma was approximately 50% at a PCP concentration of 5×10^{-7} M. Giles *et al.* [19] reported fractional binding to human plasma

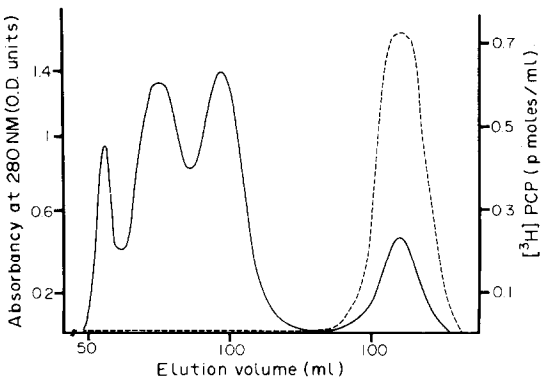

Fig. 4. Elution pattern of 2 ml of human serum that had been equilibrated with 10 pmoles of [^{3}H]PCP for 2 hr at 37° prior to chromatography on a Sephadex G-200 column. Protein (——absorbancy at 280 nm) and radioactivity (- - -) were eluted from the column with buffer.

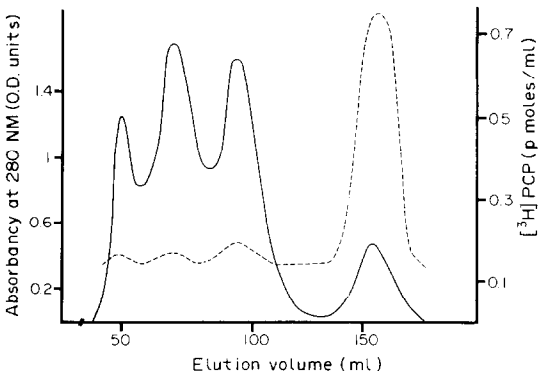

Fig. 5. Elution pattern of 2 ml of human serum (equilibrated with 10 pmoles of [^{3}H]PCP for 2 hr at 37°) from a Sephadex G-200 column with buffer containing 0.1 nM [^{3}H]PCP. Key: absorbancy (——); radioactivity (- - -).

proteins to be somewhat higher (~78%) at 4×10^{-7} M PCP. It should be noted that plasma was dialyzed against 4 vol. of buffer in their experiments rather than 200 vol. as in our experiments. Both studies support the notion that PCP plasma binding is readily reversible, which was unexpected due to the fact that PCP is a lipophilic drug. Most lipophilic drugs bind strongly to plasma proteins. Our experiments with gel chromatography (Fig. 4) demonstrated just how reversible the binding was, in that the protein–PCP complex dissociated completely. As mentioned above, the size of the protein to which PCP is bound may be an important consideration in that the rate of transcapillary passage of the bound drug is dependent on the size of the protein. The Scatchard plot suggests that albumin may be involved in binding due to the fact that each ml of human plasma bound approximately 6 nmoles of PCP. Albumin could easily accommodate PCP since there are approximately 600 nmoles of this protein per ml plasma. Identification of the protein–drug complex was made difficult by the weakness of the association. Elution of the bound drug from gel chromatography columns with buffer containing the ligand, a technique that has been used previously for identification of weak complexes [10, 20], enabled us to show some binding to macroglobulins and lipoprotein and to immunoglobulins as well as to albumins. The major portion of binding was to the latter. These data suggest that very little transcapillary passage of bound PCP occurred because high molecular weight proteins are involved in PCP binding.

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