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A Spectrophotometric Method for the Analysis of Chlorpromazine and Related Phenothiazines in Biological Tissues: Its Application to the Study, in Vivo and in Vitro, of Tissue Distribution of Chlorpromazine

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A SPECTROPHOTOMETRIC METHOD FOR THE ANALYSIS OF CHLORPROMAZINE AND RELATED PHENOTHIAZINES IN BIOLOGICAL TISSUES. ITS APPLICATION TO THE STUDY, IN VIVO AND IN VITRO, OF TISSUE DISTRIBUTION OF CHLORPROMAZINE

by

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LIFE

BERNARD GOTHELF was born on May 8, 1928 in Chicago, Illinois.

He was graduated from the John Marshall High School, Chicago, in January 1946. He majored in Chemistry at the University of Illinois, Urbana, and was graduated with his B.S. in 1950. He received his M.S. degree in Biochemistry at the State University of Iowa in 1952.

He served in the Army of the United States from 1952 through 1954.

In 1955 he worked with Dr. M. Lepper, Department of Medicine, University of Illinois, College of Medicine, doing research on mucoproteins.

In 1956 the author was a research associate at Loyola University School of Dentistry, Department of Biochemistry until he resumed his graduate studies.

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In 1960, the candidate became a National Institutes of Health Trainee, and also served as a Graduate Assistant in the Department of Pharmacology and Therapeutics.

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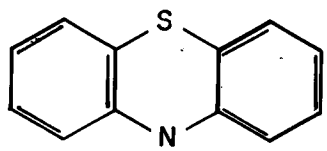
CHAPTER I

INTRODUCTION

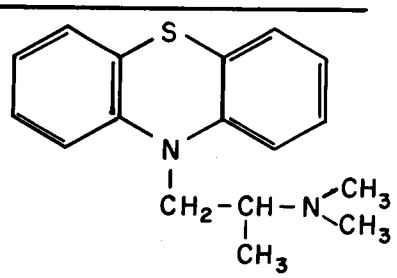
The pharmacological properties of the chemically homogenous group of phenothiazine compounds differ widely. Phenothiazine (fig. 1), the parent compound possessing the basic structure of this group, had been synthesized in 1883 and had been introduced as an antihelmintic primarily in veterinary medicine about 1933. Its clinical use in man has been restrictive because of its hemolytic action. The phenothiazines received renewed interest as therapeutic agents within the past two decades from two separate research groups. In the United States the search for more potent antimalarials during World War II led to the synthesis of various homologs of phenothiazine (1-3). The 10-dialkylaminoalkyl phenothiazines, the closely related homologs of chlorpromazine (CPZ), were first synthesized in 1944 by Gilman and coworkers (1,2). The pharmacological properties of these compounds were not investigated since these phenothiazine derivatives failed to satisfy the antimalarial interests of these researchers. Independently, the second group, headed by Halpern in France, interested in the phenothiazines as drugs exhibiting potent antihistaminic activity with lesser side effects or toxicity than the known antihistaminics, also synthesized some 10-dialkylaminoalkyl phenothiazines (4,5).

Figure 1

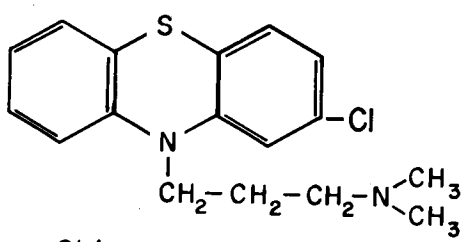
Structural Formulae of Phenothiazine, Promethazine and Chlorpromazine



Phenothiazine



Promethazine
(Phenergan)



Chlorpromazine
(Thorazine, Largactil, etc., see text)

These derivatives, for example, promethazine (phenergan, fig. 1) were found superior to the other known antihistaminic drugs.

During the clinical trials of the antihistaminic phenothiazine derivatives, for example, promethazine, marked hypnotic effects were noted. Halpern et al. (6) reporting on the local anesthetic action of various antihistamine drugs observed that certain of the phenothiazine derivatives exhibited a distinctive type of central action. Winter also recognized a "central" component of action of a phenothiazine compound, promethazine, from his study of drugs causing the potentiation of hexobarbital sleeping time in mice (7) and the depression of the conditioned reflex in rats (8).

A chemical congener of promethazine which would exhibit more potent central action was sought. It was during this search that the synthesis of 10-(3-dimethylaminopropyl)-2-chlorophenothiazine (R.P. 4560) was accomplished in 1950 at Rhone-Poulenc Specia Research Laboratories and reported by Charpentier et al. (9) in 1952. The generic name of chlorpromazine (CPZ) was proposed by the French group (9). Prior to the publication of the synthesis of CPZ by Charpentier studies of the pharmacological properties had been in progress (10) and reports of clinical trials of R.P. 4560 had been published (11-16). The drug had been introduced (17) by various trade names in different countries, for example, Thorazine (United States), Largactil (France, England, and Canada), Megaphen (Germany), Hibernal

(Sweden), Amplictil (Brazil), Aminiazine (Russia), and Ampliactil (Argentina).

Courvoisier et al. (10) reported in detail on the pharmacodynamic properties of R.P. 4560 (CPZ), a new compound utilizable to potentiate anesthesia and artificial hibernation. Promethazine and CPZ were used in Europe as components for the "cocktail lytique", a mixture used for the production of artificial hibernation during surgery. This mixture usually consisted of an analgesic, a parasympatholytic, an antihistaminic, and a hypothermic agent, and was administered as a pre-anesthetic preparation. Promethazine and CPZ were used in the preparation to produce the latter two effects. Courvoisier and her associates demonstrated that CPZ enhanced the action of general anesthetics, analgesics, and narcotic drugs, and exhibited sympatholytic, spasmolytic, hypotensive, hypothermic, sedative, hypnotic, anticonvulsant, anti-emetic, antifibrillatory and analeptic activities. The drug was capable of exerting a depressive action on the conditioned reflex activity and of suppressing the psychomotor activity of the alcoholic; it reduced capillary permeability, exerted some protective action against hemorrhagic and traumatic shock; and it prevented blood coagulation, but at a concentration not attained in therapy; CPZ was found to be practically devoid of antihistaminic activity. However, CPZ was introduced into medicine as an antiemetic and preanesthetic agent.

In addition to the introduction of CPZ as a useful potent anti-emetic agent (18,19) capable of blocking the action of apomorphine on the chemoreceptive emetic trigger zone (20-22), and as an agent useful in the potentiation of anesthetics (15, 23, 24) analgesics (25,26), and hypnotics (15), early clinical reports (13, 16, 27, 28) made it evident that CPZ exerted a non-hypnotic depressant action on the central nervous system. Animals given CPZ displayed a calming or docilizing effect; they became lethargic and motionless (10). Thus, the usefulness of CPZ in alleviating anxiety, agitation and hyperactive motor states in psychotic and psychoneurotic individuals became apparent. Patients suffering from simple or neurotic depressions and certain melancholias responded well to CPZ treatment (28). The anxiety and tension of neurotics were reduced (27-30). Agitated behavior was calmed without the production of narcosis. The observed calmness experienced differed from that following the use of sedatives. The somnolent patient could readily be aroused without exhibiting the stuporous state usually displayed by sedatives. A new term was introduced to describe the pharmacological action of CPZ --"tranquilization", and the era of tranquilizers was introduced with the advent of CPZ. The introduction of CPZ as a useful agent in this field of medicine has done much to revolutionize concepts of mental health. The usefulness of CPZ as an anti-emetic and preanesthetic agent was overshadowed by its greater usefulness in psychiatry.

The effectiveness of CPZ in its application in psychiatry was reviewed by Viaud (31) in 1954, less than two years following the report of the synthesis of this compound. Since that time the literature pertaining to the application of CPZ in mental health and psychiatric treatment grew continually and is, at present, voluminous. The drug has found application in the treatment of severe agitation, acute and chronic schizophrenia, manic states, depression, where anxiety and agitation predominate, psychoneuroses and psychosomatic disorders, addiction to alcoholic and narcotic drugs, and delusional, belligerant and anxiety reactions in organic mental disorders (32). CPZ has been used successfully as an antiemetic and a pre-anesthetic agent.

Several review articles pertaining to the pharmacological and tranquilizing actions of CPZ are available (33-38). A rather thorough comprehensive review, containing 6800 references, on the chemistry and pharmacology of CPZ and related phenothiazine tranquilizers was presented recently by Schenker and Herbst (38). Killam and Killam (34,35) cite the available evidence indicating the involvement of the reticular system as a site for CPZ action. Domino (33) reviewed the data on the mode and sites of CPZ action. Involvement of the hypothalamus and limbic system in the action of CPZ has also been indicated. Considering the multifacet pharmacological responses produced by CPZ administration, it appears that the mechanism of action of CPZ may be complex.

Tissue distribution of CPZ following its administration in vivo has been reported for dogs (39,40), mice (41), rabbits (40), rats (40,42), and monkeys (42). However, there appears to be a lack of agreement among these investigators as to the distribution of CPZ within the tissues, particularly as to its localization within the brain. Wechsler and Roizin (42) stated that after the administration of massive doses (100 microgm. per gm.) of CPZ, only traces of this compound could be found in the brain. Low concentrations of S^{35} CPZ in brain were also reported by Fyodorov (40), whereas Salzman and Brodie (39) reported that CPZ accumulates in the brain. Their findings essentially agreed with those of Christensen and Wase (41). Other differences exist between that data obtained by Salzman and Brodie and those obtained by Yasuda and Aoki (43).

Methods have appeared in the literature pertaining to the chemical analysis of CPZ from biological fluids (44-47) and from tissues (39,48). The procedures describing the quantitative determination of CPZ in biological tissues present certain disadvantages. Salzman and Brodie's procedure (39), based on an extraction of CPZ from an alkalinized biological solution into an isoamyl alcohol-heptane mixture, and subsequent re-extraction into dilute hydrochloric acid, requires an ultraviolet spectrophotometer for the determination of CPZ. Thus, a correction for tissue blanks is necessary because the blanks read high in the ultraviolet light. Moreover, they report that by their method,

only 40% of CPZ was recovered after the addition of the drug to brain homogenate. Wechsler and Forrest (48) modified the Salzman and Brodie method; they changed the composition of the organic extraction mixture, substituted glacial acetic acid for the hydrochloric acid, and added hydrogen peroxide. They claimed 95% CPZ recoveries from brain tissue. However, utilizing the modified procedure, Wechsler and Roizin (42) reported very low concentrations of CPZ in the brains of monkeys and rats following massive doses of the drug. The data of Wechsler and Roizin suggest that their recovery of CPZ from tissues, in vivo, was incomplete.

CHAPTER II

PURPOSES AND APPROACHES TO THE PROBLEM

The methods available for CPZ analysis from biological tissues appear inadequate, primarily because of insufficient recovery and of the necessity of corrections for the high tissue blanks. One of the purposes of the research described here is to present a chemical procedure which can eliminate these inconveniences.

The chemical procedure based upon the oxidation of CPZ with sulfuric acid, previously described by Dubost and Pascal (44) for the determination of CPZ in biological fluids, constituted the basis for the present method. However, the oxidation was performed in the presence of ferric ion. This procedure provided the advantages of obtaining spectrophotometric readings in the visible range and eliminating the necessity of correcting for tissue blanks and for inadequate recoveries. The average recovery of CPZ added to brain homogenates was about 90%.

Drugs are usually distributed throughout the body once they are administered into the system. Certain tissues can exhibit definite affinities for that compound and can accumulate the drug either in high or significant concentrations. Information about the distribution of a particular drug may provide some insight pertaining to the site of action for that drug. Often the localization of the drug in an organ or tissue may be

associated with the concept of the target site of action, that is, the organ characterized by high concentrations of drug is, at the same time, responsible for the pharmacological effect. The accumulation of iodine in the thyroid gland for the treatment of goiter (49) may be cited as an example. Concentration of a drug in a particular tissue is not always a necessary criterion for the site of drug action. A drug may concentrate in large amounts in some organs or tissues and produce no therapeutic or pharmacological activity. The deposition of high concentrations of thiopental in the fat, is marked even after the animal has recovered from the hypnotic effect of the drug (50-52). It becomes apparent then that the fat serves as storage sites rather than the site of action. Digitalis, a drug whose effect is directly on the myocardium, also does not selectively localize in heart muscle (49).

Pharmacologic effect of a drug may often be related to its concentration in plasma (52-54) as well as to its deposition or accumulation in the tissues. That both these factors play a role in drug action would be little disputed. Brodie et al. (50,51) interpreted the deposition of thiopental into body fat as being related to the termination of its anesthetic activity. Almost a decade later, Goldstein and Aronow (52) extended the earlier experiments and showed that the termination of thiopental activity could be more correctly associated with a critical concentration presented in the areas of the brain affected.

In this consideration, the question arises whether the drug action is associated with the target organ or with a receptor site or sites on that particular organ. That is, a drug may be selective in its action. It may affect a specific tissue or a specific site on that tissue, perhaps a single cell or group of cells which are highly specialized or sensitive to that particular drug. These may be considered as receptor cells. Furthermore, the sensitivity of that receptor cell or group of cells to the drug may be limited to a small area located on the surface or the interior of the cell, that is, some specific site of the cell. Since A.J. Clark focused attention upon the importance of the molecular organization of the cell, present concepts imply that molecules of compounds, or drugs, do not react with the cell as a whole, but with other molecules of which the cell may be composed or which it contains. These cellular molecular components become of importance once the site of action for that particular drug is determined.

Distribution studies often help provide some indication of the drug activity. If not from the aspect of site of action, these studies may provide the necessary information of absorption, storage, metabolism, excretion patterns, toxicity, usefulness in therapy, and perhaps dosage requirements in relation to effects.

Since the reports of tissue distribution of CPZ following its administration to animals appear inconclusive, it was of interest to study tissue distribution of CPZ in cats. In

view of the paucity of data on the fate of CPZ in animal tissues, its distribution was traced for forty-eight hours after its administration. Certain behavioral and pharmacologic observations were correlated with tissue levels of CPZ.

From the observations obtained in vivo, it was considered interesting to study whether significant uptake of CPZ by the tissue can occur in vitro. Many methods (54-56) are available for studying protein interaction with relatively small ions, such as drugs. Goldstein (54) classified the techniques employed in vitro under three basic principles: 1) Those processes which study protein interactions based upon the reduction of the thermodynamic activity of the drug whereby the concentration and the biological action of the drug may be reduced; 2) Those processes which measure alteration of drug properties, for example, solubility, diffusion, stabilization. Reduction of thermodynamic activity may also explain some of these effects; and 3) Those processes which observe changes in the properties and behavior of the protein.

The first two principles, thus, deal with techniques which involve changes in the interacting molecule, that is, the drug. Equilibrium dialysis, an example of the first group, results in a reduction in thermodynamic activity of the smaller molecule. Osborne (57), almost sixty years ago, demonstrated the usefulness of this technique for protein interactions with salts. The application of the equilibrium dialysis technique to

the study of organic ions was made by Klotz, Walker, and Pivan (58) who reported on the binding of sulfonate anions of methyl orange and azosulfathiazole by bovine serum albumin. Since then, many significant equilibrium dialysis studies with organic compounds have been reported (54,59-64). Most of the investigations utilizing this technique have been on purified proteins, for example, bovine serum albumin and globulin.

The equilibrium dialysis technique in the following experiments was performed with CPZ dialyzed against the tissue homogenate. The primary purpose of the study utilizing the equilibrium dialysis procedures was to determine whether interaction of CPZ with tissue homogenates can occur and whether various tissues in this system would exhibit differences in uptake of CPZ somewhat similar to that observed in vivo. Thus competition by the tissues for the uptake of CPZ in vitro was investigated. Some preliminary experiments pertaining to the strength, or affinity, of the tissue binding of CPZ were made.

Interactions of small molecules, for example, drugs, with macromolecules, the cellular constituents, are of interest in pharmacology from the standpoint of drug action, storage, metabolism, excretion, and transport. Of the many available methods for studying small ion interaction with proteins, in vitro, the equilibrium dialysis technique was selected because it is felt that the results obtained by this technique may be used to provide some information for the mechanism of CPZ action.

These results may be considered preliminary work toward the considerations of CPZ action at the molecular and possibly the "receptor" level. Investigations of the characterization of the cellular component or components involved in the interaction with CPZ were not pursued in the present work.

From the application of the law of mass action to the multiple equilibria involved in ion-protein interactions, results obtained from the equilibrium dialysis technique employing purified proteins can be extended for the calculation of the energy of association of each successive ion to a protein as well as to the number of sites available for the binding of the ion (55). While this application is beyond the scope of the present experiments, it would indeed be of interest for a future investigation.

CHAPTER III

MATERIALS AND METHODS

Principle of the Chlorpromazine Method

The chemical procedure based upon the oxidation of CPZ with sulfuric acid, previously described by Dubost and Pascal (44) for the determination of CPZ in biological fluids, constituted the basis for the present method. However, the oxidation was performed in the presence of ferric ion. This procedure provided the advantage of obtaining results from spectrophotometric readings in the visible range.

CPZ was extracted by diethyl ether from alkalinized solutions of tissue homogenates or of whole blood. The drug was then extracted from the ether into 2N sulfuric acid. An aliquot of this aqueous layer, to which ferric ion had been added was mixed with an equal volume of concentrated sulfuric acid to produce a cherry-red color which showed an absorption maximum between 520 and 530 mμ (Fig. 2) in the Beckman B spectrophotometer. Maximum absorption in the ultraviolet range at about 275 mμ is shown in figure 3.

The intensity of color read at 530 mμ depended upon the concentration of sulfuric acid and of ferric ion. Figure 4 denotes the relation between sulfuric acid concentration and color development at two concentrations of CPZ. In the absence of ferric ion, little color was produced even at 30 microgm./ml

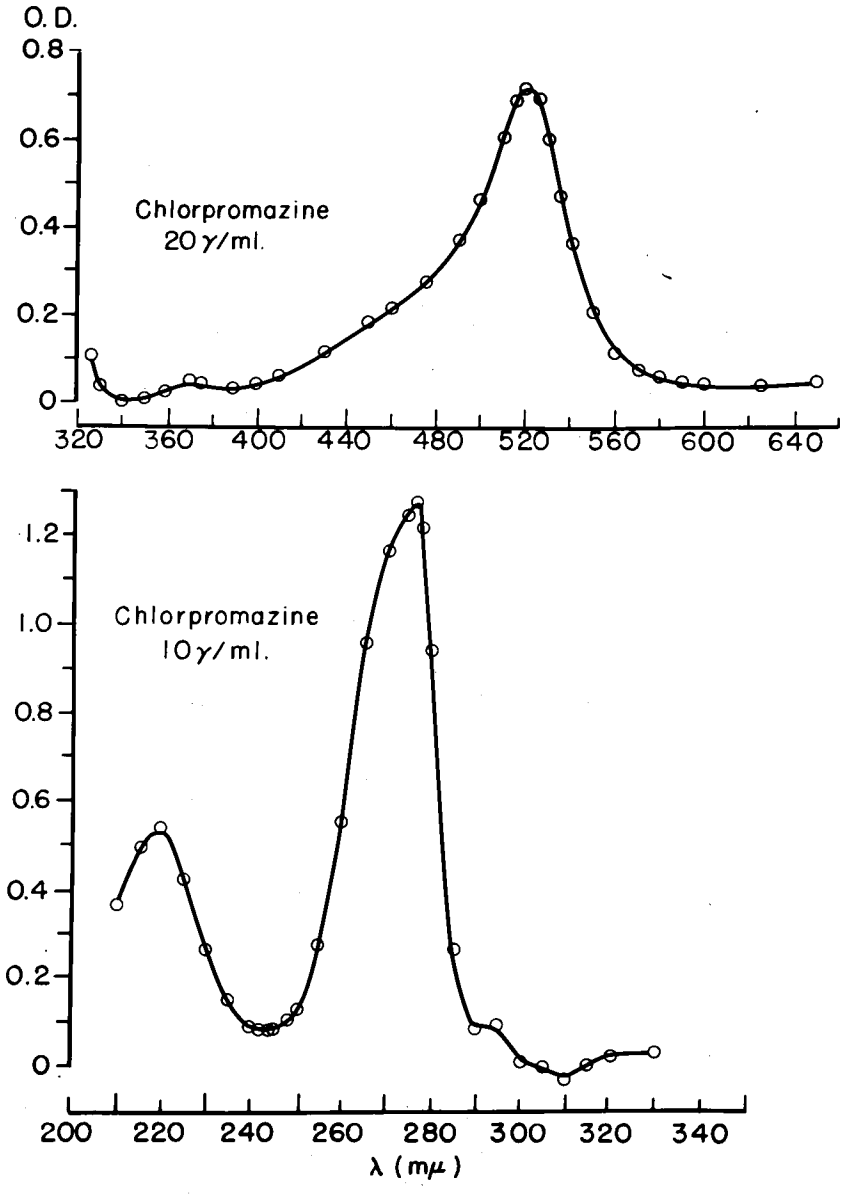

O. D.
0.8
0.6
0.4
0.2
0
Chlorpromazine
20 γ/ml.
320 360 400 440 480 520 560 600 640
1.2
1.0
0.8
0.6
0.4
0.2
0
Chlorpromazine
10 γ/ml.
200 220 240 260 280 300 320 340
λ (mμ)

Figure 2: Upper Half. Absorption spectrum in visible range for chlorpromazine. Abscissa: wavelength (λ) in mμ. Ordinate: optical density (O.D.). Beckman B spectrophotometer was employed. Chlorpromazine concentration, 20 microgm. per ml.

Figure 3: Lower Half. Absorption spectrum in ultraviolet range for chlorpromazine. Abscissa: wavelength (λ) in mμ. Ordinate: optical density (O.D.). Beckman DU spectrophotometer was employed. Chlorpromazine concentration, 10 microgm./ml.

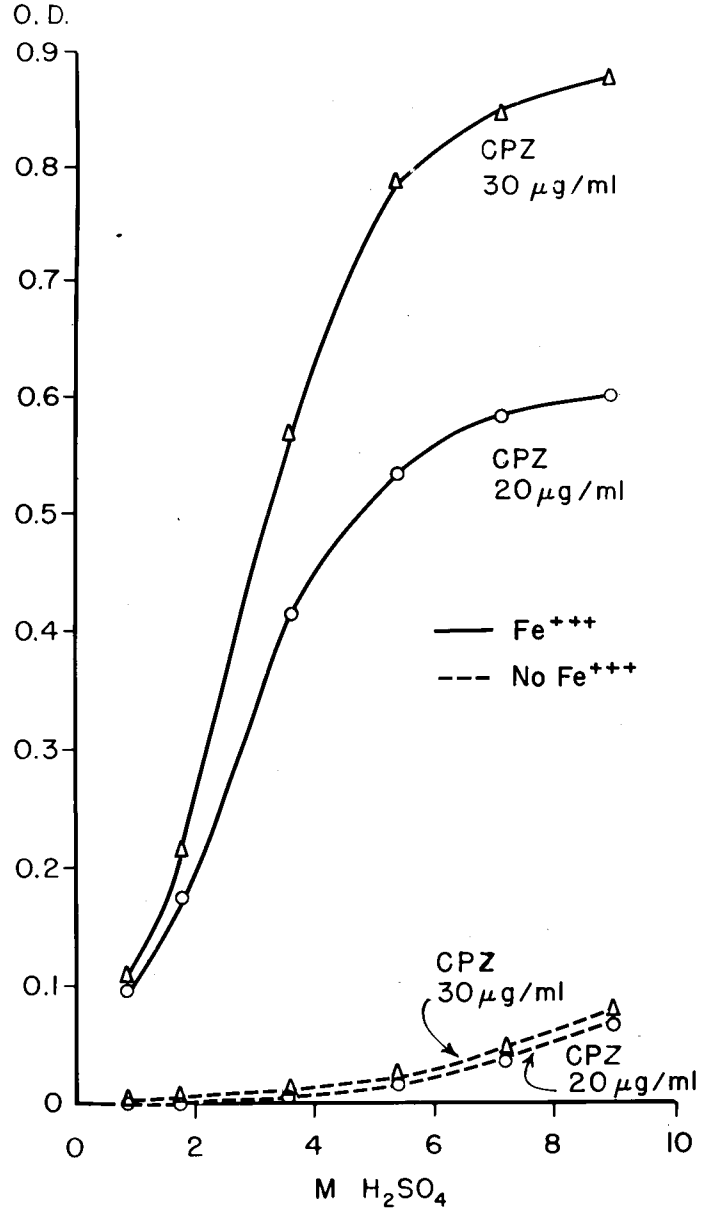

O. D.
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
M H_2SO_4
CPZ
30 μg/ml
CPZ
20 μg/ml
Fe^{+++}
No Fe^{+++}
CPZ
30 μg/ml
CPZ
20 μg/ml

Figure 4: Effect of sulfuric acid concentration upon color reaction of chlorpromazine in presence and in absence of ferric ion. Solid lines represent color development in presence of ferric ion; broken lines refer to color development in absence of ferric ion. Circles and triangles denote 20 and 30 microgm. per ml., respectively, of chlorpromazine. Abscissa: molarity of final sulfuric acid concentration. Ordinate: optical density at 530 mμ.

of CPZ in the final reaction mixture, and the difference in optical density between 20 and 30 microgm./ml of CPZ was insufficient to lend itself for an analytical procedure. In the presence of ferric ammonium sulfate (200 microgm./ml), intense color was produced by stronger (from 4 to 9 M) concentrations of sulfuric acid; color intensity seemed to approach a plateau with 8-9 M concentrations of sulfuric acid. Figure 4 also shows the dependence of the color upon ferric ion in the presence of sulfuric acid.

Figure 5 shows the effect on color development when ferric ion concentration was varied and the concentration of CPZ was 20 microgm./ml in the final reaction mixture. In the absence of sulfuric acid, the color was slight even when concentrations of ferric ammonium sulfate were as great as 1000 microgm./ml. Furthermore, the color was unstable, and it faded to a turbid orange within ten minutes. The presence of sulfuric acid (Fig. 5) both intensified and stabilized the color.

Thus ferric ion alone, or sulfuric acid in the absence of ferric ion, were not satisfactory for quantitative analysis of CPZ. For concentrations of ferric ion and sulfuric acid employed in the procedure, the relationship between CPZ concentration and optical density followed Beer-Lambert's Law over the concentration range of from 1 to 35 microgm./ml of CPZ (Fig. 6).

Oxidation to the red chromophore did not occur with the cupric, mercuric, zinc, molybdate, cobaltic, manganic, or

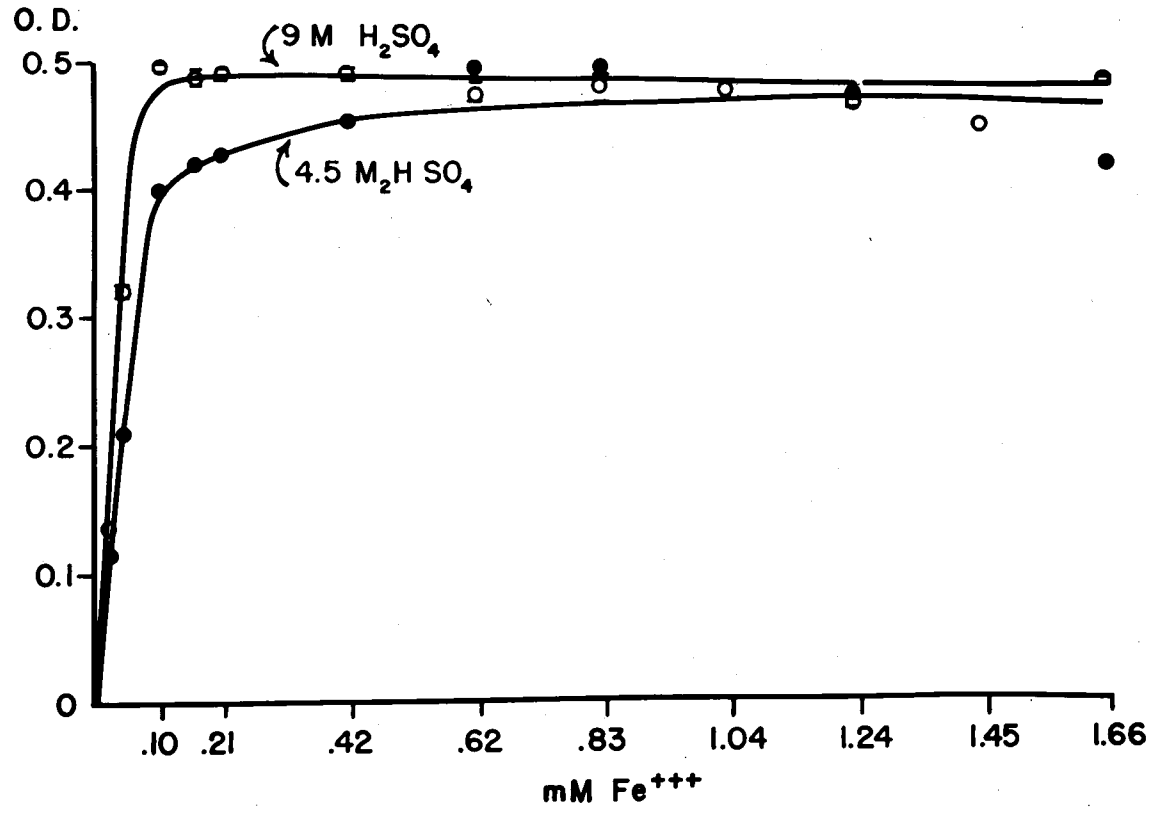


Figure 5: Oxidation of chlorpromazine by ferric ion in the presence of sulfuric acid. The two curves refer to color development with increasing concentrations of ferric ion in 4.5 and 9 M H_2SO_4. Each point represents: one experiment, duplicate readings, in the case of 4.5 M H_2SO_4; two experiments with duplicate readings in the case of 9 M H_2SO_4 (vertical bars represent range of optical densities). Abscissa: mM Fe^{+++}. Ordinate: optical density at 530 mμ.

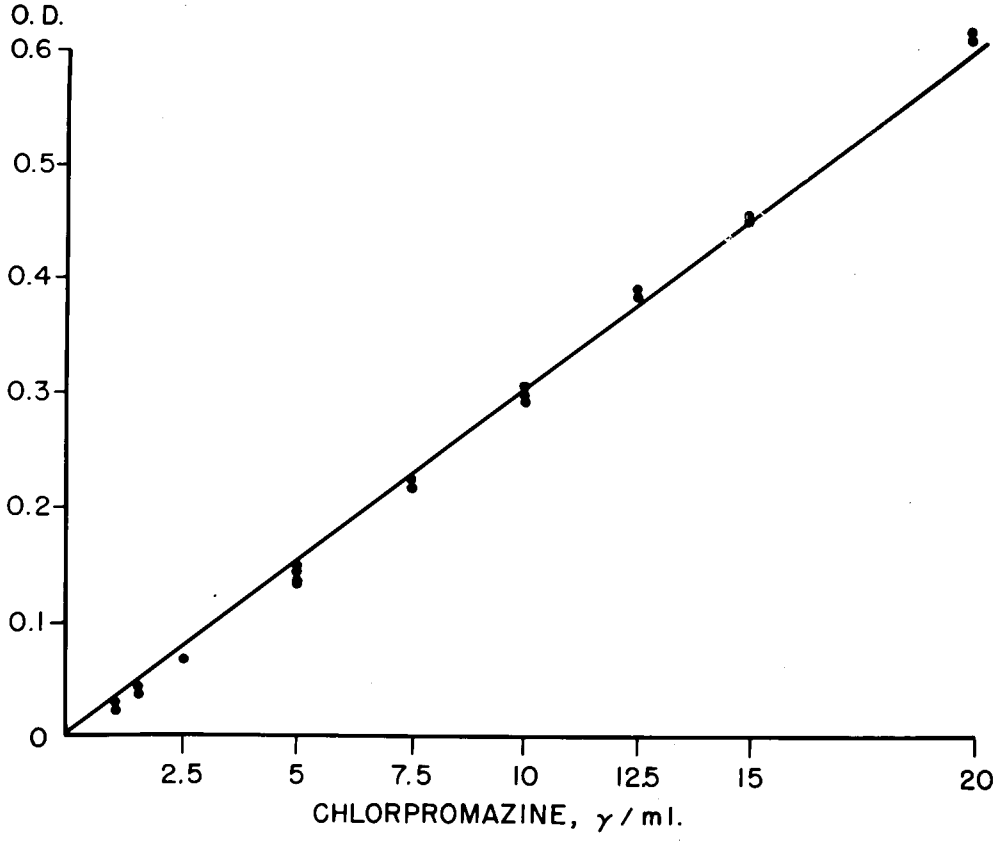


Figure 6: Oxidation of chlorpromazine to a cherry-color chromophore in the presence of sulfuric acid and ferric ion. Abscissa: concentration of chlorpromazine, microgm. per ml. Ordinate: optical density at 530 mμ. From two to four experiments per chlorpromazine concentration indicated; each experimental point represents duplicate readings.

chromic ions, nor did it occur when the ferric ion was complexed as in ferricyanide or ferrocyanide. The ceric ion, however, was found as effective as the ferric ion (65).

Determination of CPZ in Blood and Other Tissues

Homogenates of brain, liver, muscle and other tissues were prepared in a ratio of 1.0 gm of tissue to 2.0 ml water. Whole blood, or 1.0 ml of whole blood diluted with water to 3.0 ml, was used. To 3 ml of the homogenate or of the blood sample, 20 ml of anhydrous diethyl ether was added, followed by 2 ml of 5 N sodium hydroxide. The mixture, in a tightly stoppered bottle, was shaken mechanically for thirty minutes. The aqueous layer was discarded and the ether layer was washed once with 5.0 ml of water. CPZ was then extracted from the ether by shaking for sixty seconds with three separate portions of 3.0 ml of 2 N (4 M) sulfuric acid. The traces of ether trapped in sulfuric acid were removed overnight in an air oven at 50-55 °C. Subsequently, 0.2 ml of 2 per cent (0.0415 M) ferric ammonium sulfate was added, the volume adjusted to 10 ml with 0.1 N (0.2 M) sulfuric acid, and a 5.0 ml aliquot of this solution was mixed with an equal volume of concentrated sulfuric acid cooled in an ice bath. After recooling, the mixture was permitted to equilibrate for thirty minutes in the dark at room temperature and then read at 530 mμ in the Beckman Model B Spectrophotometer. A blank, consisting of 3.0 ml of distilled water, as well as standard solutions containing 50, 100, or 200 microgm. of CPZ hydrochloride,

were carried through the entire procedure.

Methods Employed for Animal Procedures

Fresh aqueous 2 per cent solutions of CPZ hydrochloride were employed for the intravenous injection into the saphenous or femoral vein of adult cats. The doses were 40 microgm. per gm. (30 cats) and 20 microgm. per gm. (2 cats).

Rapid intravenous injection of 40 microgm. per gm. of CPZ is usually fatal within two minutes. Slow and rapid administration was, therefore, employed to determine tissue levels of CPZ after prolonged time intervals and immediately after its administration, respectively. In the former case, injection intervals of five to fifteen minutes were used. Tissue levels of CPZ were determined at intervals timed from the start of the injection until the animal was sacrificed by intravenous injection of air (27 cats). Tissue samples were then removed as rapidly as possible. Blood levels of CPZ were determined immediately; other tissues were frozen on dry ice, and stored until analyzed.

To determine CPZ concentrations in whole blood and in other tissues immediately following its administration, polyethylene cannulae were inserted at least one week before CPZ injection. Cannulae, inserted while the animals were under ether anesthesia, were placed into the right femoral vein for rapid (15 second) injection of CPZ and into the left femoral artery for rapid withdrawals of blood (3 cats). All blood samples were collected in heparinized syringes.

Rectal temperature, reaction to pain, righting reflex, pupillary diameter, and the nictitating membrane were observed in seventeen animals. In the course of preliminary experiments on the hypothermic action of CPZ, it was noticed that immobilization resulted in a fall in body temperature even in unmedicated animals. To distinguish between the hypothermic actions caused by the drug and by immobilization, the following procedure was employed: five cats were restrained in a supine position for two to twenty-four hours, and their rectal temperatures measured. After permitting at least four days for recovery, CPZ was administered (40 microgm. per gm., intravenously), the cats were restrained again for various time intervals, and rectal temperatures were recorded hourly for forty-eight hours. Analgesia was measured by observing the reaction of the cat to pain produced by applying a hemostat clamp on the tail or the toe pad for fifteen seconds.

Principle of Equilibrium Dialysis

In essence, in the equilibrium dialysis technique, two compartments are established by the presence of a membrane, permeable to smaller ions but impermeable to larger molecules such as proteins and various structural cell components. Tissue homogenates, prepared in a suitable buffer, constituted the inner compartment; known concentrations of CPZ in the same buffer were in the outer compartment. Several variants of this basic procedure are described below. Following designated intervals, during

which the penetration of CPZ through the membrane may occur, aliquots were analyzed for CPZ content. Interaction of CPZ with cellular components of the tissue homogenate may be considered to have occurred if, at equilibrium, the amount of CPZ per unit volume found in the inner chamber was greater than the concentration of CPZ in the outer chamber. However, if CPZ concentrations in both chambers, at equilibrium, were approximately equal, it may be assumed that interaction of CPZ with cellular components was negligible.

Two inherent sources of error in the equilibrium dialysis method have been recognized (55). For one, there may be asymmetry in the distribution of the small molecule, if it is ionic, as a consequence of the Donnan membrane effect. Either corrections can be employed to minimize any discrepancies in distribution due to the Donnan effect, or it can be reduced to negligible proportions by the addition of an innocuous electrolyte (66). The assymmetric distribution of the small ions due to the Donnan effect may also be eliminated if the pH selected is at the isoelectric point. In the present experiments, the phosphate buffer was considered as providing an innocuous electrolyte. Secondly, absorption of the small molecule (CPZ) to the membrane may occur. Correction or possibly elimination of this source of error can be made by employing a control dialysis, in which the buffer solution replaced the homogenate in the inner chamber and CPZ is measured in both compartments. This procedure

was adapted in these experiments.

For meaningful equilibrium dialysis results, the volumes of the two chambers should remain constant. Osmotic pressure effects may affect the experimental results obtained from equilibrium dialysis by causing shifts in the volume of water between the two compartments. Low concentrations of homogenate or of the test drug tend to minimize or eliminate osmotic pressure effects. To ascertain that osmotic pressure effects did not enter as a variable during these experiments, volume measurements of the inner compartment were performed periodically. No significant changes in volume of the inner compartments were observed with the concentrations of homogenate or of drug employed.

Solutions Employed for Equilibrium Dialysis

CPZ Stock Solutions

Stock solutions of 500 mg. CPZ hydrochloride per 50 ml water were prepared and kept refrigerated in the dark until used. Aliquots were removed and diluted with phosphate buffer on the day of use.

Thimerosal Solution

A commercial preparation of thimerosal (Eli Lilly and Co.), 1:1000 solution, was employed. 1 ml of the stock solution was diluted to 10 ml with water. 0.5 ml of the thimerosal dilution was added to each 30 ml of buffered CPZ solution.

Phosphate Buffer Solutions

Sodium monohydrogen phosphate, Na_2HPO_4, Fisher

Scientific, A.R. (Lot #720607), F.W. 141.965 was used. 9.48 gms. per liter of water was prepared as stock solutions.

Potassium dihydrogen phosphate, KH_2PO_4, Fisher Scientific, A.R. (Lot #722191), F.W. 136.091 was used. 9.08 gms. per liter of water was prepared as stock solutions.

Designated proportions of each were mixed to prepare the phosphate buffer employed in the experiments (see appendix).

Preparation of Tissue Homogenates

The initial experiments were performed with the inner chambers containing homogenates prepared in a ratio of 1.0 gm. of tissue to 2.0 ml of phosphate buffer (that is, 1:3 tissue homogenates similar to that described in the previous section for the analysis of tissue distribution of CPZ in vivo). The 1:3 homogenate dilution had been selected for the analysis of tissues obtained from the cat which had been administered CPZ because of convenience of volumes since at instances CPZ might be present in concentrations insufficient to be analyzed from less than 1 gm. of tissue. This homogenate preparation, 1:3, was undesirable for the dialysis procedure because the tissue was too concentrated. Furthermore, the 1:3 homogenate preparation exhibited an uptake of CPZ during equilibrium dialysis which required high dilutions for the analysis of CPZ. The level of CPZ in the inner compartment when homogenates of 1:10 or 1:100 tissue dilution were employed was at a sufficient concentration for convenient analysis. Therefore, equilibrium dialysis experiments were performed

utilizing the tissue homogenates prepared as 1:10 dilutions (1 gm. of tissue plus 9.0 ml solution) or 1:100 dilutions in the phosphate buffer used for that particular experiment. The preparation of the latter dilution was effected by diluting aliquots of the 1:10 tissue homogenate to the appropriate volume with the phosphate buffer.

Boiled Homogenate

Tissue homogenate (1:100) prepared as described above was placed on a hot water bath at 90 °C for two hours. The tubes were covered with glass condensation bulbs during this procedure. After cooling to room temperature, suitable aliquots were pipetted into the dialysis bag.

Aged Tissue Homogenate

Aged tissue refers to tissue homogenate (1:100) which had been prepared as described above, stored in stoppered containers at 0-4 °C for a period of two to four weeks.

Preparation of the Dialysis Tubing

For equilibrium dialysis, the membrane should be permeable to water and the drug under investigation, and impermeable to the binding substance such as, the protein, or, in this instance, the non-diffusible constituents of the tissue homogenate. Cellophane dialysis tubing appeared to be suitable. The tubing was a seamless product prepared from regenerated cellulose by a viscose process. Beside cellulose, the manufacturer states that

the tubing may contain glycerine, water and traces of sulfur compounds (approximately 0.1 per cent). Apparently this small quantity of sulfur left from the manufacture of the casing can interfere during binding studies of various metallic ions. Interference in studies of protein complexes of some metals by the sulfur left in the casings has been reported (55). Therefore the removal of sulfur prior to the use of the tubing is indicated.

The method suggested by Hughes and Klotz (55) for the preparation of tubing for equilibrium dialysis constituted the basic procedure used in these experiments. Lengths of about ten inches of the cellophane casing were immersed in a beaker of distilled water, and heated in a boiling water bath at 86° C for 45-60 minutes; the strips were transferred to fresh distilled water and the heating period was repeated. The strips were then transferred through several washings of distilled water at room temperature and kept in distilled water until ready for use. A sufficient number of strips were removed from the distilled water and soaked overnight in the appropriate phosphate buffer used for the equilibrium dialysis procedure.

Excess buffer was removed by pulling the strip between the fingers three or four times. A double knot was tied on one end and the tissue homogenate in the appropriate buffer, or, when used for controls, the buffer solution alone was pipetted into the dialysis bag. The top was then tied into a double knot, and the tips of the bag were cut close to the knots at both ends. A

thread, permitting for freedom of motion of the bag, was tied to one end in order to facilitate easy removal of the dialysis bag from the test tube.

Dialysis Procedure

Thirty ml aliquots of the phosphate buffered CPZ solution were pipetted into 40 ml polyethylene tubes. Thimerosal (0.5 ml of 1:10,000 solution) was added to eliminate bacterial growth during the experimental interval. A dialysis bag containing 10 ml of the tissue homogenate prepared as described above was immersed into the CPZ solution. Parafilm layered beneath the rubber stopper was used to tightly stopper the tube. The inner compartment of the control assemblies contained 10 ml of buffer solution but no homogenate. Aliquots of both compartments were analyzed for CPZ. Control dialysis assemblies were prepared to assure that sufficient time to attain equilibrium elapsed and to provide a check against errors due to absorption of CPZ onto the cellulose dialysis tubing. Equilibrium was determined by employing a series of similar chambers from which at varying intervals samples were removed for analysis of CPZ. Thus, equilibrium was ascertained when the concentration of CPZ of the chambers showed no further changes or for control, CPZ concentration in one inner and outer chamber were approximately equal.

According to Hughes and Klotz (55) adequate stirring should be maintained to hasten the equilibrium time. Mixing was accomplished by either of two procedures. In the case of one

method, a magnetic stirring bar was placed in the outer compartment; consequently, the tissue homogenate settled during the dialysis procedure. The question was raised that the settling of the homogenate may reduce the surface area of the tissue components exposed to the CPZ molecule. In the other technique therefore, the whole assembly was placed onto the Eberbach shaking machine. No settling of homogenate occurred in this latter case. Both mixing procedures were performed so that the tubes were enclosed in a darkened container in order to reduce the production of oxidation products formed by CPZ from exposure to light.

At the designated intervals which constituted the conclusion of the experiment for that particular assembly, the dialysis bag was removed and blotted dry with tissue paper. One end of the bag was cut just below the knot and the contents poured into a test tube. In the situations where settling of the homogenate occurred, the bag was inverted several times to assure adequate resuspension of the homogenate prior to removing the aliquot for analysis of CPZ.

At the termination of the experiment the pH of the buffer, the buffered CPZ solutions, and the solutions of the outer compartment of each reaction mixture were read on a Beckman Zeromatic pH instrument. Changes in pH were not observed in any reaction vessel even forty-eight hours after the start of the experiment.

Three approaches of the equilibrium dialysis procedure were designed to study the uptake, affinity, and competition between the tissues.

1) For the measurement of uptake of CPZ, the procedure as described above was employed, that is, CPZ in the outer solution was dialyzed against the tissue homogenate.

2) Similar experiments were performed using a single outer chamber and two or more tissues in separate dialysis bags to show whether competition for CPZ would occur between tissues. In these latter experiments, the ratio of total homogenate volumes in the inner compartments to that of the buffered CPZ volume in the outer compartment was identical with the ratio which obtained in the measurement of uptake. For example, when four homogenates of cerebrum, heart, liver, and lung, were used to observe whether competition would occur between these tissues, 8.0 ml of each homogenate in separate dialysis bags were suspended in 96 ml of buffered CPZ solution; or 2.5 ml homogenates of each tissue were suspended in 30 ml of solution. Hence a constancy of a 1:3 ratio of inner to outer compartment volumes was maintained in the systems. In some instances, only two tissues were employed for competition experiments. Again the 1:3 ratio of combined inner to outer chamber volume was utilized.

3) For an indication of the affinity of CPZ for the tissue, two experiments were carried out. a) A dialysis bag was carried through the procedure 1 for uptake. After equilibrium,

the bag was removed, blotted dry with tissue paper, and transferred to a fresh chamber containing only the buffer. Dialysis exceeding the equilibrium time period was repeated. b) A tissue with know CPZ content obtained from an animal administered CPZ, was dialyzed against the buffer, and also against a buffered CPZ solution.

CHAPTER IV

RESULTS

Recovery of Chlorpromazine from Biological Tissues

The extent of recovery of CPZ incubated with brain homogenates was investigated. CPZ, 250 microgm. (13 experiments) and 25 microgm. (8 experiments), was incubated in 1.0 gm of brain homogenate. The average recoveries were 87.6 ± 10.8 and 86.95 ± 6.51 per cent, respectively (Table 1).

Specificity of CPZ Method

This chemical procedure was reported in this laboratory as being specific for the intact molecule of CPZ and its sulfoxide (67). More extensive studies of specificity were carried out at present. The method proved specific for the intact phenothiazine ring with the chlorine atom in the number 2 position, and for the corresponding sulfoxides (Table 2). Alterations in the 10-dimethylaminopropane side chain did not alter the colored reaction product. The method did not distinguish between CPZ, chlorpromazine-N-oxide (CPZ-NO), secondary (Nor_1 CPZ, monomethylchlorpromazine) and primary amine (Nor_2 CPZ) analogs of CPZ, prochlorperazine, perphenazine, and thiopropazate (Table 2). The earlier results (67) suggest that sulfoxides of these compounds would react in a similar manner.

Absorption spectra of several phenothiazines were investigated over ultraviolet (Fig. 2,7) and visible (Figs. 8,9)

Table I

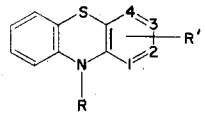
Recoveries of Chlorpromazine from Brain Homogenates

(CPZ was added to brain homogenates 1:3 for analysis)

CPZ added (microgm.)	CPZ recovered* (microgm.)	% recovered	Mean % recovered ± S.D.
250	225	90.0	
250	221	88.2	
250	159	63.8	
250	192	76.7	
250	239	95.9	
250	234	93.9	
250	215	86.0	87.6 ± 10.8
250	215	86.0	
250	236	94.5	
250	262	105.6	
250	232	92.9	
250	229	91.5	
250	186	74.2	
25	20	80.0	
25	18.75	75.0	
25	22.8	91.9	
25	21.1	84.5	86.95 ± 6.51
25	22.8	91.1	
25	22.2	89.0	
25	23.1	92.4	
25	23.	92.5	

*Values are averages of duplicate determinations

TABLE 2 COLOR FORMATION BY 10-SUBSTITUTED PHENOTHIAZINES AND RELATED COMPOUNDS IN PRESENCE OF H_2SO_4 AND Fe+++



E_{530} values read at 10 to 20 microgm/ml.

Drug	*R*	*R*[1]	*Color*	E_{530}
Chlorpromazine	$(CH_2)_3NMe_2$	2-Cl	Cherry-red	$1{\cdot}096 \times 10^4$
Chlorpromazine 2° Amine (SKF 4514 A, Nor$_1$ CPZ)	$(CH_2)_3NHMe$	2-Cl	Cherry-red	$1{\cdot}208 \times 10^4$
Chlorpromazine-N-oxide maleate	$(CH_2)_3\overset{\uparrow}{N}(O)Me_2$	2-Cl	Cherry-red	$1{\cdot}092 \times 10^4$
SKF 6100 A	$(CH_2)_4NMe_2$	2-Cl	Cherry-red	$1{\cdot}079 \times 10^4$
Prochlorperazine	$(CH_2)_3N$ N-Me	2-Cl	Cherry-red	$0{\cdot}927 \times 10^4$
Perphenazine	$(CH_2)_3N$ $N(CH_2)_2OH$	2-Cl	Cherry-red	$1{\cdot}192 \times 10^4$
Thiopropazate	$(CH_2)_3N$ $N(CH_2)_2OCOMe$	2-Cl	Cherry-red	$1{\cdot}177 \times 10^4$
Chlorprothixene†	$=CH(CH_2)_2NMe_2$	2-Cl	Orange	$0{\cdot}206 \times 10^4$
Imipramine HCl*	$(CH_2)_3NMe_2$	H	Colorless	0·0
2-Hydroxypromazine maleate	$(CH_2)_3NMe_2$	2-OH	Rust	$0{\cdot}754 \times 10^4$
Methoxypromazine maleate	$(CH_2)_3NMe_2$	2-OCH_3	Purple	$0{\cdot}419 \times 10^4$
Promazine	$(CH_2)_3NMe_2$	—	Orange	$0{\cdot}404 \times 10^4$
Triflupromazine	$(CH_2)_3NMe_2$	2-CF_3	Orange	$0{\cdot}247 \times 10^4$
Fluphenazine	$(CH_2)_3N$ $N(CH_2)_2OH$	2-CF_3	Salmon	$0{\cdot}226 \times 10^4$
Thioridazine	$(CH_2)_2$ N Me	2-S-CH_3	Purple	$1{\cdot}005 \times 10^4$
SKF 5915	$(CH_2)_3NMe_2$	3-Cl	S1 Purple	$1{\cdot}142 \times 10^4$

†In the case of chlorprothixene, C replaces N.
*In the case of imipramine, a CH_2-CH_2 group replaces S.

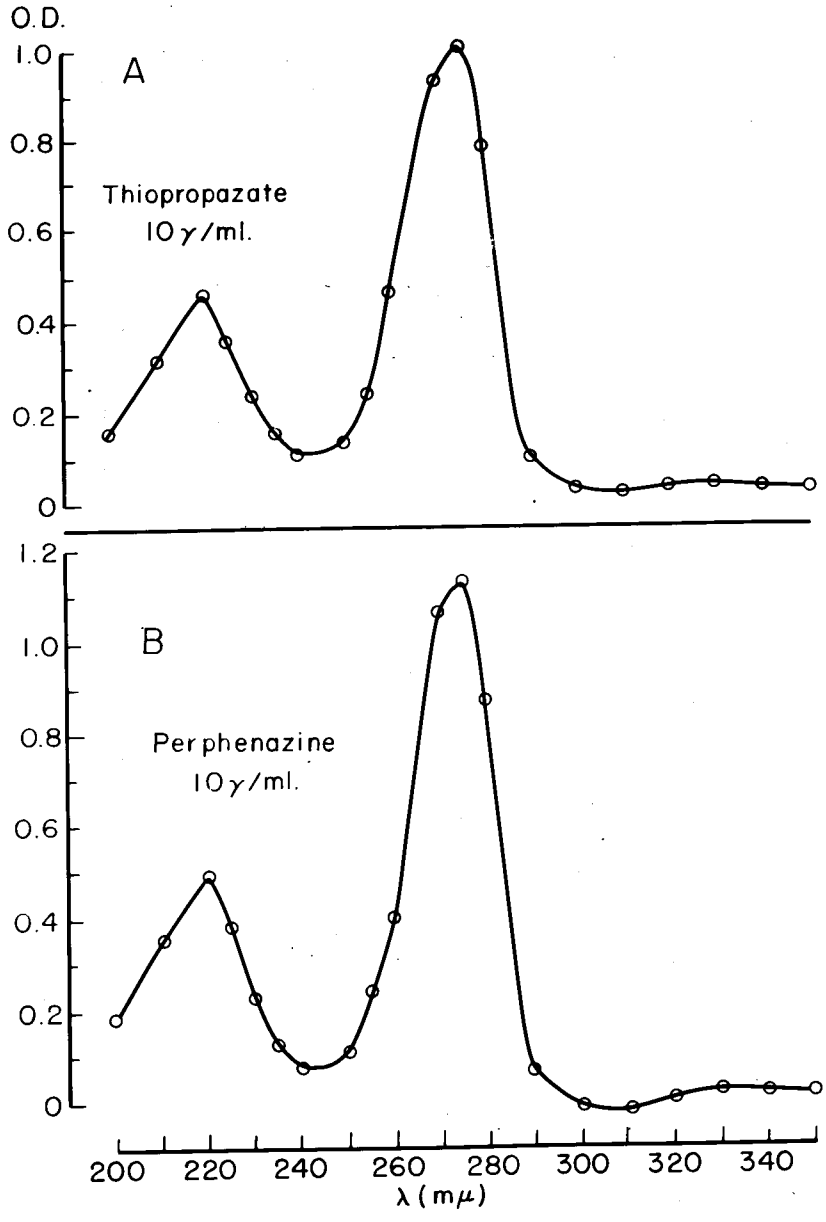


Figure 7: Absorption spectra in ultraviolet range for some phenothiazines: A. Thiopropazate; B. Perphenazine. Abscissa: wavelength (λ) in mμ. Ordinate: optical density (O.D.). Beckman DU spectrophotometer was employed. Drug concentrations, 20 microgm. per ml.

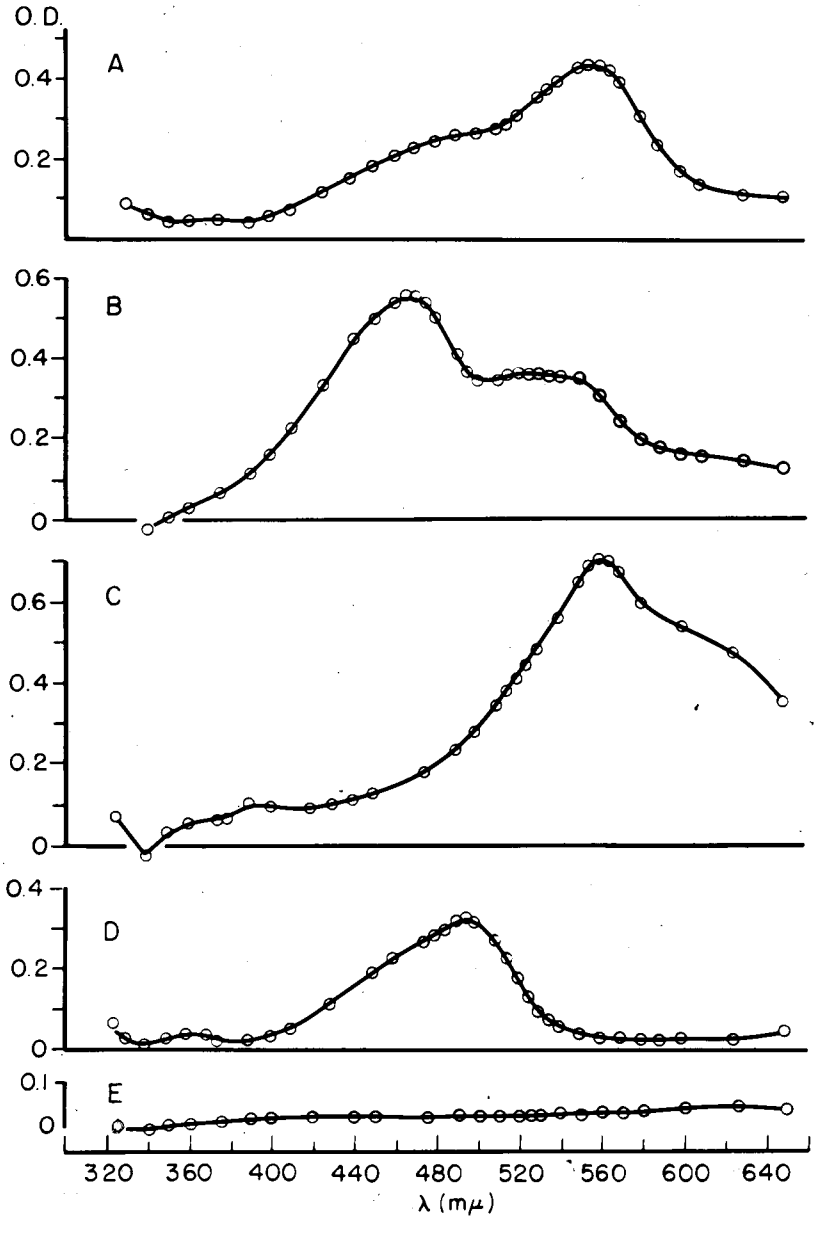


Figure 8: Absorption spectra in visible range for some phenothiazines: A. 2-Methoxypromazine; B. 2-Hydroxypromazine; C. Thioridazine; D. Fluphenazine; E. Imipramine. Abscissa: wavelength (λ) in mμ. Ordinate: optical density (O.D.). Beckman B spectrophotometer was employed. Drug concentrations, 20 microgm. per ml.

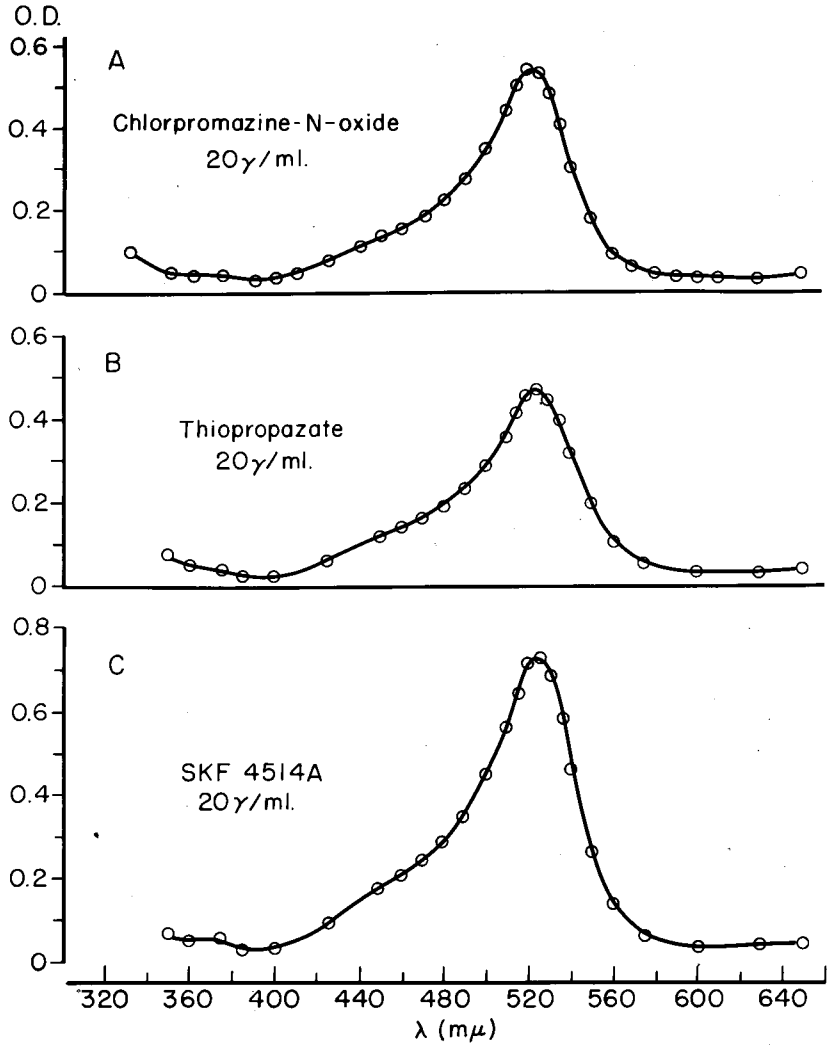


Figure 9: Absorption spectra in visible range for some phenothiazines: A. Chlorpromazine-N-oxide; B. Thiopropazate; C. SKF 4514A. Abscissa: wavelength (λ) in mμ. Ordinate: optical density (O.D.). Beckman B Spectrophotometer was employed. Drug concentrations, 20 microgm. per ml.

wave range (350-650 mμ). CPZ-NO, thiopropazate, SKF 4514-A (Nor_1 CPZ) and perphenazine (Figs. 7,8) which differ from CPZ only in substitutions on the N-alkyl chain (Table 2) exhibited spectra similar to that of CPZ (Fig. 2); their colored reaction products were identical, by visual observations, with the cherry-red chromophore of CPZ. Figures 9A and 9B show absorption spectra for two phenothiazines, which while having the 10-dimethylaminopropyl group as the side chain on the ring nitrogen, differed from CPZ in that the chlorine atom in the 2 position is replaced by either a methoxy or hydroxy group. It is apparent that the respective absorption spectra differed in the visible range. Other substitutions for the chlorine atom in the 2 position also resulted in differently colored reaction products. For example, thioridazine (Fig. 9C) yielded a purple color, while promazine, fluphenazine (Fig. 9D), and triflupromazine produced various shades of orange (Table 2). Also, substitutions of chlorine elsewhere in the phenothiazine nucleus led to reaction products differing in color from CPZ (Table 2). Finally, alterations in the molecular structure of the phenothiazine nucleus, such as removal of either nitrogen or sulfur atom, altered the chromophore. Chlorprothixene produced orange color. Imipramine (Fig. 9E) remained colorless in the procedure (Table 2), and its presence did not interfere in the determination of CPZ.

Since it appeared that the method was specific for 2-chlorophenothiazines with a N_{10}-side chain (2-CPN), this method

could be expected, in vivo, to assay CPZ and some metabolites structurally related to it. Accordingly, pertinent data pertaining to the tissue distribution are expressed in terms of 2-CPN, rather than CPZ, even when under certain conditions, only the CPZ could be expected to be present.

The results indicate also that the extraction and analysis method described here can lend itself, after suitable studies, to differential analysis of clinically important phenothiazines other than CPZ.

Tissue Distribution of Chlorpromazine

Thirty seconds after rapid intravenous injection of CPZ, 40 microgm. per gm., 2-CPN concentrations in whole blood varied between 300 and 500 microgm. per ml (Fig. 10). Ninety seconds later, concentrations were reduced to 120-140 microgm. per ml (Fig. 10). Respiratory and cardiac arrest occurred about this time. In the case of cats given CPZ by slow injection, the levels of 2-CPN in the blood decreased to 10-20 microgm. per ml within seven minutes after its administration (Fig. 11). This level of 2-CPN in the blood remained essentially unchanged during the subsequent forty-eight hours (Fig. 11).

The earliest analysis of brain (cerebrum), heart, liver, and lung was carried out fifty seconds after rapid administration of CPZ. High concentrations (0.8 and 1.1 mg, respectively) of 2-CPN were found in the heart and in the lung, where 2-CPN was present in amounts greater than those found in the blood at that

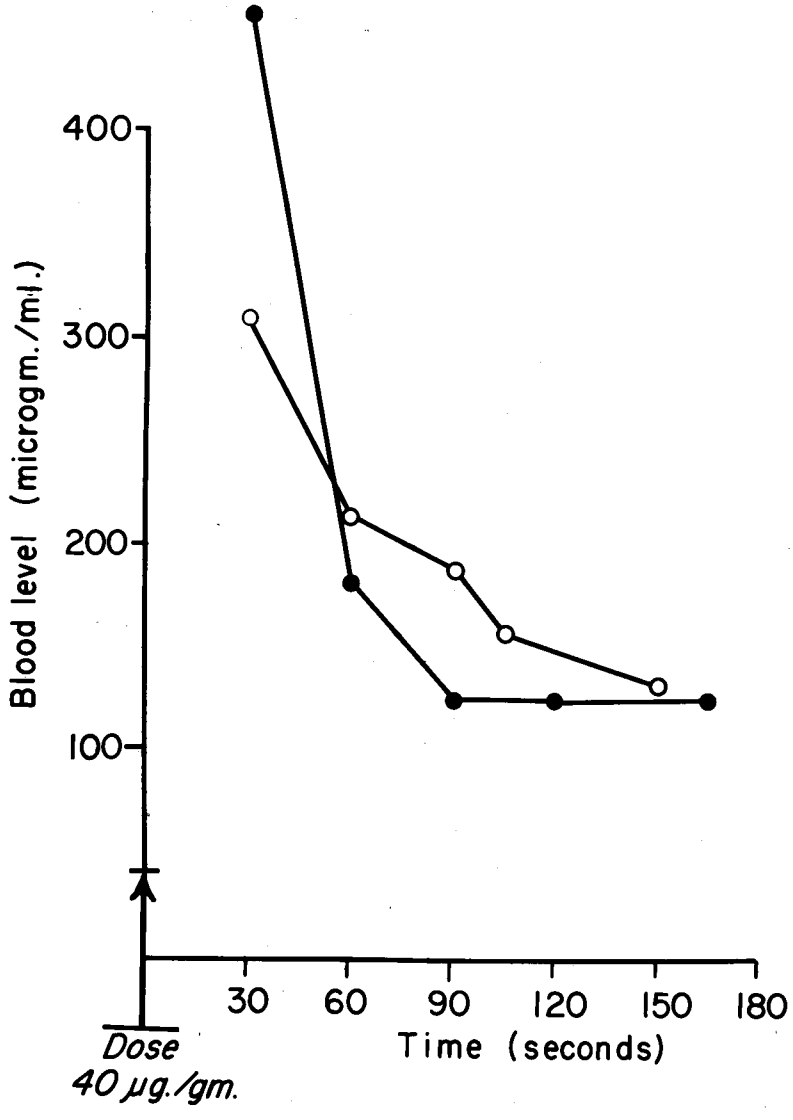


Figure 10: Blood levels of CPZ after rapid intravenous injection of 40 microgm. per gm. Abscissa: time in seconds; ordinate: whole blood level in microgm. CPZ per ml of blood. Data obtained in two cats, each point represents duplicate readings. First two readings obtained in a third animal, which died 50 sec. after medication, were in line with those illustrated in this figure.

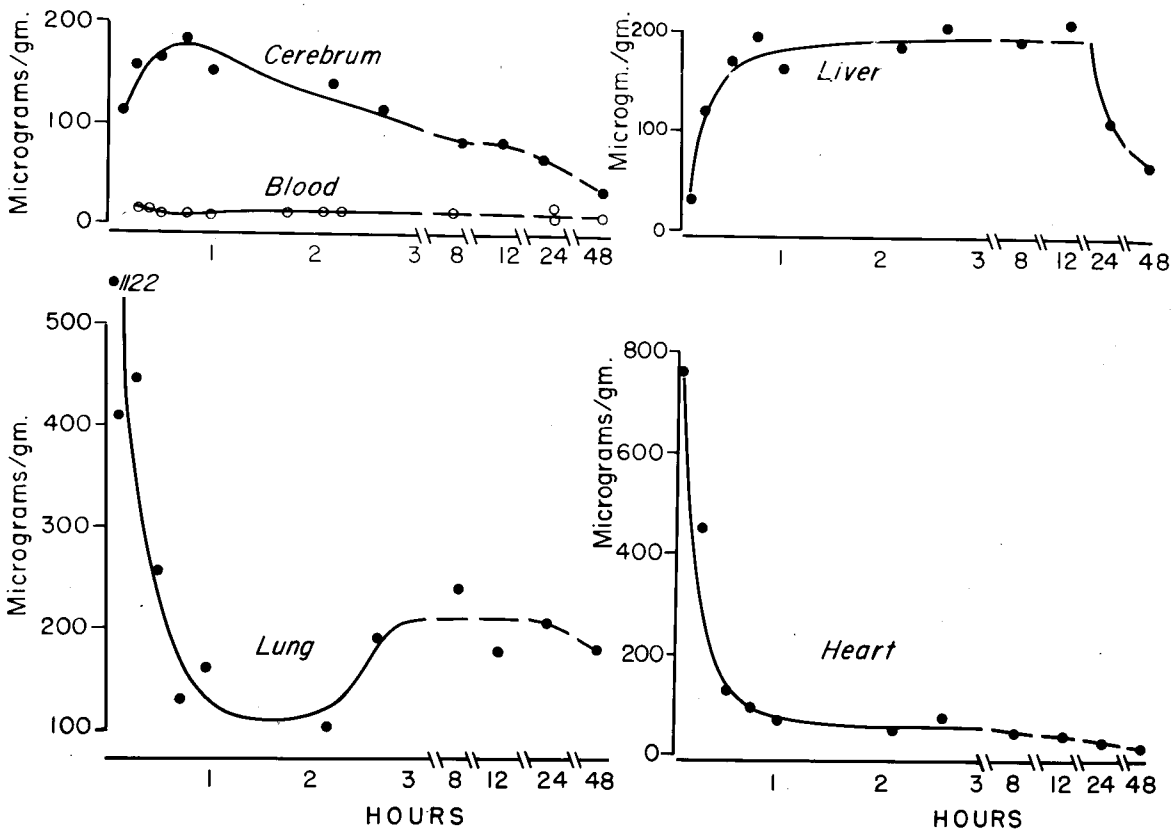


Figure 11. Levels of 2-CPN in five selected tissues after slow intravenous administration of 40 μg/gm. In all four plots, abscissa represent hours (notice five changes in time scale) and ordinate--μg/gm of tissue (notice that the concentration scale changes from plot to plot). Each point represents averages obtained from duplicate readings in at least two cats (Table 3).

time (Table 3). Liver and cerebrum exhibited levels of 2-CPN of 64 and 168 microgm. per gm. respectively (Table 3); these levels were lower than those found at fifty seconds in the blood. At four and six minutes, 2-CPN levels in the cerebrum (Table 3) and other parts of the brain (Table 3) were much higher than in blood; thus, 2-CPN content of the blood, present in the tissues investigated, could not appreciably contribute to the total 2-CPN found in the tissues. The level of 2-CPN in the cerebrum exhibited a transitory decrease from 168 microgm. per gm. at fifty seconds to 111 microgm. per gm. at four to five minutes (Table 3, Fig. 11). Maximum concentration of 2-CPN for the cerebrum appeared at about forty-five minutes. Thereafter, 2-CPN content of the cerebrum declined slowly. At twenty-four and forty-eight hours, concentrations of 2-CPN in brain were about 70 microgm. per gm. and 30 microgm. per gm. respectively.

Besides cerebrum, several other brain areas were analysed for 2-CPN at various time intervals following CPZ administration (Table 4). The cerebrum, hippocampus and various brain stem areas had consistently higher CPZ concentrations than the cerebellum; the differences, while statistically significant, never exceeded 50 per cent, and generally, were much less (Table 4); the differences between cerebrum, brain stem and hippocampus were even less marked.* Also, the general pattern of the slow decrease of 2-CPN level, following its 30-60 minute peak, was

* See appendix

TABLE 3 TISSUE LEVELS OF 2-CPN AT VARIOUS TIMES AFTER ADMINISTRATION OF 40 μG/GM OF CPZ
Average values in μg/gm. Numbers of cats per determination in parenthesis. Determinations carried out in duplicate.

	Min								Hr				
Tissue	0·83	4–6	15	30	45	60	132	160	8	12	18	24	48
Cerebrum	168	111	157	164	182	151	140	116	83	81		67	32
	(1)	(2)	(1)	(3)	(1)	(2)	(2)	(1)	(2)	(2)		(2)	(2)
Heart	779	459	426	129	96	69	58	80	36	39	44	25	18
	(1)	(2)	(2)	(3)	(1)	(2)	(2)	(1)	(2)	(2)	(1)	(2)	(2)
Lung	1122	420	448	258	128	160	101	19[illegible]	242*	181		210	182
	(1)	(2)	(2)	(3)	(1)	(2)	(2)	(1)	(1)	(2)		(2)	(2)
Liver	64	34	120	170	194	162	185	205	190	210		113	68
	(1)	(2)	(1)	(2)	(1)	(2)	(2)	(1)	(2)	(2)		(2)	(2)
Kidney		83		199		86	102	83					25
		(1)		(2)		(1)	(2)	(1)					(2)
Fat		18	48	22	11	96	145		30	94	54	48	25
		(1)	(1)	(1)	(1)	(3)	(2)		(2)	(1)	(1)	(2)	(2)
Spleen		53					128	87					34
		(1)					(1)	(1)					(2)
Pancreas		131											
		(1)											
Testes				80		66							
				(1)		(1)							
Sciatic Nerve				48		59							
				(2)		(1)							
Diaphragm Muscle		116		66									
		(1)		(1)									

*An additional cat showed an unexpectedly low (40 μg/gm) lung content of 2-CPN. Values for its other tissues were as expected and are included in this table.

TABLE 4. BRAIN LEVELS OF 2-CPN AT VARIOUS TIMES AFTER ADMINISTRATION OF 40 μG/GM OF CPZ (in μG/GM)
All values represent duplicate determinations in single cats. Values for the cerebrum contribute to averages in Table 3, except for the 85 min value.

	Min								Hr	
Tissue	4	6	30		60		85	132	160	24	48
Cerebrum	112	110	171	154	130	171	184	146	116	63	29
Cerebellum	67	71	120	119	96				102	45	27
Brain Stem	85	77						131	103		47
Mesencephalon						166	148				
Pons				126							
						118					
Medulla				108							
Hippocampus and Thalamus					116				132		40

similar for the four brain areas investigated.

A high level of 2-CPN persisted in the heart for fifteen minutes after CPZ injection (Table 3). This level dropped abruptly after thirty minutes (from more than 400 to about 130 microgm. per gm.) and more slowly thereafter (Fig. 11). After forty-eight hours, 2-CPN content of the heart was 18 microgm. per gm. (Table 3, Fig. 11).

Lung, like heart, exhibited very high levels of 2-CPN immediately after its administration (1.1 mg/gm). The level was still very high ten minutes later; it decreased abruptly to the level of about 100 microgm. per gm. within the next two hours. However, unlike in the heart, this decline was reversed; the level of about 200 microgm. per gm., reached at about 160 minutes, remained essentially unchanged for the subsequent forty-eight hours (Fig. 11, Table 3).

Comparatively little 2-CPN appeared in the liver during the first six minutes after CPZ intravenous administration (Table 3). Subsequently, the liver content of 2-CPN increased to a peak which was some six times higher than the six minute value (Table 3, Fig. 11). After one hour, the level of 2-CPN in the liver was as high as that in the cerebrum or lung. It remained relatively constant during the subsequent twelve hours; as 2-CPN levels in cerebrum declined during that time interval, the liver became, at twelve hours, the organ with the highest 2-CPN content. Thereafter, the amount of 2-CPN in liver decreased rapidly; the

forty-eight hour value was approximately 70 microgm. per gm. (Fig. 11, Table 3).

During the first forty-five minutes after CPZ administration, fat levels of 2-CPN were relatively low (Table 3). Higher levels were recorded thereafter; a peak value of 145 microgm. per gm. was reached after two hours. Then the fat content of 2-CPN declined; the values of 48 and 25 microgm. per gm. were recorded at twenty-four and forty-eight hours, respectively (Table 3).

Further analysis demonstrated that 2-CPN was distributed to practically every tissue (Table 3). The spleen contained 128 microgm. per gm. after two hours, and the kidney contained almost 200 microgm. per gm. after thirty minutes (Table 3). After forty-eight hours both these tissues showed levels of CPZ of about 30 microgm. per gm.

The relative level of 2-CPN in tissue may depend on the dose of CPZ; this possibility deserved special attention in view of the fact that the dose of 40 microgm. per gm. would correspond to the total dose of about 2.8 gm. in the human, which is high for a single dose in a human. Levels of 2-CPN were measured in two cats, one hour after the intravenous administration of 20 microgm. per gm. of CPZ. The average 2-CPN concentration in various tissues was (in microgm. per gm.): lung, 124; liver, 97; kidney, 75; heart, 49; blood, 10; inguinal fat, 14; cerebrum, 94; cerebellum, 91; midbrain and pons, 76; medulla, 63. These levels

were generally somewhat higher than those that could be expected on the basis of results obtained with the larger dose. The general trend was similar with both doses; 2-CPN accumulated in the lung, liver, kidney and the brain after the smaller dose as it did after the larger dose. Cerebrum exhibited the highest 2-CPN level among the parts of the brain investigated; as in the case of 40 microgm. per gm. dose of CPZ the differences were not marked.

Pharmacologic Findings

The gross pharmacologic effects were observed in thirteen cats. Shortly after the start of the injection of CPZ, 40 microgm. per gm., the animals became relaxed, the respiration depressed, and the pupils dilated. Application of the hemostat clamp onto the tail or the toe pad produced no response. Thus, the animals resembled those in plane 2 or 3, stage III, of ether anesthesia. Twelve hours after CPZ administration, painful stimuli caused no immediate response. Upon repeated stimulation a delayed response occurred; the cat withdrew the paw or the tail, and emitted a weak cry. Even thirty hours after CPZ administration, the cat still responded to pain stimuli with a characteristic 3-5 second delay. Forty-eight hours after CPZ administration, the response was immediate, but the slight cry and attempt to move the afflicted limb were not accompanied by aggression.

Co-ordinated motor activity was absent ten to eleven

hours following the injection of CPZ. The righting reflex was poor or absent for approximately twenty-four hours. However, at twelve hours, two of the thirteen cats made attempts to lap milk or to walk. Interest in these acts waned rapidly. Food placed into the cage usually was not eaten forty hours after the injection of CPZ. Even forty-eight hours after receiving medication, the animals exhibited no sustained motor activity, and mainly rested or slept; agression and resistance to rough manipulations or handling were absent. Relaxation of the nictitating membrane was apparent for more than forty-eight hours.

In the study of the hypothermic action of CPZ, four unmedicated cats, restrained for seven to twenty-four hours, revealed maximal reduction in rectal temperature of 4.2 ± 0.9 F, (Fig. 12), which occurred after immobilization of about four hours. Immediately upon their release, the temperature rose. Levels recorded prior to the restraint were attained in about an hour. CPZ, 40 microgm. per gm. intravenously, was administered to these animals restrained in a harness. The cats were released from the harness two hours (1 animal), eight hours (2 animals), and forty-eight hours (1 animal) after medication (Fig. 12). Maximal hypothermia was 8.6 ± 1.06 F; it occurred independently of the time of the termination of the restraint, eight to twelve hours after medication. Altogether, CPZ hypothermia was about double that produced of restraint; since at the end of hypothermic effects (24-32 hours after medication) the cats, while motile,

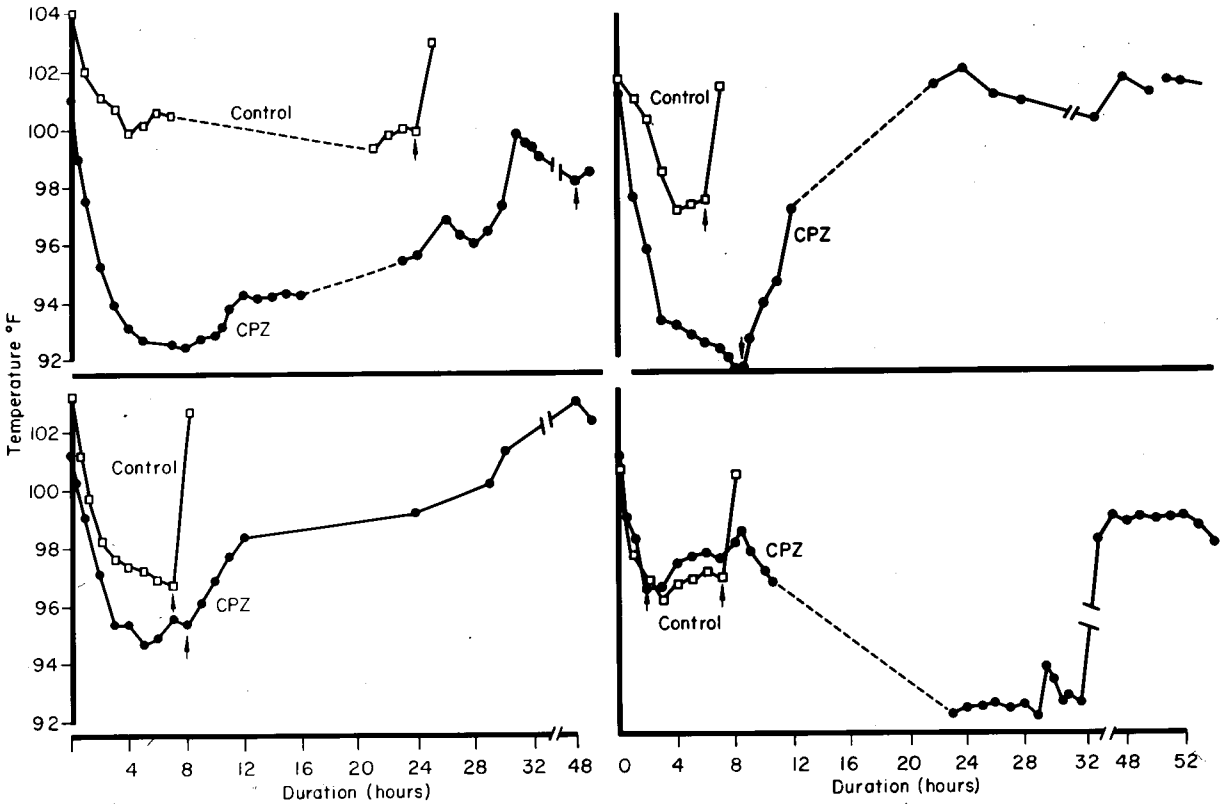


Figure 12. The effect of intravenously administered chlorpromazine, 40 mg/kg, on the rectal temperature of the cat. The curves refer to the control period ⊡ and to the chlorpromazine effect period ● for that cat. The arrow indicates the time that the cat was released from the restraining apparatus. Abscissa: time in hours. Ordinate: temperature in degrees Fahrenheit (°F).

were still sedated, it is difficult to say whether or not hypothermia was secondary to sedation.

Equilibrium Dialysis Study of Tissue Binding of CPZ

For purposes of clarification pertaining to the observations obtained by equilibrium dialysis it is necessary to define the terms "unbound", "bound", and "uptake". Unbound drug refers to the drug which has not entered into a complex with either the tissue homogenate proteins, enzymes, or cellular components, or the cellulose casing employed in these experiments. Unbound drug concentrations are those found from analysis of the outer compartment. The usage of the term "unbound" drug as opposed to "bound" drug shall be employed in this text. The term "bound" drug shall refer to the difference in concentration (microgm. per ml) of the drug in the inner and outer compartments, providing of course, that the inner chamber exhibits a higher concentration than the outer chamber. This applies only if equilibrium has been attained. The per cent bound drug will refer to the ratio of the amount of bound drug to the amount of drug found in the inner compartment at the end of the experiment. The term "uptake" employed in the text refers to the amount of CPZ, bound and unbound, which had been found inside the dialysis bag at the termination of that particular experiment.

Effects of Thimerosal

Aliquots plated from the preliminary equilibrium

dialysis experiments revealed a number of bacterial cultures which grew in the outer chamber of the reaction vessels. Identification of these bacteria was not attempted. Since bacterial growth can introduce unknown factors and may result in the metabolism or oxidation of the CPZ molecule, it was deemed advisable to select some suitable substance or technique to eliminate possible bacterial interference. Performance of experiments at low temperatures reduces the rate of bacterial growth. However, the inaccessibility of cold-room facilities required the utilization of other techniques. The introduction of thimerosal at the final concentration of 0.16 mg % (0.00016%) eliminated bacterial growth in these experiments. Table 5 shows that full recovery of CPZ was effected in the presence of thimerosal. Tests for bacterial growth after the introduction of thimerosal solution in the reaction vessel were negative. Therefore, bacterial plating was not performed routinely.

Thus, thimerosal did not change recovery as shown in table 1. The lower CPZ recovery from the experiments lacking thimerosal (Table 5) may possibly be suggestive that CPZ may be metabolized in the presence of bacterial strains. This inference must be interpreted with caution since no attempt was made to pursue this observation; furthermore, phenothiazines have been known to reduce bacterial growth (68). Recoveries observed in the presence of bacterial growth (Table 5) were not significantly different from the lower limits of the recovery recorded in

table 1.

Table 5

The Effect of Thimerosal on the Recovery of Chlorpromazine during Equilibrium Dialysis

Brain homogenates (1:3) were dialyzed for 20 hours at room temperature, pH 6.8. Volume of solution in outer chamber, 30.5 ml; inner chamber volume 4.5 ml. Initial CPZ content, 8790 microgm.

Thimerosal (ml)	CPZ Recovered (µg)			% Recovered
	Inner Chamber (A)	Outer Chamber (B)	Total (A+B)	
NONE	3546	3360	6906	78.6
0.5	5148	3812	8960	101.9

Binding to Dialysis Bag

Consideration was given whether CPZ would bind significantly to the dialysis bag. To ascertain the error which may be introduced by the binding of CPZ to the cellophane bag, determinations of CPZ from both, the inner and outer, chambers were performed, total CPZ estimated and the recovery calculated. Table 6 shows that the recoveries obtained by analysis of both inner and outer solutions from a series of experiments averaged 98.57 per cent. This indicated that the binding of CPZ to the cellulose casings was negligible or reversible.

Table 6

Recovery of CPZ Following Equilibrium Dialysis

Brain homogenates 1:10 were dialyzed for varying time periods at room temperature; pH 7.12. Volumes of solution in outer chambers equal 30.5 ml.; inner chambers volumes, 8.0 ml. Initial CPZ content 10560 microgm. in each tube. Mixing produced by magnetic stirrer.

Dialysis Duration (Hours)	CPZ Recovered (microgms)			Difference from initial concentration	% Difference
	Outer Chamber (A)	Inner Chamber (B)	Total (A+B)		
1	8889	1381	10,270	-290	-2.74
2	8697	2130	10,827	267	2.53
4	6780	3624	10,404	-156	-1.48
6	6039	4512	10,551	- 9	- .08
8	3715	6372	10,087	-473	-4.48
16	3556	6657	10,213	-247	-2.31

Effect of Varying CPZ Concentrations

High initial concentrations of the drug could establish an equilibrium which would saturate the inner homogenate solution to the extent that the existence of binding would not be reflected. The effect of increases of the CPZ concentration on the binding by cerebrum homogenates (1:100 dilution) resulted in a linear increase in uptake at four hours (Fig. 13A) and at six hours (Fig. 13B) for CPZ concentrations investigated. No experiments were carried out in which the initial CPZ concentration was increased to the level at which further increments resulted in no greater uptake of CPZ by that particular homogenate concentration. Since a concentration of 1 mM did not appear to be near the limiting concentration for CPZ uptake, this concentration was employed in subsequent experiments.

Effect of Different Mixing Techniques

Equilibrium in the control experiments was attained at approximately six hours when the magnetic stirring bar (Fig. 14A) or the Eberbach shaker (Fig. 14B) constituted the method for mixing. Comparison of the two shaking methods for the binding of CPZ, employing 1:100 brain homogenates, revealed that a slight difference occurred in the time to attain equilibrium (Figs. 14A, B). By means of the magnetic stirring mechanism the uptake proceeded somewhat slower than that obtained by the Eberbach shaker. Almost eight hours were required to attain equilibrium by the

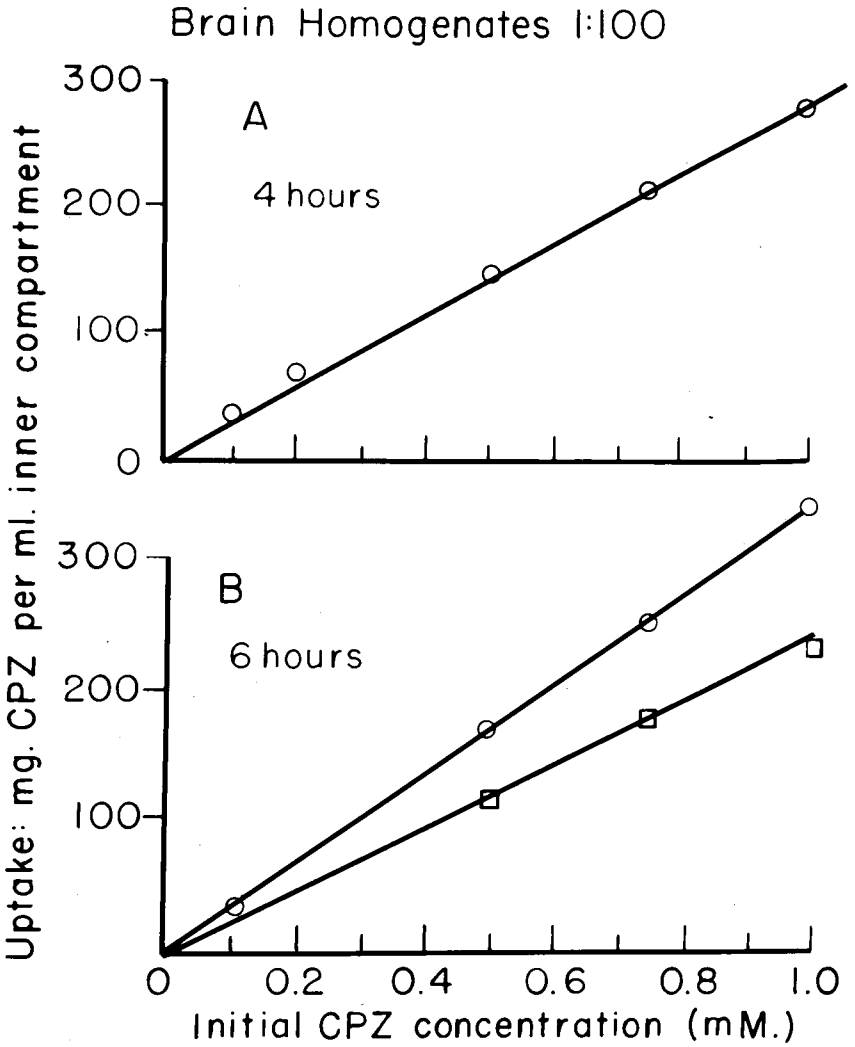


Figure 13: Effect of various CPZ concentrations on uptake by cerebrum homogenates (1:100). A. Uptake at 4 hrs. (pre-equilibrium). B. Uptake at 6 hrs. (equilibrium). The curves represent controls □ and homogenate ○ . Room temperature, pH 7.12. Abscissa: Initial CPZ concentration in mM. Ordinate: Uptake, microgm. CPZ per ml inner compartment.

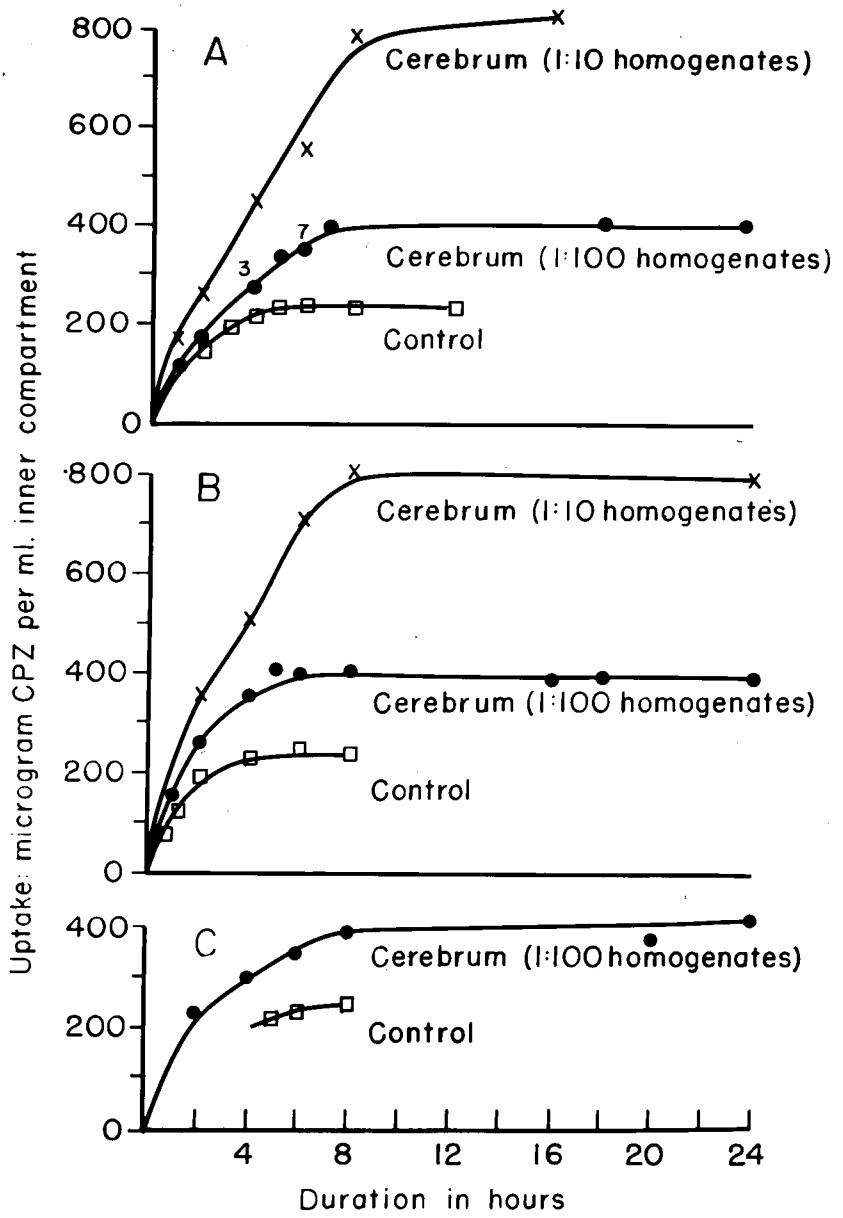


Figure 14: Uptake of CPZ by cerebrum homogenates: A. Room temperature, mixing by magnetic stirring bar; B. Room temperature, mixing by Eberbach shaker; C. At 0-4 ° C, mixing by Eberbach shaker. The curves represent control assemblies □, homogenates 1:100 ●, and homogenates 1:10 x, at pH 7.12. Abscissa: Duration in hours; Ordinate: Uptake; microgm. CPZ per ml inner compartment.

former technique whereas equilibrium was attained within six hours using the latter shaking mechanism. From examination of figures 14A and 14B, it may appear that the magnetic stirring technique resulted in a greater uptake of CPZ; however, this difference was within the range of variability of the analytical method employed.

Effect of Temperature on CPZ Uptake

The selection of lower temperatures to perform equilibrium dialysis by most investigators stems from the knowledge that lower temperatures tend to limit or reduce protein hydrolysis and denaturation or enzyme destruction. The uptake of CPZ by tissue (cerebrum) homogenates was measured in the presence of thimerosal at 0-4 °C and at 24-26 °C (room temperature).

Appropriate controls were performed in both experiments. The controls at the two temperatures attained equilibrium at approximately the same time. Equilibrium for cerebrum homogenates (1:100) occurred at about eight hours at 0-4 °C (Fig. 14C), and within six hours at 24-26 °C (Fig. 14B). Little difference in the uptake of CPZ at equilibrium was evident at these two temperatures (Figs. 14B and 14C), although the period to attain equilibrium was longer at the lower temperature. Somewhat similar effects of temperature upon the binding of other organic compounds had been reported by Klotz et al. (58) and Davison and Smith (60). Evaluation of the kinetics of temperature effect was not attempted.

Effect of Tissue Homogenate Concentration

An increase in the tissue homogenate concentration in the inner chamber resulted in a greater uptake of CPZ (Table 7). A ten-fold increase of homogenate concentration resulted in an uptake that was almost doubled. Thus, cerebrum homogenates, 1:10, exhibited a mean uptake of 773 ± 53.5 microgm. CPZ per ml as compared to 407.3 ± 21.6 microgm. CPZ per ml for cerebrum homogenates 1:100. The per cent bound CPZ also increased at higher concentrations of tissue within the homogenate. Thus, 1:10 cerebrum homogenate dilutions exhibited approximately 87.2 ± 2.95 per cent bound CPZ as compared with 55.1 ± 2.02 per cent for 1:100 cerebrum homogenates (Table 7).

Effect of Aged and Boiled Cerebrum Homogenates

Cerebrum homogenates of two cats which had been subjected to heating at 90 °C revealed no apparent difference in the uptake of CPZ compared to unheated homogenates (Table 8). Also, cerebrum homogenates aged from two to four weeks exhibited uptake of CPZ essentially similar to that of the freshly prepared homogenates (Table 8).

Effect Exhibited by Various Tissue Homogenates

Uptake of CPZ occurred for all the tissues studied, including blood. It was found with the present experimental conditions that 1:100 homogenates of liver, brain, heart, and lung were capable of interacting with CPZ to approximately the

Table 7

The Effect of Varying Concentrations of Tissue Homogenate Preparation Upon the Uptake of CPZ

Outer solution volumes 30.5 ml; inner solution volume 10.0 ml; pH 7.12. Cerebrums from two cats were used. Each dialysis duration exceeds the equilibrium period.

Homogenate Concentration		Dialysis Duration	CPZ concentrations microgm. per ml*		% Bound CPZ
Dilution	gm. wet wgt. tissue/ml		Outer Chamber	Inner Chamber (Uptake)	
1:10	0.1	6	109	716	84.8
1:10	0.1	8	122	792	84.7
1:10	0.1	8	73	717	89.8
1:10	0.1	8	73	808	91
1:10	0.1	16	117	832	85.9
		MEAN ± S.D.		773 ± 53.5	87.2 ± 2.95
1:100	0.01	6	192	402.5	52.4
1:100	0.01	8	194	410	52.5
1:100	0.01	16	172	390	56
1:100	0.01	18	175	399	56.1
1:100	0.01	24	175	401	56.4
1:100	0.01	24	192	454	57.7
1:100	0.01	24	179	394.5	54.7
		MEAN ± S.D.		407 ± 21.6	55.1 ± 2.02

*Each figure is the average of duplicate CPZ analysis

Table 8

Uptake of CPZ by Cerebrum Homogenates Subjected to Various Treatment

Brain homogenates 1:100 were used. Outer solution volumes, 30.5 ml; inner solution volumes, 10.0 ml; pH 7.12. Initial CPZ concentration averaged 346 microgm. per ml.

Source of Homogenate	Experiment Duration (Hours)	Conditions of Experiment		Uptake of CPZ microgm/ml *
		Temp.	Stirring	
Cat #1				
Fresh	6	Room	Magnetic	350 ± 3
Aged	6	Room	Magnetic	324 ± 27
Boiled	6	Room	Magnetic	386
Cat #1				
Fresh	24	Room	Magnetic	454 ± 1
Boiled	24	Room	Magnetic	470 ± 1
Cat #2				
Fresh	2	Room	Eberbach	264 ± 5
Boiled	2	Room	Eberbach	238 ± 4
Cat #2				
Fresh	4	Room	Eberbach	358 ± 2
Boiled	4	Room	Eberbach	371
Cat #2				
Fresh	6	Room	Eberbach	401 ± 9
Boiled	6	Room	Eberbach	402
Cat #2				
Fresh	24	0-4°C	Eberbach	417 ± 2
Boiled	24	0-4°C	Eberbach	422

*Figures include average ± range (a minimum of 2 analyses for each figure).

same extent (Figs. 14C, 15A, B, C). With all tissues, the maximum uptake at room temperature occurred at about six to eight hours.

Whole blood, diluted 1:10, which contained heparin as the anticoagulant, exhibited an uptake of CPZ which differed from that observed with the other tissues. Table 9 compares the uptake and the per cent bound CPZ of the blood obtained from one cat to that of cerebrum 1:10 and cerebrum 1:100 homogenates prepared from another cat. When whole blood, diluted 1:10, constituted the inner medium, blood exhibited an uptake less than that found for a similar 1:10 dilution for brain homogenate. A much lower uptake was anticipated, because of the results in vivo, indicating that little CPZ was retained in blood. The uptake and per cent bound CPZ observed in blood, diluted 1:10, in vitro, may be comparable to the lower dilution (1:100) of the tissue homogenates (Table 9).

One experiment was performed whereby undiluted heparinized blood, to which CPZ was added, constituted the solution of the outer chamber. The design of the experiment was similar to that described for competition between the tissues for CPZ, that is, four tissue homogenates constituted the inner chambers. Thus, 10.0 ml homogenates (1:100) of cerebrum, heart, liver, and lung were suspended in separate dialysis bags in 96 ml of the heparinized blood. For each tissue, the uptake per ml of homogenate (Table 10) was, in this experiment, approximately 10 per cent of

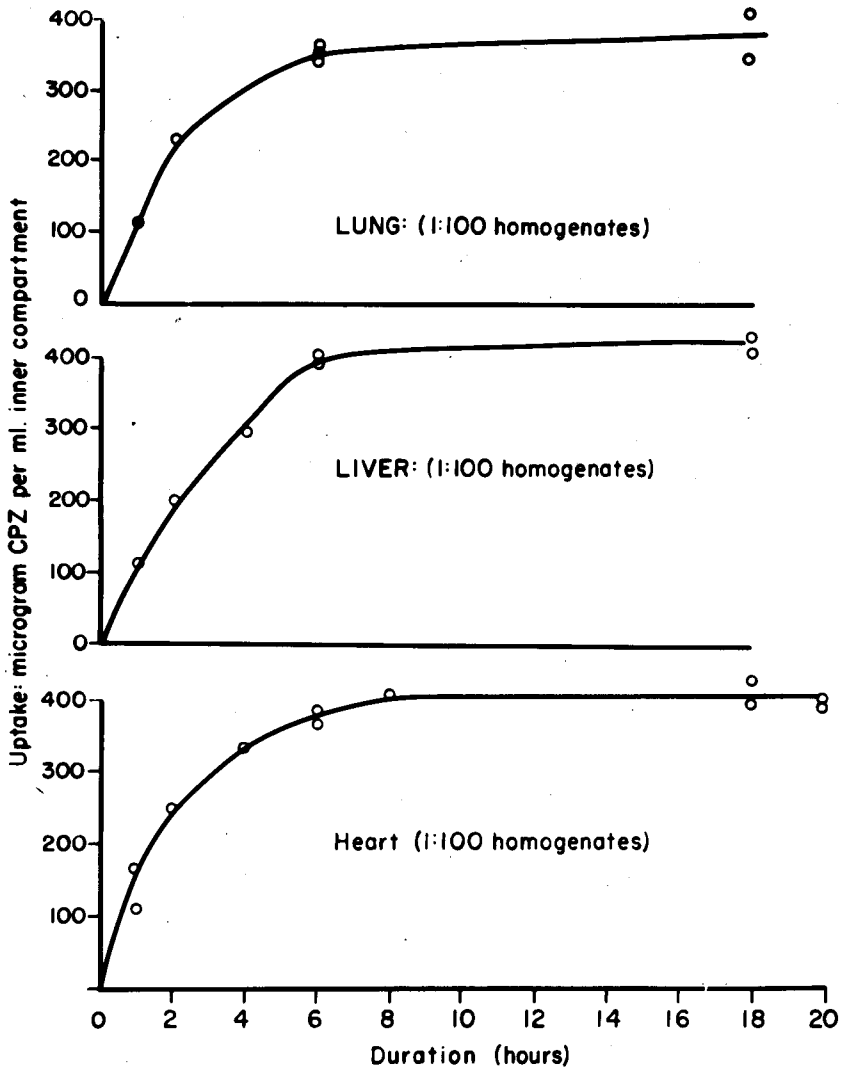


Figure 15: Uptake of CPZ by tissue homogenates 1:100; A. Lung; B. Liver; C. Heart. Mixing effected by Eberbach variable speed shaker. Room temperature, pH 7.12. Abscissa: Duration in hours; Ordinate: Uptake; microgm. CPZ per ml inner compartment.

Table 9

Uptake of CPZ by blood in comparison to cerebrum homogenate

Outer solution volumes, 30.5 ml; inner solution volumes, 10 ml. pH 7.12. Initial CPZ concentration 350 microgm. per ml. Mixing by magnetic stirring bar.

Tissue	Experiment Duration (Hours)	Uptake of CPZ (microgm/ml)*	% Bound CPZ
Control (Equilibrium)	6.	232 ± 13	
Whole blood 1:10	6	334 ± 18	47.4
Cerebrum 1:10	6	564 ± 18	64.3
Cerebrum 1:100	6	350 ± 3	40.4
Whole blood 1:10	18	407 ± 3	60.3
Cerebrum 1:10	16	832	85.9
Cerebrum 1:100	18	399 ± 10	56.1

*Figures include average ± range (2 analyses per each figure).

the initial CPZ concentration of the blood--a level far less than that expected from the comparison of the results obtained in vivo where very little CPZ was found in the blood because the tissues exhibited significant uptake (see results p. 44). Thus, CPZ was not bound by the tissues when undiluted whole blood constituted the outer medium of the equilibrium dialysis chamber.

Table 10

Uptake of CPZ by Various Tissue Homogenates in the Presence of Blood

Outer solution containing 368 ± 7 microgm. CPZ per ml consisted of 96.0 ml heparinized whole blood plus 2.0 ml thimerosal. The dialysis bags contained 10.0 ml of the respective tissue homogenates (1:100) in phosphate buffer, pH 7.12. Duration of experiment 6 hours at room temperature. Mixing effected by magnetic stirring bar.

Tissue Homogenate	Final CPZ Concentrations (microgm. per ml)	
	Outer Solution	Uptake
	312	
Cerebrum		37.2
Heart		33.0
Liver		40.4
Lung		32.0

Since 1 mM concentrations of CPZ precipitated in phosphate buffer at pH 7.4, CPZ may have precipitated in the blood (pH 7.4), an effect which would affect the results. However, under the experimental conditions, the pH of the blood at the termination of the experiment was 7.2.

Effect of Competition of Tissues

To determine whether competition between the various tissues occurred in vitro, dialysis bags containing the tissue homogenate were suspended in a beaker of buffered CPZ solution so that the ratio 1:3 total homogenates to buffered CPZ prevailed as in the uptake experiments (see Methods, p. 33). At the designated intervals aliquots were analyzed as described under procedures. The results of these experiments are shown in Table 11. Uptake of CPZ by each of these tissues appeared to be similar. Further experimentation is necessary to determine whether the slightly larger uptake of CPZ exhibited by liver homogenates is statistically significant. Thus, in these experiments, the differences in tissue uptake observed in vivo did not occur.

Affinity of Cerebrum Homogenates for CPZ

To test for affinity of the tissue for CPZ, duplicate equilibrium dialysis assemblies were prepared. One assembly served as a control. The bags were removed at the same time; one of the bags was blotted dry on tissue paper and resuspended in a chamber of fresh phosphate buffer lacking CPZ. This new

Table 11

Uptake of CPZ by Various Tissue Homogenates in Competition with each other

All tissues were prepared as 1:100 homogenates. Buffered CPZ solution, pH 7.12, constituted the outer chamber (see methods).

Tissue Homogenate	Experiment Duration	Conc'n of CPZ (microgm/ml)*		
		Outer Medium		Inner Chamber
		Initial	Final	
Expt. 1	6	325	197 ± 1	
Cerebrum				384 ± 2
Heart				358 ± 2
Liver				414 ± 16
Lung				344 ± 32
Expt. 2	6	336 ± 8	178 ± 4	
Cerebrum				316
Heart				384 ± 6
Liver				398 ± 2
Lung				362 ± 14
Expt. 3	18	372 ± 6	168	
Cerebrum				451 ± 9
Heart				462
Liver				566 ± 26
Lung				417 ± 5
Expt. 4	18	372 ± 6	172 ± 1	
Cerebrum				458 ± 21
Heart				444 ± 25
Expt. 5	20	336 ± 8	163 ± 3	
Cerebrum				404
Heart				398 ± 1
Liver				430 ± 4
Lung				334 ± 14
Expt. 6	20	336 ± 8	168	
Cerebrum				399 ± 5
Heart				402 ± 5
Liver				410 ± 3
Lung				374 ± 10

*Figures include average ± range (results for 2 analyses per each figure).

assembly was dialyzed for another period (see table 12). Buffer control assemblies, that is, the inner compartments containing buffer solution only, were subjected to the same procedure. The buffer control showed the establishment of a second equilibrium following the initial uptake experiment. This indicated that the dialysis bag was not damaged by the procedure which may have resulted in an interference with the movement of the CPZ outward from the bag.

The results of four experiments containing cerebrum homogenates are shown in table 12. From these assemblies it was observed that the unbound CPZ, present in the physical solution of the tissue homogenate, which constituted the amount of CPZ in equilibrium with that of the outer solution of the first assembly, moved across the membrane of the dialysis bag to reestablish an equilibrium. The remainder of the CPZ within the confines of the tissue compartment, that is, the bound CPZ (Table 12, column D) must have exhibited an affinity to the tissue (Table 12, column H). Otherwise, since a concentration gradient was evident, equal amounts of CPZ should have been found in the inner and outer solution of the second dialysis assemblies. The latter did not occur (Table 12, compare columns F and G).

Furthermore, when the inner compartment composed of a homogenate (1:10) prepared from the cerebrum of a cat given CPZ in vivo was dialyzed against phosphate buffer in the outer compartment, it was observed that the already bound CPZ was not

TABLE 12

AFFINITY OF CEREBRUM HOMOGENATES FOR CPZ.
OUTER SOLUTION VOLUMES OF BOTH ASSEMBLIES WERE 30.5 ml.;
INNER HOMOGENATE VOLUMES WERE 10.0 ml. ROOM TEMPERATURE. pH 7.12

DIALYSIS BAG CONTENTS	INITIAL DIALYSIS				SECOND DIALYSIS					
		CPZ CONC'N. (µg/ml)				CPZ CONC'N (µg/ml)			TOTAL AMTS OF CPZ (µg)	
	DURATION (HOURS) (A)	*UPTAKE INNER CHAMBER (B)	*OUTER CHAMBER (C)	BOUND CPZ (D)	DURATION (HOURS) (E)	*INNER CHAMBER (F)	*OUTER CHAMBER (G)	BOUND CPZ (H)	INNER CHAMBER (J)	OUTER CHAMBER (K)
Cerebrum 1:100	***4	358								
	***4	357	193	164	16	172	60.6	111	1720	1848
Cerebrum 1:100	6	344.5	231.5	113						
	6	**362	235	127	16	176	61	115	1760	1860
Cerebrum 1:100	6	345.5	205.8	140						
	6	**352.6	203	150	16	188	53.8	134	1880	1641.1
	6	**351.3	204	147	18	188	53.7	134	1880	1638
Cerebrum 1:100	18	**408	169.7	238	6	257	49.7	207	2570	1512
Buffer	6	**230	234	--	16	53.3	52.4			

* Each figure includes the average of two analysis for CPZ.
** These figures are estimated from analysis of inner and outer chambers of the second dialysis assembly. These figures multiplied by 10 (the total volume of the inner chamber) are equal to columns J plus K of the respective experiments.
*** This experiment was terminated prior to equilibrium.

released into the outer compartment (Table 13). This particular cerebrum contained pharmacological significant amounts of CPZ; that is, 104 microgm. CPZ per gm. was found following the intravenous injection of CPZ (analysis of the homogenate, 1:10, prepared from this tissue, indicated 10.2 microgm. CPZ per ml of homogenate). This cerebrum was also capable of further concentration of CPZ, in vitro (Table 13). The uptake of CPZ after twenty-four hours of dialysis was 791 microgm. per ml. It appeared that the combinations formed by CPZ and the tissue were rather firm.

Table 13

Dialysis of Cerebrum from Cat which had been Administered Intravenous CPZ

Outer solution volumes were 30.5 ml; inner solution volumes of homogenates, 1:10, were 10.0 ml. pH 7.12, room temperature.

Source	Dialysis Duration (Hours)	Outer Medium		CPZ Conc'n Inner Solution (microgm per ml)
		Contents	Initial Conc'n (microgm per ml)	
Cerebrum	16	Buffered CPZ	352	832
*Cerebrum	24	Buffered CPZ	344	791
*Cerebrum	24	Buffer	0	10.1

*Initial CPZ content 104 microgm. per gm. Analysis of tissue homogenate, 10.2 microgm. per ml.

CHAPTER V

DISCUSSION

Specificity of the CPZ Method

Recoveries of CPZ of almost 90 per cent were obtained by means of the described procedure for the quantitative analysis of CPZ from biological tissues based upon the extraction of CPZ from alkalinized ether and the subsequent oxidation of CPZ by ferric ion in the presence of sulfuric acid. The advantages of this procedure were: 1) spectrophotometric readings may be obtained in the visible range, thereby eliminating the corrections for tissue blanks which are required in the ultraviolet methods; 2) recoveries of CPZ from animal tissues, especially brain, were greater than those previously reported (39,48).

The procedure appeared specific for CPZ and other 2-chlorophenothiazines possessing a side chain on the ring nitrogen. One point to be clarified is whether or not the material referred to in the RESULTS as 2-chlorophenothiazine with a N_{10}-side chain (2-CPN) may represent chlorpromazine itself, or include also the metabolites of CPZ.

Metabolism of CPZ has not been fully clarified. Sulfoxides (69); compounds altered in the N_{10}-alkyl chain (70-72) and and N-oxide of CPZ (73); monophenols (74,75) and conjugated materials (47,74,76) were identified as metabolites of CPZ. These compounds represent, depending on species, from 22 to 35 per cent

of administered CPZ (77,78). Relatively little CPZ is excreted unchanged (77,78); the fate of from 60 to 80 per cent of CPZ is not known.

The method employed reveals, besides CPZ, chlorinated phenothiazines differing from CPZ in the N chain, as well as their sulfoxides; it does not assay compounds with major changes in the phenothiazine ring, with substitution for chlorine in the 2 position, or with chlorine present elsewhere in the ring. Thus, the assay may have measured, besides CPZ, the metabolites described above and perhaps unidentified CPZ metabolites, provided they are close analogs of CPZ.

Since glucuronide conjugation does not occur in the cat (79,80), the presence of CPZ-glucuronides in these analyses is unlikely. Reduced pharmacological activity exhibited by some of the CPZ metabolites have been reported. The sulfoxidation products of CPZ exhibited decreased pharmacological activity (81-84). Nor_1CPZ showed only slightly lesser activity than CPZ, but the primary amine analog, Nor_2CPZ, exhibited pharmacologic action about equal to that of CPZ. CPZ-NO was found less effective pharmacologically than Nor_1CPZ (83,84). Since reduced pharmacologic effects may be anticipated from some of these derivatives, there appears less likelihood for their presence.

On the other hand, metabolites generally do not remain in the tissues for long and they can be expected to appear rapidly in the blood. The fact that only low levels of 2-CPN were

found in the blood, suggests relative absence of such metabolites. Furthermore, there is no indication that CPZ is metabolized in the brain, muscle, or lung. Altogether, it is likely that the 2-CPN figures reported here for blood, brain, muscle or lung, refer mostly to CPZ. This seems certainly true for short time intervals after the administration of CPZ. Admittedly results referring to liver may bear a different connotation, since liver constitutes the major site of CPZ metabolism (39, 85-87).

Tissue Distribution of CPZ

It is interesting that these data indicate rapid transfer of intravenously administered CPZ from the blood to other tissues. Within ten minutes the blood levels of CPZ were consistently low and did not reflect the levels of the compound in other tissues. It should be stressed that immediately after injection very high levels of 2-CPN were found in the heart and in the lung. However, this accumulation was only temporary, and subsequently heart and other muscle exhibited relatively low levels of 2-CPN, while 2-CPN concentrations in the lung levelled off at a high plateau.

Data on the concentrations of CPZ in the brain after in vivo administration vary extensively (39-43, 88-90). This variation may be due to the fact that, as in the case of the present method, methods employed may not have been specific for CPZ, even though the data were expressed in terms of the latter. On the whole, the present results agree with those of Salzman and Brodie

(39), who used an ultraviolet spectrophotometric method for CPZ determination in dogs, and with those of Christensen and Wase (41), who employed S^{35} CPZ in mice; both these groups reported accumulation and concentration of CPZ in the brain.

On the other hand Fyodorov (40) who employed S^{35} CPZ in rabbits, rats and dogs; Wechsler and Roizin (42) who used an ultraviolet spectrophotometric method for rats and monkeys; and Yasuda and Aoki (43) using rats and a spectrophotometric technique, obtained low levels of CPZ in the brain. In fact, Wechsler and Roizin (42) reported "traces" of CPZ following intramuscular administration of 100 microgm. per gm. of this compound to rats. Wechsler and her associates (42,48) suggested that the results of Salzman and Brodie (39) can be explained by inadequate recovery obtained by these investigators; this argument cannot hold with regard to our results, since CPZ was quantitatively recovered from the brain by the present method. Furthermore, deJaramillo and Guth (88) found recoveries from the Wechsler and Forrest method were about 50 per cent from the brain.

Still another problem arises with regard to distribution of CPZ within the brain. Wase et al. (89), using the radioisotope method reported great differences between CPZ concentrations in various brain areas, with the highest levels found in the thalamus after single administration. A 300-fold difference was found between concentrations in the thalamus and the cortex, and a 3-fold difference between those in the thalamus and the cerebellum.

Guth and deJaramillo (90) reported in a preliminary study that the levels of CPZ in the hypothalamus and in the medulla of dogs were 7-10 times higher than those in the cerebellum. deJaramillo and Guth (88) also reported negligible amounts of CPZ in the temporal and frontal cortex of dogs forty-five minutes following CPZ injection intravenously. However, Wechsler and Roizin (42) administered 150 microgm. per gm. of CPZ to monkeys and found little difference in CPZ concentrations in the basal ganglia and in the cortex, and only traces of CPZ in the cerebellum and pons. In the present study, no such pronounced differences were found between CPZ concentrations in the brain stem, cerebrum, hippocampus and cerebellum, although the brain stem and the hippocampus retained 2-CPN (or CPZ) somewhat better than the other parts of the brain.

A particularly interesting facet of this study is that tissue levels of CPZ or 2-CPN were measured over a forty-eight hour period; this is a longer time period than that covered by earlier investigations. In several tissues 2-CPN was retained for a long time. After it accumulated in the brain 4-5 fold in one hour, its level decreased slowly; even at forty-eight hours it did not fall significantly below that corresponding to the dose administered (40 microgm. per gm.). Retention of 2-CPN in the liver was even more pronounced; moreover, the pattern of the disappearance of 2-CPN from the liver was different from that observed in the brain. After a high level of 2-CPN was maintained

in the liver for twelve hours after its administration, it decreased subsequently. While Fyodorov (40) and Salzman and Brodie (39) also found high levels of CPZ in the liver, they reported that liver CPZ content decreased rapidly in rabbits, rats, and dogs within three hours of its administration.

The third tissue which accumulated and stored CPZ (or 2-CPN) was the lung. The lung differed from brain and liver by accumulating 2-CPN more extensively, and retaining it for a longer period. Indeed, in the lung, there was no decrease in 2-CPN concentration from its peak at three hours till forty-eight hours after its administration.

Altogether, contrary to the earliest investigations, (39,43) this study indicated that the 2-CPN concentrations do not decrease uniformly in all tissues. Any tissue, therefore, might exhibit the highest CPZ or 2-CPN concentration at any specific time.

No explanation for these shifts can be given at present. Since the liver is implicated in the metabolism of CPZ by in vitro studies (47, 85-87) it may be speculated that CPZ metabolism may be induced in the liver after a latent period. Another factor may be the release of CPZ or 2-CPN from various tissues, and its storage in fat, where its level is high in one and two hours. However, fat does not seem as important a storage site for CPZ as for thiopental and pentobarbital (50-52, 91).

Correlates of Pharmacologic Activity and Brain CPZ Levels

An attempt was made in the course of this study to correlate the changes in concentrations of 2-CPN in brain which occur with time with the intensity of the pharmacologic action of CPZ. It should be stressed, first of all, that the levels of CPZ in whole blood could not be used to predict the pharmacologic actions of this compound since blood concentrations remained low and relatively constant from seven minutes to forty-eight hours after the administration of CPZ. The temperatures of CPZ-treated cats rose above those of restrained controls after twenty-four to thirty-two hours, while cerebrum concentrations of 2-CPN decreased to about 60 microgm. per gm., and thus was two or three times lower than the peak level. At that time the analgesic action of CPZ, ascribed to the depression of the midbrain reticular formation (92), was still evident, but the righting reflex had largely recovered. The analgesic effect of CPZ, tranquilization, and anorexia persisted even when the level of 2-CPN in the cerebrum decreased to 30 microgm. per gm. This, too, suggests that measurements of 2-CPN in the brain actually refer to CPZ itself, or less probably, to an equipotent, unidentified, metabolite of CPZ. Thus, not all neuropharmacologic actions of CPZ are terminated simultaneously. This could be explained if not all brain parts lost CPZ--or 2-CPN--equally rapidly; it may be suggested that tranquilization at forty-eight hours depends upon relatively high levels of CPZ at that time in the brain stem and the hippo-

campus, since the latter was associated with emotional behavior (93). This suggestion is all the more speculative since the behavioral significance of various brain areas is still obscure.

Interaction of CPZ with Tissue Homogenates

Interaction of CPZ with homogenates of different tissues was observed by means of the equilibrium dialysis technique utilizing a phosphate buffer system. The preferential uptake shown by the tissues in the intact animal was not evident by equilibrium dialysis in the buffer system employed. Homogenates of each tissue, cerebrum, heart, liver, and lung, prepared in dilutions of 1 gm. wet weight tissue per 100 ml exhibited respectively similar uptakes at equilibrium of about 400 microgm. CPZ per ml of homogenate. Approximately 225 microgm. of this CPZ or 55 per cent was bound to the tissue.

Employing cerebrum homogenate as a representative of these tissues, an increase of the homogenate concentration to 1:10 reflected in a greater uptake of CPZ by the tissue to more than 800 microgm. CPZ per ml of homogenate at equilibrium. Of this CPZ uptake, almost 700 microgm. or 87 per cent was bound CPZ. Whether 1:10 or 1:100 homogenates were used during equilibrium dialysis, the bound CPZ far exceeded the concentrations of CPZ found in the animal tissues in vivo.

It was observed from the competition experiments, that is, those experiments in which single dialysis assemblies contained the four tissue homogenates in separate cellulose bags,

that each of these tissues, cerebrum, heart, liver, and lung, exhibited essentially similar uptakes of CPZ and thus, each tissue displayed similar binding of CPZ. These experiments provided support that the differential uptake of the tissues in vivo was not evident by the technique employed in vitro.

Since similar uptake and binding of CPZ by equilibrium dialysis in the phosphate buffer system were exhibited by the four tissues, cerebrum was selected as a representative tissue to investigate some characteristics of this system for the CPZ interaction with tissue homogenates. Varying the initial CPZ concentration of the outer chamber from 0.1 to 1.0 mM resulted in a linear increase of the uptake of the CPZ by the cerebrum homogenates (1:100). The 1 mM CPZ concentration did not appear to be at the limiting level for the CPZ uptake. Negligible effects by temperature changes were observed on the equilibrium level of CPZ uptake by the cerebrum homogenates (1:100)--similar uptakes were exhibited at 0-4 °C and 24-26 °C, although the lower temperature prolonged the period to attain equilibrium, occurring at about eight hours as compared to six hours at room temperature. Heating the tissue homogenate at 90 °C for two hours or the ageing of the homogenate also showed no significant variations of the uptake or the amount of bound CPZ at equilibrium.

The combination of CPZ with the tissue homogenate appeared firm. Transfer of the dialysis bag to another assembly after an uptake of CPZ in the first dialysis assembly was

established, or the performance of the dialysis experiment upon cerebrum homogenates prepared from an animal administered CPZ, resulted in very little loss of the bound CPZ (See results section Table 12, compare columns D and H; also Table 13). Thus, cerebrum exhibited a rather strong affinity for the bound CPZ.

Many factors may have contributed to the difference of the results obtained in vitro as compared to the preferential uptake of CPZ exhibited by the tissue in vivo. Firstly, the blood flow to the various organs investigated differs markedly (94,95). Blood flows through the heart and lungs and then the blood is distributed in various percentages to the organs. In normal human subjects at rest the blood flow has been estimated (94) in per cent of cardiac output as follows: cerebral, 13, coronary, 4, splanchnic, 24, and renal, 19. Estimates available for the dog (95) indicate that the blood flow to the heart, liver, and kidneys in percentage of cardiac output was 5.2, 30.0, and 11.1, respectively. Thus, different amounts of CPZ are delivered, in unit time, to each tissue. During equilibrium dialysis, the external medium of the phosphate buffer exposed the same amount of CPZ to each of the tissue homogenates.

Secondly, some structural components or the cellular compartmentalization which may be responsible for the differential uptake and the binding of CPZ in the tissue in vivo may have been destroyed during the homogenization procedure. The presence of a blood brain barrier (96) or other membranes (97) has been

involved in the movement of drugs into and out of an organ. Freeman and Sprites (98) have shown that human erythrocytes can combine with CPZ and they have attributed CPZ effects to alterations of membrane permeability. Perhaps these alterations play a role, in vivo, preventing the further influx or outflow of CPZ; the membranes are disrupted during the homogenization procedure and thus do not participate in pertinent processes. In the absence of these elements various biochemically identified, or as yet unidentified, units of the cell present in the homogenate may then bind indiscriminantly with CPZ.

Several cellular substances or components have been reported as capable of interaction with CPZ, in vitro (98-103). Harris, Saifer and Volk (100) reported CPZ bound significantly to the ganglioside strandin, and also to chrondroitin sulfuric acid and heparin. Yagi et al. (101) reported a complex formation between CPZ, at a concentration of 10^{-3} M, and the isoalloxazine component of flavins. Dingell et al. (102) reported that CPZ was bound primarily to the particulate matter of cells. Mitochondria isolated from brain were found to exhibit greater affinity than liver mitochondria for CPZ (103). The type of combination of CPZ with a cellular constituent appeared to be firm. The results obtained from these experiments indicated that the tissues had a strong affinity for CPZ. In the absence of the structural components and differential blood flow present in vivo the cellular components may be capable of interacting in vitro to a different

extent than that seen in vivo.

Ehrenpreis (99) had reported that CPZ exhibited binding capabilities to the protein he isolated by fractionation with ammonium sulfate and which at first (61) was considered as a "receptor" for acetylcholine but later (104) as the "drug binding component of conducting membrane only". Histochemical results obtained by Ehrenpreis (104) indicated that the "receptor" protein was present in close proximity to, or a part of, the membrane. He suggests the possibility that this protein may be of membranous origin. Spirtes and coworkers (98, 105-107) have also implied that CPZ effects were related to alterations of membrane permeability. The location of CPZ activity on the membrane may be related to the fact that cell membranes are composed primarily of lipid, that is, phospholipid.

Some physical and chemical characteristics described by Ehrenpreis (104) for the drug-binding protein were that it did not gel on heating and that it contained a significant amount of phospholipid. In the equilibrium dialysis experiments reported at present, brain homogenates heated in phosphate buffer at pH 7.1 showed no apparent alterations of the binding of CPZ. Perhaps the cellular structure described by Ehrenpreis may be implicated in the binding of CPZ in the present experiments.

Some consideration may also be given that CPZ, a lipophilic compound, may have become bound to the lipoproteins or to the sphingolipids, for example, the cerebrosides or gangliosides.

Cerebrosides and gangliosides occur in tissues other than in brain. The gangliosides have been found in most parenchymatous tissues, for example, spleen and erythrocytes. However, the tissues investigated exhibit different ganglioside concentrations; on the basis of phosphorus per 100 gm. fresh tissue, rat liver contained one-half the amount of sphingomyelin found in rat heart and about one-thirthieth that of cat brain (108). Therefore, it is unlikely that the binding effects in vitro may be attributed solely to the binding to gangliosides.

An explanation offered by Harris et al. (100) for the significant binding of CPZ to strandin, a rather specific ganglioside from the brain, was that the reaction occurred between the carboxyl group of the neuraminic acid in the ganglioside and the overall positive charge of CPZ. The view that N-acetyl neuraminic acid may be involved in the role of the gangliosidic binding of CPZ is possibly supported by the work reported by Irwin et al. (109). These investigators found that gangliosides prevented the blocking activity of CPZ on acetylcholine stimulated frog rectus muscle. Partial destruction of the ganglioside activity against the CPZ action occurred when N-acetylneuraminic acid was removed by acid hydrolysis from the ganglioside.

When the role of mucopolysaccharides, for example, hyaluronic acid, heparin, and the chondroitin sulfates, which constitute integral components of the mucoproteins, is elucidated, perhaps the indiscriminate binding exhibited by the tissues in

vitro may be clarified. Chondroitin sulfate has been found present in the heart valves, the skin, the cartiage, tendons and bones. Heparin is also present in various animal tissues (110). The homogenization procedure may release these substances from certain forces existing in vivo which exert a differentiating effect for the combination of CPZ with that particular organ.

The results obtained with blood in vitro could possibly be extrapolated with the results obtained in vivo. Uptake and binding of CPZ by blood, when whole blood (1:10) constituted the inner compartment during equilibrium dialysis, was much less than that exhibited by comparative amounts of the other tissues. Thus, the low uptake of CPZ by blood in vitro may be considered as favorably comparing with that found in vivo.

However, when undiluted blood containing CPZ constituted the outer compartment which was used against tissue homogenates as the inner chamber; very little CPZ penetrated the dialysis bag--that is, not even the equilibrium concentration in the inner compartments was attained. This is unlike the findings in vivo, in which the tissues displayed a rapid uptake of CPZ from the blood. CPZ may have been, however, precipitated by the blood and this would prevent dialysis of CPZ from the outer to inner chamber. CPZ, at 10^{-3} M, was precipitated in phosphate buffer at pH 7.4--the approximate normal pH of blood. The white crystalline precipitation of CPZ was not observed in the blood, in vitro. At the termination of the experiment the blood had a pH of 7.2--

a pH at which CPZ is soluble. Furthermore, CPZ has a pKa of 8.2 (111). Thus, even at pH 7.4 enough CPZ should have been ionized to effect an inward migration to the binding sites of the tissues.

It is conceivable then that the whole blood, in vitro, could bind the CPZ, if CPZ were presented to it first, and if the factors present in vivo, which induce the release of CPZ from the blood to the tissues, were not active in vitro. In vivo, blood was capable of initially concentrating approximately 300 microgm. CPZ per ml (see Fig. 10, Results), even though for a short duration.

Also, it should be remembered that in vitro, undiluted whole blood containing CPZ was dialyzed against tissues at 1:100 dilution. This tissue concentration difference may in itself account for the failure of the blood to release CPZ in vitro and may be a factor which could be reconciled by comparing blood concentration versus tissue concetration in vivo.

The blood used in these experiments contained heparin which had been added to prevent its coagulation. The binding of CPZ by heparin has been reported by Harris et al. (100). Perhaps the presence of heparin may explain some of the effects toward the uptake and binding of CPZ by the blood in vitro. However, a conclusive interpretation of the heparin effect on the blood-CPZ interaction is precluded by the influence which may be exerted by the following observations:

Firstly, the gradual loss of anticoagulant and anti-complementary activities of heparin incubated with a washed red blood cell suspension was considered as indicative of a disappearance and possible absorption of heparin (112). Thus, the binding of CPZ observed in these experiments with the blood may not have been influenced by the presence of heparin.

Secondly, heparin is normally found in varying concentrations in the liver, lung, spleen, muscle, heart, and other tissue, especially the mast cells (110). The destruction of the mast cell by CPZ reported by LeBlanc (113) may cause the release of a rich supply of heparin. But, homogenates of liver and lung, both known to contain large amounts of heparin (110), exhibited similar uptake and per cent bound CPZ as that obtained by the other tissues which contain lesser amounts of heparin. This consideration tends to minimize the influence that the endogenously produced heparin may have had toward the effect of the binding of CPZ by the blood or the tissue.

Thirdly, although under normal conditions, heparin is not in the free state in the body, it is known that free heparin may appear in some abnormal conditions. Dogs in anaphylactic shock can release heparin from the liver (110). The withdrawal of the large volume (about 110 ml) of blood from the cat, a small animal, produced severe shock. Conceivably, heparin may have been released from the liver of the cat so as to attain a significantly high titer in the blood. The levels of the endogenously

produced heparin plus that added to prevent the coagulation of the blood may have been significantly high so that the binding of CPZ by the blood may be attributed to the presence of heparin in these experiments.

Further interest in binding effects of CPZ with heparin could possibly center on the glucuronic acid or other acid moieties present in heparin. Since in these experiments, homogenates, and not purified biochemically indentified constituents were employed, perhaps an explanation for the indiscriminate in vitro binding observed here may be due to the action of carboxyl groups of various compounds such as the N-acetylneuramic acid, and the glucuronic acids of heparin, hyaluronic acid and the chondroitin sulfates which may combine with the overall positive charge of CPZ; these groups may be indiscriminately distributed in all tissues studied.

Another consideration pertaining to the in vitro study of blood is that CPZ can, in vitro, combine with the red blood cells and alter the osmotic permeability of the erythrocytic membrane (98). Perhaps, once the red blood cells have taken up CPZ, the altered osmotic permeability prevented the release of CPZ to the tissue homogenates. The explanation of the binding of CPZ by the erythrocytes in vitro merit further investigation.

Thus, many questions are presented from the results obtained, in vitro. It is interesting to attempt to identify the component or components that exist in vitro and in vivo which

bind CPZ. Extension of results obtained from equilibrium dialysis upon purified proteins isolated from the tissue could provide information to calculate the energy of association of each ion as well as the number of sites available for the binding of the ion. Approaches through the combination of CPZ with purified substances have been reported (100). The attempt to isolate the CPZ--receptor by fractionation of the CPZ bound tissue presents many difficulties, perhaps the most important is that through means of chemical differentiation the CPZ may separate from its receptor component. Some preliminary experiments have been attempted in this direction.

CHAPTER VI

SUMMARY

1. A spectrophotometric procedure for the quantitative analysis of CPZ from biologic tissues based upon the extraction of CPZ into alkaline ether and subsequent oxidation by ferric ion in the presence of concentrated sulfuric acid is described. Recoveries of CPZ from brain by means of this method amounted, in vitro, to 86.6 ± 6.6 per cent.

2. The procedure was specific for CPZ and other 2-chlorophenothiazines possessing a side chain on the ring nitrogen. Changes in the phenothiazine nucleus or substitutions for chlorine in position 2 resulted in colors which differed from the cherry-red chromophore. Spectra for many of these compounds were established. Thus, besides CPZ, other related compounds can be analyzed in tissues by means of this method.

3. Tissue levels of CPZ following its intravenous injection into cats were determined by this method. Immediately following the administration of CPZ, 40 microgm. per gm., levels near 1 mg. per gm. could be determined in the heart and in the lung. There was a rapid shift of CPZ from the blood to other tissues.

4. CPZ concentrated four or five fold in the lung, liver, and brain at one hour after its administration; at which

time the content of the heart and other tissues was relatively low. CPZ content remained high in the liver and in the lung for twelve and forty-eight hours respectively.

5. Pharmacological actions of chlorpromazine did not terminate simultaneously. Hypothermia and analgesia seemed associated with the 30-50 microgm. per gm. CPZ levels in various parts of the brain; tranquilization appeared to require less CPZ.

6. Utilizing equilibrium dialysis, the interaction of CPZ with the various tissue homogenates occurred in a phosphate buffer system.. The preferential uptake for CPZ exhibited by the tissues in vivo was not evident in vitro, and dialysis of the four tissues in single assemblies did not show competition for CPZ uptake.

7. Cerebrum homogenates exhibited a strong affinity for the CPZ. Negligible amounts of the bound CPZ were released from the tissues when cerebrum homogenates containing CPZ were dialyzed against phosphate buffer.

8. A discussion of the various structural components to which CPZ may bind was presented.

APPENDIX I

Preparation of Phosphate Buffer Solutions Used for the Equilibrium Dialysis Experiments

Stock solutions of the sodium monohydrogen phosphate, (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) prepared as described in the text were mixed in the proportions indicated below. Th pH of the resulting solution was checked on the Beckman Zeromatic pH meter.

Na_2HPO_4 (ml)	KH_2PO_4 (ml)	pH
9.00	1.00	7.648
8.00	2.00	7.347
7.00	3.00	7.146
6.00	4.00	6.976
5.00	5.00	6.813

This table is a portion of that found in Gortner, R.A. and Gortner, W.A.: Outlines of Biochemistry, 3rd Ed., John Wiley & Sons, Inc., New York, 1949.

APPENDIX II

The differences, for all time periods investigated, between the cerebrum and the cerebellum, and between various brain stem areas and the hippocampus on one hand, and the cerebellum on the other, were found significant at $p > 0.05$ level.

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APPROVAL SHEET

The dissertation submitted by Bernard Gothelf has been read and approved by five members of the faculty of the Graduate School of Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

12/30/1964
Date

Signature of Advisor