UVEAL PIGMENT AND PHENOTHIAZINE COMPOUNDS*

BY *Albert M. Potts*, M.D.

I. HISTORICAL REVIEW

PHENOTHIAZINE DERIVATIVES were introduced to medical practice when methylene-blue (Figure 1, II) was introduced as an analgesic by Paul Ehrlich in 1890.[1] Much later the parent substance itself (Figure 1,I) was used as a not particularly effective urinary antiseptic[2] and as an anthelminthic.[3] The present-day avalanche of phenothiazines did not begin until the French firm of Rhône-Poulenc prepared a compound in its long anti-histamine series which was highly effective against histamine effects and possessed a phenothiazine nucleus.[4] This substance, phenergan (3277 RP) (Figure 1, III), was first reported in 1946, and in the same year it was recognized that another compound in the series, diethazane (2987 RP) (Figure 1, IV), was useful in combatting the tremor of Parkinsonism.[5] With both applications the side effects of somnolence and diminution of motor activity were recognized, and phenergan plus a new substance, 4560 RP (Figure 1, V), were utilized in 1951 as constituents of the "lytic cocktail" of Laborit and Huguenard to produce effective hypothermia.[6] Finally, in 1952, this last compound (now known to us as chlorpromazine) was used for the treatment of agitated psychotics and found to be highly effective in dispelling agitation and excitation.[7]

Rapid cognizance of the unusual psychotropic action of these substances was followed by wide acceptance and human consumption in undreamed-of quantities. Nevertheless, no serious eye effects from phenothiazines were reported until 1956 when Kinross-Wright[8] described profound loss of vision accompanied by pigmentary retinopathy following the use of a new phenothiazine designated NP-207

*This research was supported in part by grants from the National Institute for Neurological Diseases and Blindness, USPHS (Grant #B2523); the National Council to Combat Blindness (Grant #G262); and the American Cancer Society, Illinois Division.

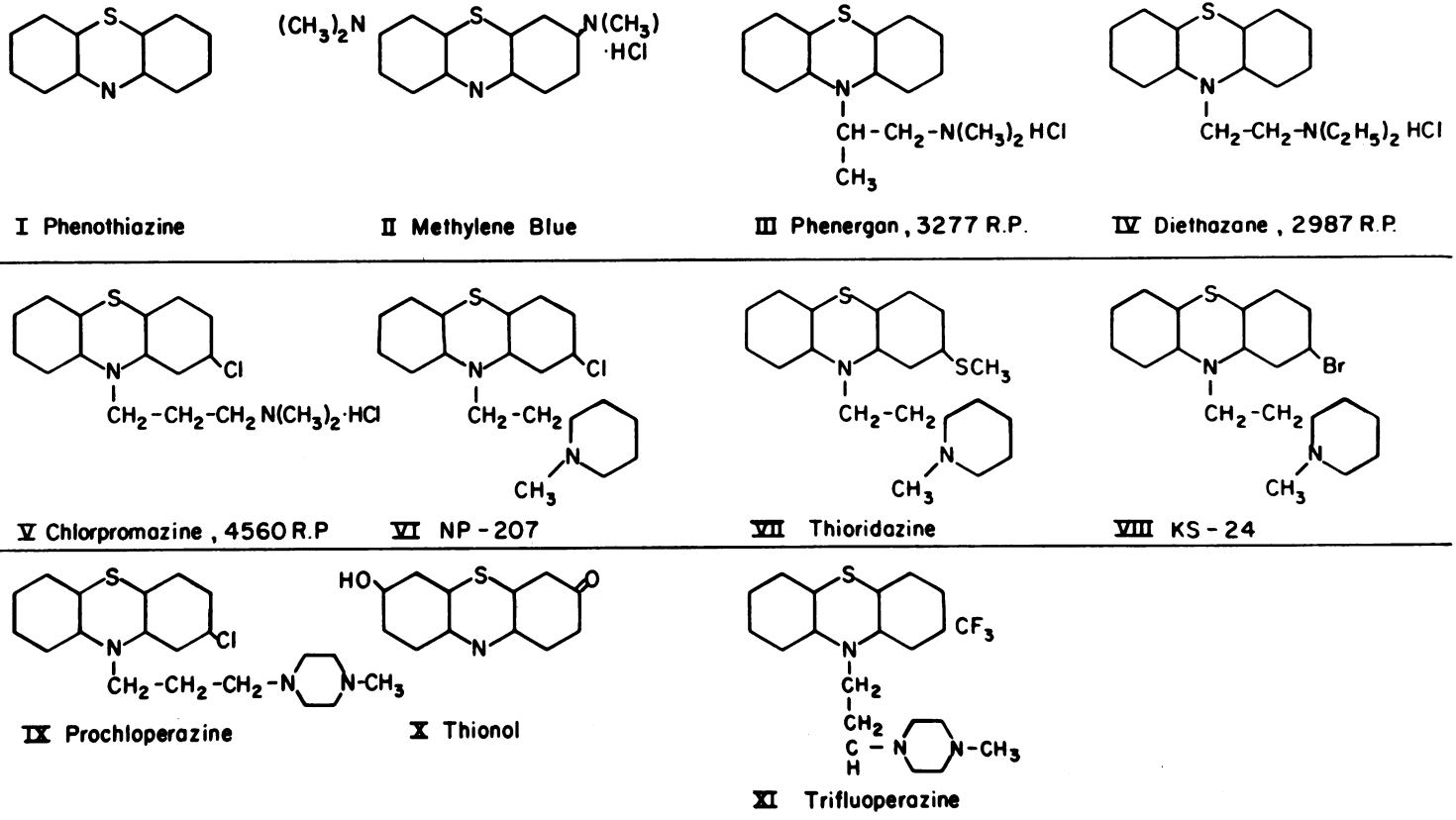


FIGURE 1. STRUCTURES OF PERTINENT COMPOUNDS

(Figure 1, VI). Eye findings in these patients were reported by Goar and Fletcher.[9] There was little doubt that the drug was responsible, for 28 of 32 patients developed retinopathy. Further reports from Verrey in Zurich[11] (24 of 78 patients) and Rintelen in Basel[12] (24 of 62 patients) fortified the conclusion. Symptoms included loss of visual acuity, night blindness, and late development of "pepper and salt" pigmentary retinopathy. Eye findings were observed in a patient who had as little as 9 g. of the drug at an average rate of 400 mg. per day. In all cases there was marked tendency for recovery after cessation of the drug, but in the more severe cases recovery was only partial. A lack of abnormal electroretinographic findings was reported by Burian and Fletcher.[10] However, the electroretinograms were performed two years after administration of the drug.

Four years later a second phenothiazine tranquilizer, thioridazine (Figure 1, VII), was found to produce a pigmentary retinopathy comparable to that of NP-207, although much higher doses—in the neighborhood of 4 g. per day—were required. Four cases were reported from Cleveland,[13,14] and at least two others have appeared elsewhere.[15,16]

As yet no eye from a case of phenothiazine retinopathy has been examined histologically, so any inferences on the site of the involvement must be based on indirect evidence. Such indirect evidence is from the extinguished electroretinogram in the early stages of thioridazine toxicity[14] indicating early and widespread involvement of the outermost retinal layer—the receptor cells. A second piece of evidence is the demonstration of early pigment changes exterior to the retinal pigment epithelium by inspection with the television ophthalmoscope and deep red monochromatic illumination at 650 mμ.[14] Thus a logical interpretation of the late ophthalmoscopic findings is that we are dealing with a primary choroiditis and subsequent retinal involvement.

Shortly after the findings of clinical toxicity the laboratories of the Sandoz Corporation in Basel began a series of attempts to reproduce the toxic phenomena in lower animals.[17] Changes in fundus appearance of cats were, indeed, produced with NP-207 and its 2-brom analog designated KS-24 (Figure 1, VIII). However, these changes in appearance were always confined to the area of the tapetum lucidum, and no significant histopathological changes were found. No such funduscopic changes could be produced with maximum tolerated doses of thioridazine, chlorpromazine, or several other related drugs.

Most striking of all were the results of injecting C^{14} labelled KS-24 and chlorpromazine into cats. It was found that after 24 hours both compounds appeared in "retina" (the specimen included choroid)

and iris in concentrations 9 to 15 times that expected on uniform distribution throughout the body. The fact of storage was most surprising and the equal storage of toxic and non-toxic phenothiazines was puzzling. It was in an attempt to throw more light on these phenomena that the present studies were undertaken.

II. STUDIES ON THE DISTRIBUTION OF PHENOTHIAZINES IN NORMAL ANIMALS

A. INTRODUCTION

The writer was privileged to visit the Sandoz laboratory in Basel in the autumn of 1959 and while there was shown radioautographs of eyes of animals which had been given labelled phenothiazines. It was immediately apparent that there was no storage of radioactive compound in the retina as described in the Sandoz report, but that all radioactivity had been localized, nearly uniformly, in the entire uveal tract. On inquiring it was learned that all animals used had been pigmented ones. It was suggested by me that the storage might be a function of the presence of uveal pigment and that experiments should be done in parallel on pigmented and on albino animals. It was agreed that such experiments would be done in both laboratories. In addition, our laboratory wanted more accurate information on normal distribution in many tissues and particularly those of the eye.

B. EXPERIMENTAL WORK

Animals used by us were Dutch Midget (black and white) rabbits, New Zealand (brown) rabbits, pure-bred albino rabbits, and Syrian golden hamsters.*

Compounds studied were prochlorperazine (Figure 1, IX) (courtesy, Smith, Kline and French, Incorporated), phenothiazine, and chlorpromazine (purchased from The Radiochemical Centre, Amersham, England). All compounds were labelled with S^{35} and had high specific activity. On receipt the prochlorperazine contained 3.60 μc/mg., the phenothiazine 52 μc/mg., and the chlorpromazine 22 μc/mg.

Prochlorperazine and chlorpromazine were used in aqueous solution at a concentration of 2 mg./ml. Phenothiazine was used at the same concentration in either commercial olive oil or 70 per cent ethanol.

In all animals the dose was 5 mg./kg. In rabbits the aqueous and alcoholic solutions were given via an ear vein; in hamsters they were injected intraperitoneally. In both animals the phenothiazine in oil was given intraperitoneally.

*Abrams Small Stock Breeders, Chicago, Illinois.

At the end of the experimental period the animals were sacrificed by injection of an overdose of pentobarbital. The eyes were enucleated. In the case of the rabbits aqueous was withdrawn with a 27-gauge needle on a 1-ml. syringe. A circumferential cut was made anterior to the equator and the posterior segment was separated. A sample of vitreous was collected by aspiration into a 1-ml. syringe without needle. The retina was then brushed together with a moist camel's hair brush, cut free at the nerve head, and lifted free with forceps. The choroid was carefully scraped from the sclera with the edge of a miniature Beaver scalpel (no. 67 blade). The lens was separated from the zonular attachment, following which iris was separated from ciliary body by careful tearing along the natural line of separation. The cornea was then cut free from the small scleral rim attached to it.

In addition to the portions of the eye, samples of other tissues were taken. Although all tissues were not taken from every animal, samples were analyzed from liver, lung, spleen, adrenal, kidney, pancreas, skeletal muscle, mesenteric fat, cerebral cortex, and choroid plexus. In addition, a sample of whole blood was taken by cardiac puncture immediately after the death of the animal.

The dissection of the hamster differed from that for the rabbit in some respects. Because of the small size of the eye, the work was done under 10 to 25 times magnification with a dissecting microscope. The eye was placed in a petri dish on Whatman no. 50 filter paper, moistened with saline to prevent loss of weight from the separate portions by desiccation. Because of the small size of the hamster eye, aqueous and vitreous samples were not taken and no attempt was made to separate iris from ciliary body.

Combustion and preparation of radioactive sulfate proceeded as follows. As each tissue sample was obtained it was placed on a previously weighed square of glass made by cutting an ordinary 24 mm. square no. 1 microscope coverslip into quarters with a diamond point. The glass plus tissue was immediately weighed by an assistant to the nearest tenth of a milligram on a torsion balance and then dropped into a 25 ml. Kjeldahl flask. The liquid samples had their volume measured in the syringe and were introduced into the Kjeldahl flask directly. Three ml. of 60 to 70 per cent perchloric acid were added and the tissue digested by boiling on a Kjeldahl rack behind a shield of safety glass. Because analysis was to be made for sulfate, the more customary (and safer) sulfuric acid containing combustion mixtures could not be used. Despite great care in handling the more than one thousand combustions, two explosions did occur for reasons which are not clear. No injuries resulted.

When combustion was complete and the solution in the flask was water-white the flask was allowed to cool and 1 ml. of a 4.5 per cent $Na_2SO_4.10H_2O$ solution (containing 20 mg. of carrier sulfate) was added. The contents of the flask were then transferred quantitatively to a special round-bottomed centrifuge tube made by shortening and putting a lip on an ordinary heavy-walled, 150-mm. test tube. Volume was made up to approximately 6 ml., and 1 ml. of a 4 per cent solution of $BaCl_2$ was added (containing twice the theoretical amount of Ba^{++}). After mixing thoroughly, the precipitate was spun down and the supernatant decanted. The precipitate was washed once by resuspending in 6 ml. distilled water and, after centrifuging, was washed again in 50 per cent aqueous acetone. The precipitate was dried in an oven at 110 degrees and was then completely dispersed in successive portions of a thixotropic gel–toluene scintillant preparation of the following composition:

1.0 liter toluene (reagent)

42 ml. "liquifluor," Pilot Chemical Company, Watertown, Massachusetts. (This is a toluene solution containing 100 g. 2,5-diphenyloxazole (PPO) and 1.25 g. p-bis [2-(5-phenyloxazolyl)] benzene (POPOP) per liter.)

25 g. thixotropic gel powder, Packard Instrument Company, La Grange, Illinois. (This is an especially pure and finely divided silica, sold under the name of Cab-O-Sil by Godfrey L. Cabot, Incorporated, Boston, Massachusetts.)

When preparing this mixture, half the toluene plus all the liquifluor and all the gel are mixed at low speed in a Waring blendor. The mixture is transferred with the aid of the remaining toluene to a stock bottle. Total volume of gel used was 17 ml. As each portion of suspension was obtained, it was transferred to a standard 22 ml. screw-cap vial (Wheaton Glass Company). Standards were prepared by combusting 0.1 ml. sample of a 1 to 100 dilution of the original radioactive material and by carrying through the precipitation and suspension described above. All samples were counted in an automatic scintillation counter at zero degrees (Packard Tri-Carb). The settings used were identical with those used for counting C^{14}. A vial containing scintillant and gel, but no precipitate, was used as blank, and the operation of the counter was checked by counting a commercial C^{14} standard in a sealed vial.

C. RESULTS

By comparison of counts in the tissue samples with the standard, all results have been calculated as micrograms of phenothiazine com-

pound per gram wet weight of tissue. A density of 1.0 was assumed for the liquid samples.

It should be appreciated that the experimental times were selected to demonstrate the fate of uveal phenothiazines. Although 5 mg./kg. is about 15 times the normal human tranquilizing dose for chlorpromazine (but within the range sometimes used for psychotic patients) and 70 times that for prochlorperazine, levels of drug in non-uveal tissue of our animals were usually two or three orders of magnitude lower than uveal levels by 24 hours. Such values are at the margin of reliability of the method. Net counts (after subtraction of background counts) for aqueous, vitreous, retina, sclera, lens, cornea, choroid plexus, pancreas, and skin were usually less than 1 times the background. Thus counts for these tissues were subject to wide statistical variation and attention should be focused on order of magnitude rather than smaller variations.

For this reason, too, presentation of results in a graphic form is difficult. On a linear scale, depiction of non-uveal tissues is impossible. A logarithmic scale tends to imply accuracy in the lower values which they do not actually possess. Thus tabular presentation must be resorted to frequently.

In all instances except two, figures represent mean values for at least 2 animals. Experiment C of Figure 2 and Table 1, and experiment C of Figure 4 represent a single animal, but even here figures for all eye parts represent the mean of two determinations.

That prochlorperazine concentrates in the uveal tract of pigmented rabbits is apparent from Figure 2. The dose of 5 mg./kg., if evenly distributed throughout the animal and if no drug were lost by excretion, would give a tissue level of 5 μg./g. of tissue (mean distribution value). The level in the choroid after a full 24 hours was 14 times that high and even after two days it was 11 times the mean distribution value. Concentrations in ciliary body and iris were lower than in choroid, but these, too, were well above the mean distribution value even after two days. One experiment to investigate how much uveal levels can be raised is shown in part C of Figure 2. The animal was given three doses of 5 mg./kg. at 48-hour intervals and was killed 24 hours after the last dose. The choroid contained 154 μg./g., more than twice the level from a single dose. Ciliary body and iris were correspondingly high.

Table 1 demonstrates how completely this phenomenon is confined to the uvea. Only in urine samples was the mean distribution value approached, presumably, because of excretion of the drug by this

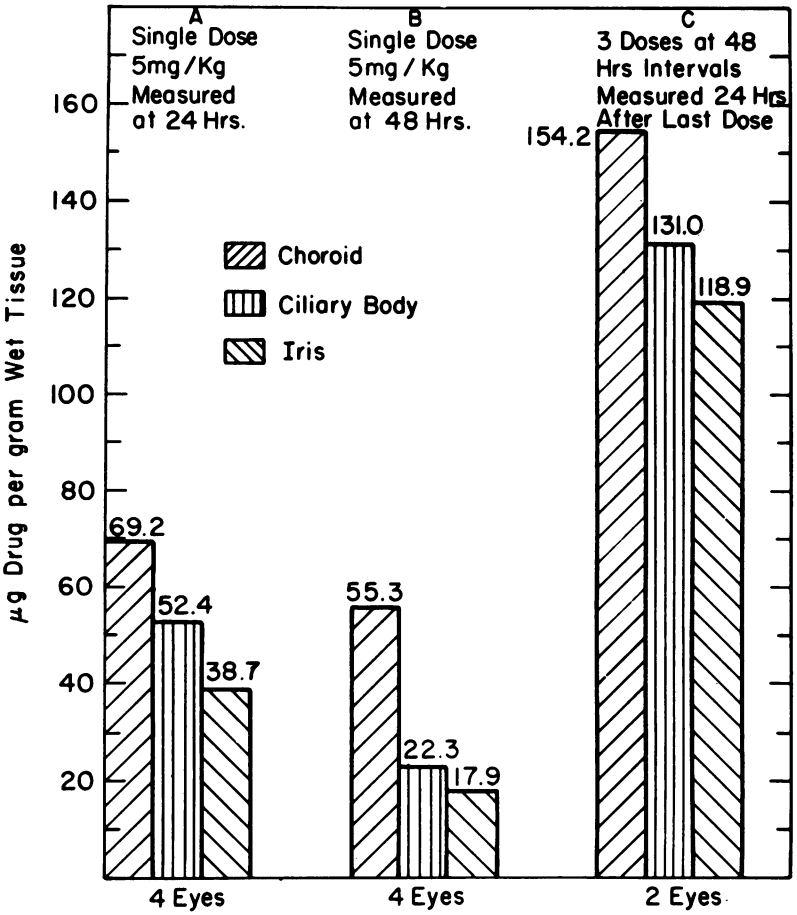


FIGURE 2. CONCENTRATION OF PROCHLORPERAZINE IN THE UVEAL TRACT OF PIGMENTED RABBITS

route. Kidney and liver although involved in excretion did not reach 5 μg./g.

Except for retina all of the non-uveal portions of the eye were well below 1 per cent of the choroidal concentration and one speculates whether the values for retina and sclera may not be due in part to contamination from adjacent choroid. In none of the other tissues recorded in Table 1 were there noteworthy findings. Brain was as low as the other tissues—a requirement if CNS action is not to be unduly prolonged.

TABLE 1. CONCENTRATIONS OF PROCHLORPERAZINE IN NON-UVEAL TISSUES OF PIGMENTED RABBITS

	(All results as μg. drug/g. wet weight tissue)		
	A	*B*	*C*
Tissue	*Single dose 5 mg./kg., measured at 24 hours, 2 animals*	*Single dose 5 mg./kg., measured at 48 hours, 2 animals*	*3 doses of 5 mg./kg. at 48-hour intervals measured 24 hours after last dose, 1 animal*
Choroid*	69.2	55.3	154.2
Aqueous	0.05	0.03	0.06
Vitreous	0.01	0.03	0.04
Retina	0.93	0.61	1.95
Lens	0.11	0.42	0.22
Cornea	0.34	0.35	0.37
Sclera	0.47	0.44	1.25
Urine	4.20	5.64	10.68
Liver	2.18	1.36	0.67
Skeletal muscle	1.27	0.43	0.49
Kidney	1.10	0.17	1.55
Choroid plexus	0.97	0.12	1.94
Brain	0.25	0.56	0.27
Spleen	0.83	0.68	1.99
Lung	0.79	0.84	1.58
Skin	0.77	0.47	0.25
Pancreas	0.48	0.22	1.05
Blood	0.06	0.09	2.57

*Choroid values given for comparison.

The striking contrast in affinity of the albino uvea for the drug is shown in Figure 3. The single dose of 5 mg./kg. and experiment time of 24 hours were identical for both groups of animals. All three uveal samples in the albino were well below the mean distribution value and were in the neighborhood of 1/50, the concentration of the corresponding tissue in the pigmented animal. Values for non-uveal tissues both in the eye and the rest of the animal did not differ in any significant manner from those given for the 24-hour pigmented animals in Table 1 (A).

In addition to the prochlorperazine experiments, concentration of chlorpromazine and phenothiazine itself was studied in pigmented rabbits. Figure 4 shows that uveal storage of chlorpromazine was even greater (1.5 to 2 times) than that of prochlorperazine, and phenothiazine was not stored selectively at all. Although Figure 4C represents a single rabbit, the lack of storage was confirmed in the hamsters (see below) and may be considered as well established.

It should also be noted that whereas for prochlorperazine ciliary body stored less than choroid but more than iris, the position of ciliary

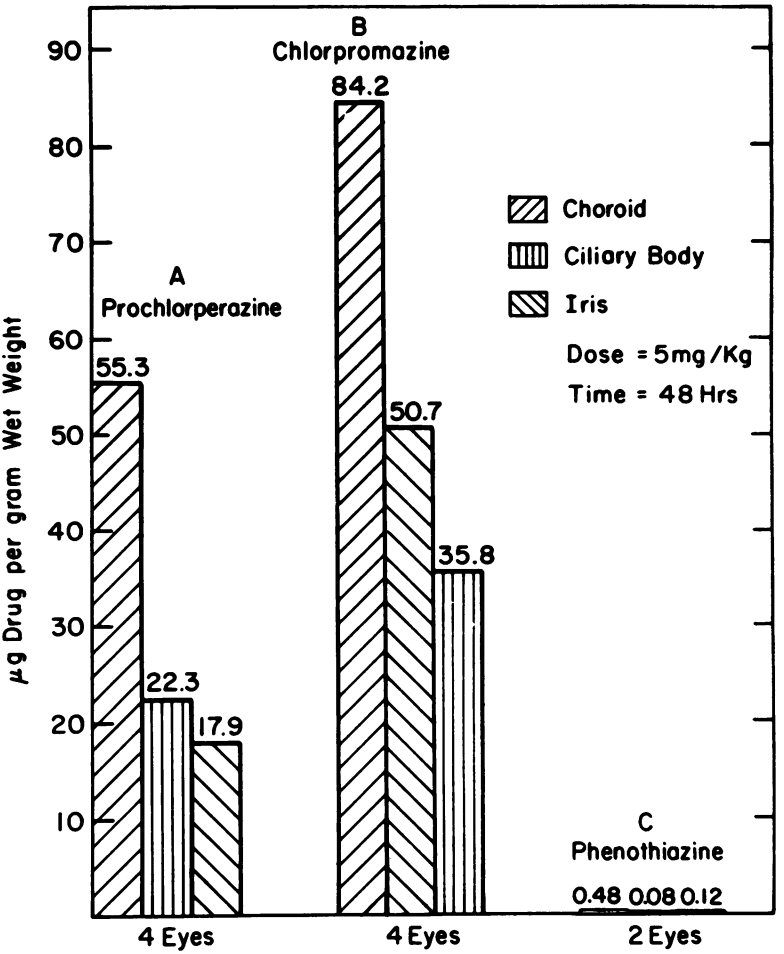


FIGURE 3. COMPARISON OF STORAGE OF PROCHLORPERAZINE IN THE UVEAL TRACT OF PIGMENTED VS. ALBINO RABBITS

body and iris was reversed for chlorpromazine. This was a consistent finding (cf. Figure 6). For behavior of other tissues see below (Table 4).

The three phenothiazine compounds were given to pigmented hamsters in the same dose of 5 mg./kg. used for the rabbits. Because of difficulties of dissection iris and ciliary body were analyzed as a single sample. Results are presented in Figure 5. Whereas the affinity of chlorpromazine and the lack of affinity of phenothiazine for uveal

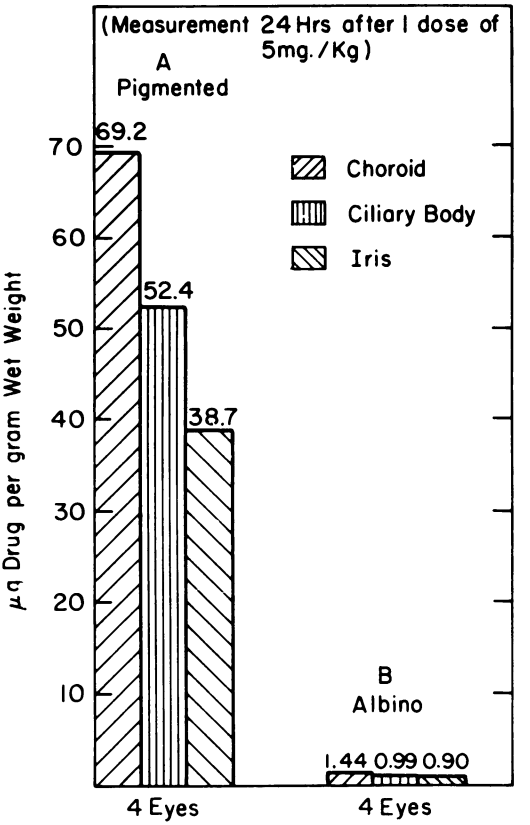


FIGURE 4. STORAGE OF THREE PHENOTHIAZINE COMPOUNDS IN THE UVEA OF PIGMENTED RABBITS

tissue is comparable in rabbits and in hamsters, the storage of prochlorperazine in the hamster reaches unusually high proportions. The mean storage by choroid in 48 hours of 280 μg./g. was 4 times that for chlorpromazine in the hamster and 5 times that for prochlorperazine in the rabbit.

Non-uveal tissues studied in the hamsters are recorded in Table 2. The retinal values were consistently high, and proximity to choroid may again explain the finding. The high values for phenothiazine in abdominal organs may be explicable on the basis of fat solubility and either coating of abdominal organs or phagocytosis. The values in group D were included but, as can be seen, the choroid level at 24 hours was lower for the same compound at 48 hours. The abdominal

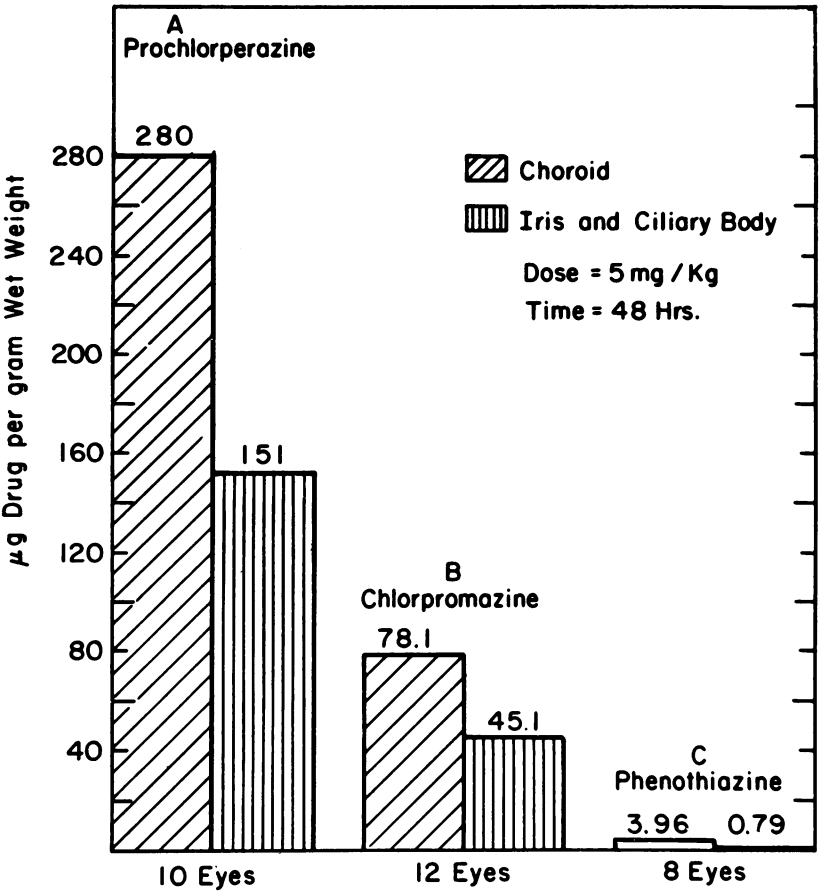


FIGURE 5. UVEAL STORAGE OF PHENOTHIAZINE COMPOUNDS IN THE HAMSTER

tumor of these animals (see part III) may make such a comparison invalid.

In one additional set of studies the long-term persistence of chlorpromazine in the pigmented rabbit was investigated. The time course of uveal concentration is shown in Figure 6. This semilogarithmic display shows that concentration in each of the uveal portions behaved in a biphasic manner. The early phase from zero time to 5 to 10 days showed a relatively rapid loss in concentration. If the straight-line portion of this curve is examined graphically, a biological half life for the compound may be calculated for the early phase (Table 3).

However after 5 to 10 days the second phase supervened. As can

TABLE 2. CONCENTRATIONS OF PHENOTHIAZINES IN NON-UVEAL TISSUES OF HAMSTERS

	(All results as μg. drug/g. wet weight tissue)			
Tissue	*A Prochlorperazine, 48 hours, 5 animals*	*B Chlorpromazine, 48 hours, 6 animals*	*C Phenothiazine, 48 hours, 4 animals*	*D* Prochlorperazine, 24 hours, 6 animals*
Choroid†	280	78.1	3.96	243
Retina	15.1	1.17	0.64	—
Lens	—	0.04	0.50	0.09
Cornea	—	—	0.69	2.42
Blood	0.35	0.03	1.03	0.15
Liver	—	—	2.35	4.21
Kidney	—	—	1.31	1.13
Lung	—	—	4.58	1.32
Spleen	—	—	15.4	0.56
Mesenteric fat	—	—	5.99	—

*These animals were bearing abdominal melanomas and may be anomalous.
†Choroid values given for comparison.

TABLE 3. BIOLOGICAL HALF LIFE OF CHLORPROMAZINE IN RABBIT UVEA (EARLY PHASE)

Dose = 5 mg./kg.	
Tissue	*Half life (Days)*
Choroid	4.0
Iris	2.8
Ciliary body	3.0

be seen from the graph this second rate of loss is extremely slow, the slope from 10 to 20 days appearing to be virtually zero. Thus a whole month after a single initial dose of the drug, no part of the uveal tract had reached a concentration as low as the average distribution value of the original dose.

In contrast to this, non-uveal tissues were down to negligible values (Table 4) and some had no detectable drug at all. At 30 days an amount of drug as small as 0.014 μg. gave a count of one times background and half this quantity was detectable with certainty.

D. DISCUSSION

It is evident from the above that the uveal tract selectively stores N-substituted phenothiazines and that uveal pigmentation is a prime factor in this storage. Confirmation of this comes via personal com-

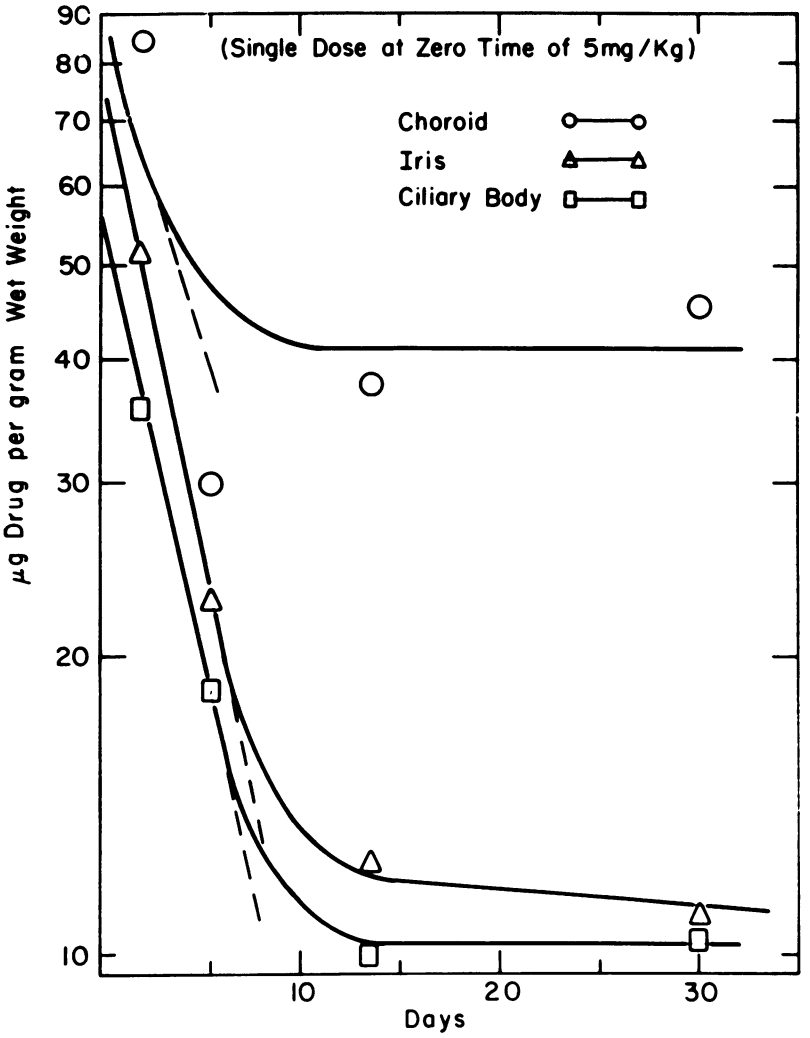


FIGURE 6. TIME COURSE OF CHLORPROMAZINE IN THE PIGMENTED RABBIT UVEA

munication from Dr. A. Cerletti in the Basel laboratory,[18] who used C^{14} labelled and S^{35} labelled thioridazine in 2 albino rabbits, 2 black-brown rabbits, and 1 cat. Choroid, iris, and retina were counted separately. Storage of thioridazine was found for both types of label and only in the uvea of pigmented animals.

TABLE 4. TIME COURSE OF CONCENTRATION OF CHLORPROMAZINE IN NON-UVEAL TISSUES OF PIGMENTED RABBITS

	(Single dose of 5 mg./kg.; 2 animals in each group)			
	(All results as μg. drug/g. wet weight tissue)			
Tissue	*A* *2 days*	*B* *5.5 days*	*C* *13.5 days*	*D* *30 days*
Choroid*	84.2	30.0	37.4	45.5
Cornea	0.18	0.27	0.09	0.05
Lens	0.12	0.15	0.05	—
Retina	0.79	0.43	0.17	0.43
Vitreous	0.11	0.30	0.10	0.0
Aqueous	0.0	0.08	0.0	0.23
Blood	0.17	0.06	0.01	0.006
Liver	2.33	1.69	0.63	0.59
Lung	4.39	2.29	0.65	0.32
Spleen	3.12	2.00	2.00	1.57
Kidney	1.92	1.27	0.61	0.46
Peritoneal fat	0.96	0.11	0.0	—
Choroid plexus	0.85	0.12	0.52	0.54
Cerebral cortex	0.22	0.22	0.0	0.0

*Choroid values given for comparison.

One puzzle is the lack of storage of the parent compound, phenothiazine. Three different substituted phenothiazines have been demonstrated to be stored in the uveal tract: chlorpromazine, prochlorperazine, and thioridazine. Thus it seems likely that the nature of the side chain or of 2-position substituent does not determine whether or not uveal accumulation will occur, although as we have seen they will modify the quantitative details. Thus the property of storage seems to reside in the phenothiazine ring. Despite this, phenothiazine itself does not accumulate in the uveal tract. One might attribute this to the fact that phenothiazine is insoluble in water and is presumably handled differently by the body. However, DeEds, *et al.*[19] describe a water soluble red dye produced in the urine after administration of phenothiazine. This compound, thionol (Figure 1, X), should certainly reach the uvea and be able to be stored, but apparently that does not happen. Thus, in addition to the phenothiazine ring, there must be an additional prerequisite for storage—either N-substitution, or 2-position substitution, or both.

Another fundamental question concerns the validity of the S^{35} label as a true marker for chlorpromazine, especially for the full 30 days of the experiment in Table 4. There are a number of pieces of indirect evidence which support the validity of the label. The Sandoz experiments were done with both C^{14} and S^{35} labelled material and, whereas

the two labels did not give identical results in single animals, both showed high storage of labelled material in the uveal tract. This is good evidence against the possibility that sulfur was selectively broken from the ring and that we are actually following SH or SO_4 and not phenothiazine.

Studies on the metabolism of phenothiazines in the body have shown that common metabolic oxidation products are the sulfoxide and sulfone, but that further oxidation with ring scission is not common.[18]

Further, the reaction between phenothiazines and uveal pigment *in vitro* (see part IV) takes place with extreme rapidity and in an environment where metabolic degradation of the compound is not possible. Thus all of the available indirect evidence supports the validity of the S^{35} tracer.

Of interest is the species variation of storage behavior, shown in the above results. The marked preference of the rabbit uvea for chlorpromazine as compared to prochlorperazine is exactly reversed in the hamster. Furthermore, with prochlorperazine, rabbit ciliary body always shows a higher concentration than iris. With chlorpromazine, iris is consistently higher than ciliary body. These differences must be based on compound specificity, and not on some simple explanation such as greater concentration of pigment granules in one part of the uvea as compared to another. Thus, it is invalid to argue that, simply because most phenothiazines are stored in the uveal tract, concentration alone cannot explain toxic choroidopathy in the human. It may well be that the answer to clinical phenothiazine choroidopathy lies in a high species specific storage of the offending compounds in man.

III. THE STORAGE OF PHENOTHIAZINE COMPOUNDS IN A TRANSPLANTABLE MELANOMA

A. INTRODUCTION

With the knowledge of pigment-dependent uveal storage of phenothiazines, as described in the preceding section, a number of applications come to mind. One of these depends on the well-known fact that the most common malignancies of the adult human globe are the malignant melanomas of the uveal tract. Diagnosis and treatment of these tumors and, indeed, of malignant melanoma elsewhere in the body are still in an unsatisfactory state. Choroidal melanoma in particular lies beneath the retinal pigment epithelium and in its early stages is not easy to differentiate from benign nevus or local hemorrhage.

An attempt to utilize radioactive tracers for diagnosis of ocular tumors was reported by Thomas, *et al.*[20] more than ten years ago. The method was based on external counting of the suspected eye after administration of P^{32} and depended upon greater incorporation of isotope in the phosphorylated compounds of rapidly growing tumor tissue than in normal ocular tissue. Despite early enthusiasm this technique has added little to the diagnosis of ocular tumors, particularly those of the posterior segment.

If a compound could be found that would combine specifically with a unique component of a tumor, and if this compound could be labelled with a gamma-emitting isotope, one would have an ideal diagnostic aid. The same compound with a short range beta-emitting isotope could provide a potentially effective therapeutic agent. Since the phenothiazine tranquilizers combine specifically with uveal pigment, it seemed that one of them might well be the hoped-for agent for diagnosis and treatment of pigmented tumors arising in the uveal tract.

A satisfactory test object for an experimental approach to this problem appeared to be the transplantable melanoma of the Syrian golden hamster, described by Greene[21] and by Fortner and Allen[22] in 1958. This tumor seemed particularly desirable, for it had been shown to be transplantable to the uvea by Burns and Fraunfelder.[23] The experiments to be reported below describe the storage of labelled prochlorperazine and chlorpromazine in transplantable melanoma in the hamster.

B. EXPERIMENTAL WORK

The tumor used was a descendant of the Greene melanoma kindly sent us by Dr. Robert Burns. It was propagated in the abdominal skin of hamsters by inserting a tiny ($1 \times 1 \times 2$ mm.) block of tumor under the abdominal skin with a trochar. Implantation in the anterior chamber was done exactly as described by Burns and Fraunfelder.[23] We found that periodic eye examination could be performed without anesthesia. A trained assistant held the animal under the slit-lamp and proptosed the eye for examination. In our animals the right eye received tumor suspension and the left eye was used as control.

When the tumors were considered sufficiently advanced (20 days) the animals were injected intraperitoneally with an aqueous solution of labelled prochlorperazine or chlorpromazine—5 mg./kg. of hamster. Twenty-four to 48 hours later the animals were sacrificed and tissues were worked up just as described in part II.

C. RESULTS

The histological appearance of an abdominal skin tumor stained with hematoxylin and eosin is shown in Figure 7. Although this strain had been selected for pigmentation and although all tumors were grossly quite dark, the relatively sparse deposits of pigment may be seen in the photomicrograph. Apparently, to begin with, the concentration of pigment granules in the melanoma is low relative to a structure like the choroid and, in addition, the tumor tended to become amelanotic on continued growth, as described by Greene.[21]

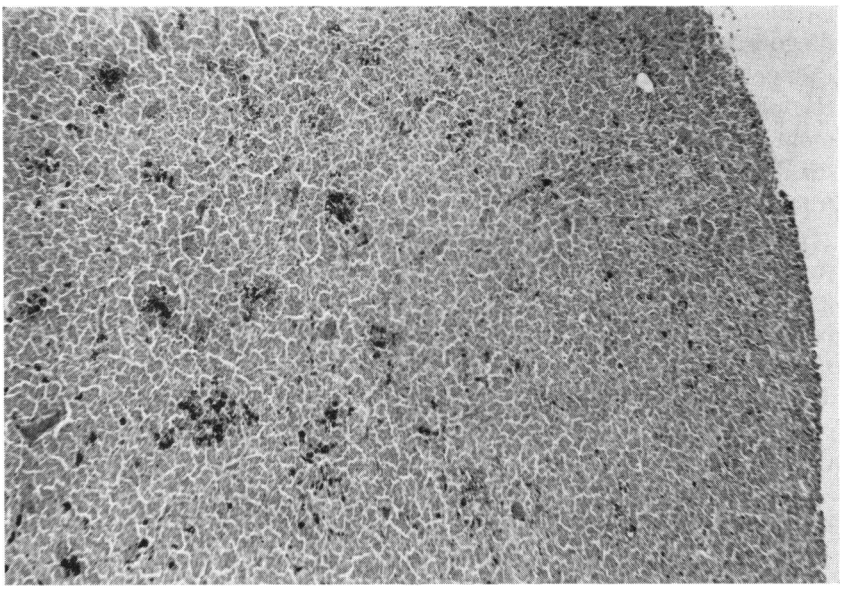


FIGURE 7. TRANSPLANTABLE HAMSTER MELANOMA CARRIED IN ABDOMINAL SKIN (× 100) (H & E)

This tendency was even more marked in the tumor grown on iris (Figure 8) as compared to the normal chamber angle of the opposite eye (Figure 9). It can be seen that the normal iris pigment has been disrupted and surrounded by growing tumor but that the tumor has produced virtually no pigment of its own. Nevertheless this tumor, too, was grossly dark.

Results of storage experiments on prochlorperazine in transplants to abdominal skin are shown in Table 5. Injection of the compound was done on the 21st day after implantation and the animals were sacrificed 24 hours later. As can be seen, concentration of the drug in the tumor

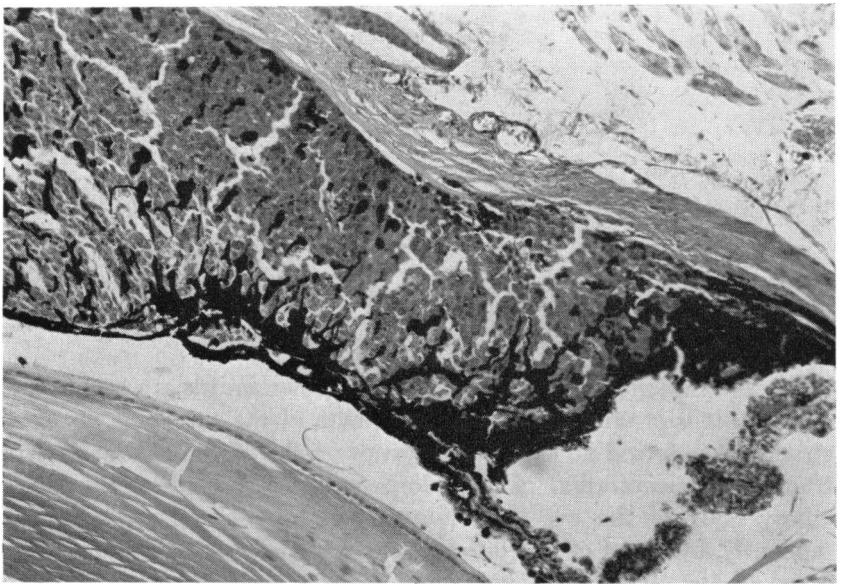


FIGURE 8. HAMSTER MELANOMA IN CHAMBER ANGLE (× 165) (H & E)

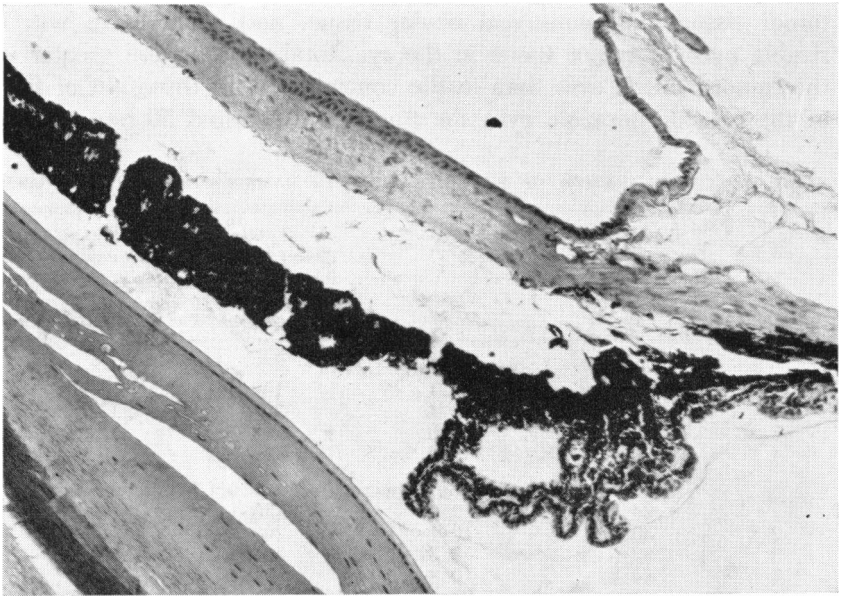


FIGURE 9. NORMAL CHAMBER ANGLE OF HAMSTER (× 165) (H & E)

TABLE 5. CONCENTRATION OF PROCHLORPERAZINE IN HAMSTER ABDOMINAL MELANOMAS AT 24 HOURS

Tissue	*Prochlorperazine μg./g. tissue*	*Determinations*
Melanoma	1.90	5
Metastasis (in mesentery)	3.21	2
Choroid	243	4
Liver	4.21	4

was between that of liver and kidney, reported in Table 2. It was well above the blood level but two orders of magnitude less than the concentration in choroid.

Storage experiments on the melanomas grown on iris are reported in Table 6. It is at once apparent that growth of the tumor on an uveal structure conferred no special properties on the additional tissue. It is true that concentration of prochlorperazine was higher in iris and ciliary body of the eye with tumor than in the tumor carried cutaneously, but most of this difference may be accounted for by persistence of the pigment of the anterior uvea and the exertion of its normal binding power. On average there was 10 times less drug per unit weight of tissue in the tumor eye compared to the non-tumor eye. However, since there was a modest differential of storage in favor of tumor tissue over non-uveal ocular tissue, and since there was a sizable mass of tumor tissue in the eye, total counts were greater in the tumor-bearing eyes than in the control eyes. In three out of four of the prochlorperazine eyes the tumor eye counted 50 per cent or

TABLE 6. CONCENTRATION OF PHENOTHIAZINES IN HAMSTER OCULAR MELANOMAS

Animal	*Iris and ciliary body, μg./g. wet weight*		*Total Count/minute, iris and ciliary body, tumor eye as % control*	*Weight, iris and ciliary body, tumor eye, vs. control*
	Control eye	*Tumor eye*		
		PROCHLORPERAZINE		
1	329	18.1	109	18.8×
2	98.3	15.4	215	13.8×
3	120	18.0	183	12.1×
4	100	8.2	146	18.2×
		CHLORPROMAZINE		
1	24.2	2.03	120	14.4×
2	30.0	10.1	290	8.7×
3	62.5	3.37	101	18.0×
4	84.3	10.8	96	7.5×
5	46.0	37.5	101	1.2×
6	23.3	6.76	226	7.9×

more higher than the control—an effect which would have shown equally well with a gamma emitter and external counting.

In the case of chlorpromazine and the ocular melanomas, concentration in the tumor eye was low compared to the control. However, because the concentration of chlorpromazine is less than that of prochlorperazine in normal uvea, the differential between tumor and non-tumor eye was less. Perhaps for the same reason absolute counts were less and do not differ significantly between the two eyes in each animal.

D. DISCUSSION

It seems likely from the foregoing that any attempt to use phenothiazine storage as an aid to diagnosis or treatment of melanomas would be subject to the same types of uncertainties as apply to compounds already in use. It is well known that intraocular malignant melanoma varies widely in pigment content.[24] Hence any method which relies on storage in pigment would vary in effectiveness with the amount of pigment in the tumor. A heavily pigmented tumor would be easily detected, a moderately pigmented one less easily, and an amelanotic melanoma would be found least easily. However, when even a poorly pigmented tumor accumulates sufficient mass in the eye it would become detectable as is shown in the prochlorperazine animals with ocular tumors. The real question to be settled is how great that mass must be in any given case and whether a compound even more suitable than prochlorperazine might be found.

IV. THE *in vitro* REACTION OF PHENOTHIAZINES AND OTHER COMPOUNDS WITH ISOLATED PIGMENT GRANULES

A. INTRODUCTION

On the basis of the findings of part II regarding the failure of storage of phenothiazine compounds in the uvea of albino rabbits, it seemed highly likely that the uveal pigment itself was responsible for our concentration phenomenon. In order to further investigate this possibility, a study was made of the reaction of isolated uveal pigment with a series of pure compounds.

Within recent years pigment granules from various sources have come under renewed scrutiny for a variety of reasons. It has become appreciated that the pigment granule is a unique biological unit which is extremely resistant to osmotic and other insult.[25,26] The pigment granule possesses a number of enzyme systems which are

ordinarily present in mitochondria and which are resistant to osmotic shock but which are inhibited by the same substances which affect mitochondrial enzymes.[25–27] The structure of melanin granules from various sources has been examined under the electron microscope by a number of workers.[28–31] In each report, a fine structure has been described for the melanin granule and it is either implied or stated that the dense pigment is deposited on the protein membrane framework of the pregranule. Much emphasis is placed by some workers on the relative scarcity of mitochondria in melanized cells, and the affinity of janus green B for both melanin granules and mitochondria to the point where melanin granules are actually held to be mitochondria.[32] Others offer evidence why this is not possible.[31] Whatever will be the outcome of this semantic contest, it is of significance that in the melanized cell much of the business of energy metabolism is conducted in the large population of melanin granules. Further, because of the osmotic resistance described above, it is relatively easy to isolate melanin granules and study them uncontaminated by other tissue constituents. Thus it seemed of some significance to us to learn how our water-soluble phenothiazine derivatives behave toward isolated pigment granule preparations.

B. EXPERIMENTAL WORK

Pigment was prepared in adequate quantity for our purposes from the uvea of beef eyes. Most of the experiments in this section were done on choroidal melanin, but the preparation is equally satisfactory for iris and ciliary body. Twenty grams of choroid were ground in a mortar with an equal weight of washed sea sand and with successive portions of 15 to 25 ml. of distilled water. After each grinding the water was decanted into 50-ml. glass centrifuge tubes until the choroidal stroma, originally black, had become gray and the aqueous supernatant no longer took on additional pigment. The combined supernatants were centrifuged for 7 minutes at the lowest setting of the International model I centrifuge to remove sand and large tissue particles. The supernatant was then decanted into nylon centrifuge tubes and spun for 10 minutes at 5900 $\times$ G in the Servall-refrigerated automatic centrifuge. This served to sediment the pigment granules and the supernatant was discarded. The residue from the low speed centrifugation was resuspended in distilled water and recentrifuged 6 times. Each supernatant was added to the pigment granule pellet, the pigment resuspended and recentrifuged at 5900 $\times$ G. The final residue was suspended in 25 ml. distilled water and two 2 ml. aliquots

were removed for determination of dry weight. On the basis of this determination the volume of solution was adjusted in the later experiments so that each ml. contained 10 mg. dry weight of pigment granules. Microscopic examination showed a uniform suspension of pigment granules in Brownian movement, uncontaminated by other cellular or subcellular constituents.

Exploratory experiments were done with early pigment granule preparations and radioactive prochlorperazine by mixing, centrifuging, removing a 0.1 ml. aliquot from the supernatant, and counting in an alcohol-toluene-scintillant system in the automatic scintillation counter. As a result of these experiments it was learned that large enough amounts of drug were taken up by pigment to allow the use of a simpler analytical method and finally the following method of procedure was adopted.

In a 10-ml. nylon centrifuge tube was placed: 1 ml. of pigment suspension containing 10 mg. of pigment granules (by dry weight); 1 ml. of buffer or acid; 4 ml. of water; and 1 ml. of a solution containing 2.5 μmole of the compound to be tested. Contents of the tube were mixed. After 15 minutes the tubes were centrifuged at 35,000 $\times$ G for 10 minutes in the Servall centrifuge, and an appropriate aliquot of the supernatant was removed for determination of the compound in question. All experiments were done in duplicate.

Where the substance tested was colored, as in the rather large series of dyes (see below), the aliquot was compared with a standard of known concentration in a Bausch and Lomb spectronic photometer. The wavelength was set at the absorption peak in the visible, characteristic of the substance under consideration. Colorless substances which contained aromatic rings were measured at the wavelength maximum in the ultraviolet typical of the particular ring system. The Beckmann D.U. quartz spectrophotometer was used for this purpose, and the absorption maximum was determined on the machine in a preliminary experiment.

It was found that no appreciable additional ultraviolet absorption was contributed by the extraction of the pigment granule preparation that this procedure accomplished. Hence no correction for this factor was required.

C. RESULTS

A synopsis of the yields of pigment granules from choroid is shown in Table 7. In addition to dry weight measurement it was attempted to further calibrate some of the preparations by measuring the uptake

of chlorpromazine for each preparation by the standard method described above. However, the utility of such a single point calibration beyond the qualitative indication of uniformity is open to question (see below).

TABLE 7. SYNOPSIS OF PIGMENT PREPARATIONS FROM BEEF CHOROID

Preparations	*Wet weight starting tissue grams*	*Dry weight of total yield (mg.)*	*mg. Granules per gram choroid*	*% of 2.5 μmole chlorpromazine uptake by 10 mg. pigment*
1	20	224	11.2	—
2	20	368	18.4	—
3	20	259	13.0	58.6
4	20	173	8.7	65.2
5	40	575	14.4	56.5
6	40	828	20.7	47.7

Mean yield: 14.4 mg. granules per gram wet weight of choroid.

The preliminary experiments with labelled compound showed an avidity of pigment granules for prochlorperazine *in vitro* far surpassing that of the whole choroid in live animals. From Figure 2 one finds that in the rabbit loaded with three doses of prochlorperazine the uptake was 154.2 μg. (0.41 μmole) per gram of choroid. Using the mean yield of pigment (Table 7) and carrying beef pigment granule weights over to the rabbit, the gram of rabbit choroid would contain 14.4 mg. dry weight of pigment granules. From preliminary experiments (cf., Figure 10) 14.4 mg. of pigment will take up 1.15 μmole of prochlorperazine and (from Table 7) twice this much chlorpromazine. Thus if one even considers only the pigment granules in the choroid, they are three or more times as avid *in vitro* for the phenothiazines as *in vivo*.

Other preliminary radioactive experiments showed that washing with water or N/10 HCl removed less than 1 per cent of the compound on the granules but that successive washings with N/10 NaOH or 95 per cent ethanol removed 80 per cent or more of the material. A study of the capacity of the granules for prochlorperazine was done by dispersing 50 mg. of granules in 25 ml. of solution containing 1.32 μmole of radioactive compound, separating the granules by centrifuging, and resuspending in a fresh solution containing the original concentration of compound. This procedure was repeated an additional 5 times. An aliquot of each supernatant was counted for determination of prochlorperazine. The results are plotted in Figure 10.

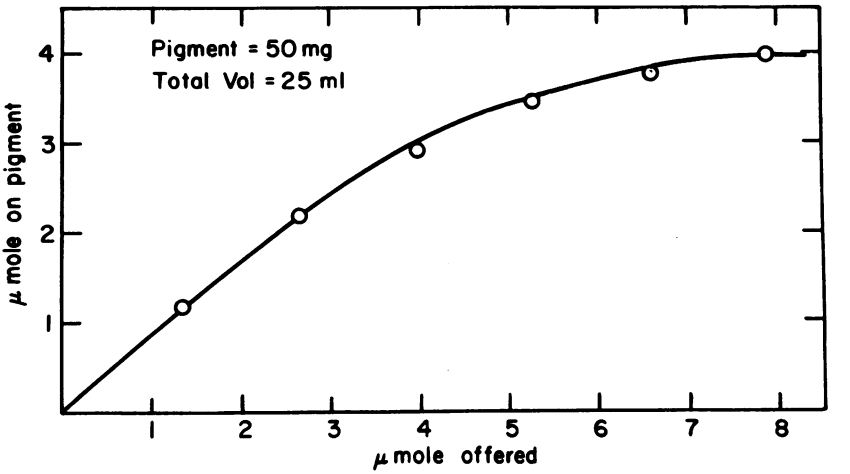


FIGURE 10. UPTAKE OF PROCHLORPERAZINE BY CHOROIDAL PIGMENT

Here it became evident that the pigment had a high enough capacity for phenothiazines so that usable results could be obtained with smaller amounts of pigment and with spectrophotometric analysis. This would make possible the determination of pigment capacity for increasing concentration of drug without re-using the pigment. In order to make such a series of tubes uniform it would be necessary to determine the effect of pH on uptake and, if buffers were to be used, this would in turn require investigation of salt concentration on uptake. For this reason the effect of increasing concentrations of sodium chloride was investigated using the standard system, described above, of 10 mg. of pigment, 2.5 μmole of chlorpromazine, and 7 ml. total volume. The pH was brought to 3.0 in each tube with 1 ml. of N/10 HCl. Results of this experiment are reported in Table 8. As can be

TABLE 8. THE EFFECT OF SODIUM CHLORIDE CONCENTRATION ON UPTAKE OF CHLORPROMAZINE BY PIGMENT GRANULES

Tube	*Final concentration NaCl*	*Uptake % of 2.5 μmole*
1	0.0	65.2
2	0.01M	65.2
3	0.1M	64.4
4	1.0M	72.8
5	10.0M	72.8

seen, although moderately higher uptakes were found with the two higher salt concentrations, the maximum difference observed was 8.4 per cent, and differences up to 0.1 M. were negligible. Once it was apparent that salt concentration as such did not effect the reaction it was possible to go on to examine the effect of pH using buffers. The system was the standard one described above. To each vessel was added 1.0 ml. of a buffer solution of ionic strength 0.05. Final pH of each solution as well as per cent uptake are shown in Table 9. Here again, although modest changes with pH did occur, there was less than 10 per cent variation in total uptake for all pH values except 9.

TABLE 9. THE EFFECT OF PH ON UPTAKE OF CHLORPROMAZINE BY PIGMENT GRANULES

Tube	*pH*	*Buffer ionic strength 0.05*	*Uptake % of 2.5 μmole*
1	3.6	Acetate	65.6
2	4.0	Acetate	68.9
3	5.0	Acetate	74.8
4	6.0	Phosphate	70.0
5	7.0	Phosphate	66.8
6	8.0	Phosphate	70.4
7	9.0	Borate	79.2

With this knowledge that a modest amount of buffer did not affect uptake over a wide pH range, it was possible to re-examine the question of how uptake varies with concentration. Once more the standard chlorpromazine system was used. One ml. of phosphate buffer was used to maintain pH at 6.0 and increasing concentrations of chlorpromazine were presented to the 10 mg. of pigment. The results of the experiment are shown in Figure 11. The result with chlorpromazine obtained spectrophotometrically was quite comparable to that with prochlorperazine obtained by radioactivity measurement. In each case, the curve convex upward resembles an adsorption isotherm. However, when the log of amount adsorbed per unit weight of pigment was plotted against log equilibrium concentration, as in Figure 12, two different straight lines resulted indicating a biphasic process. Perhaps this shows the transition from one molecule taken up per active site to uptake of a second molecule.

To get some clue to the connection between chemical structure and strength of binding, a series of water soluble cyclic compounds were reacted under the same standard conditions as the phenothia-

zines, i.e., 2.5 μmole compound, 10 mg. pigment, 7 ml. total volume, and pH = 3.0. The results of this experiment are shown in Table 10. The structural formulas of the compounds used are given in Figure 13. As can be seen, all of the dyes showed some affinity for pigment. Some 13 dyes were more avidly taken up than chlorpromazine. Only pyridine and hydroquinone had no detectable uptake.

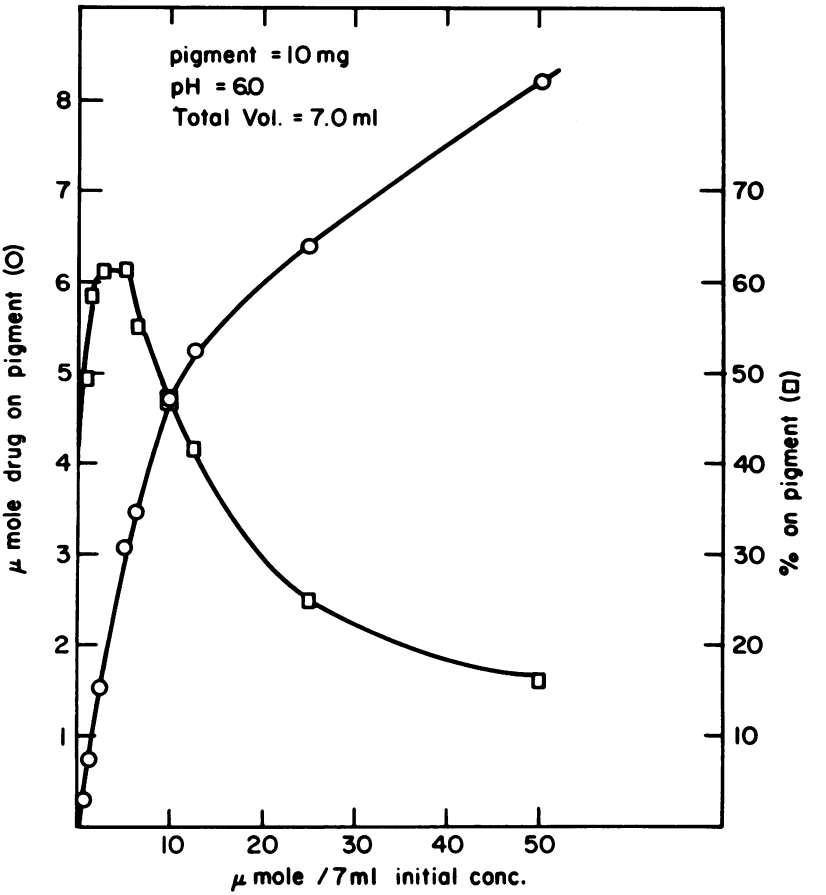


FIGURE 11. ISOTHERM FOR CHLORPROMAZINE ON UVEAL PIGMENT

D. DISCUSSION

One tacit assumption made in the *in vitro* experiments reported above is that the choroidal melanin granules can be used as a chemical reagent as if one were dealing with a pure substance. That the

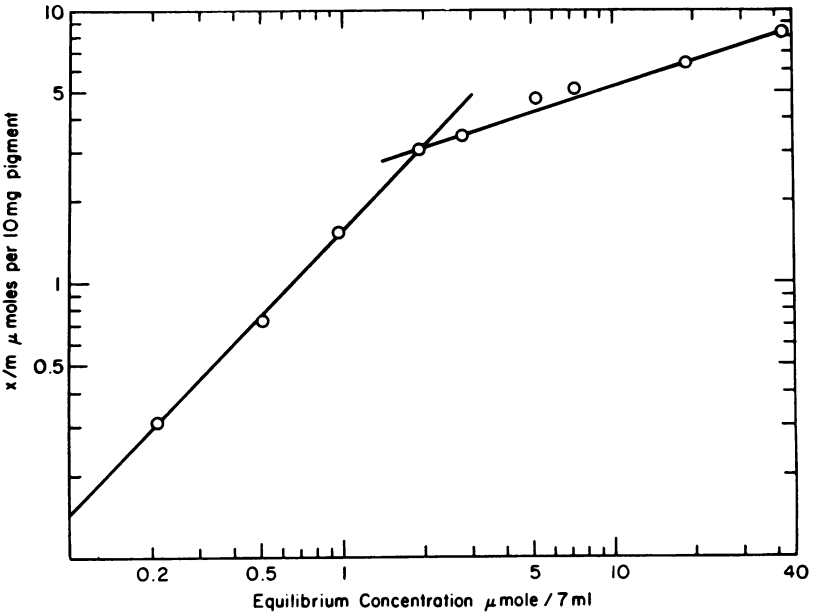


FIGURE 12. LOG-LOG PLOT OF PIGMENT-CHLORPROMAZINE ISOTHERM

granule is not composed of a singular molecular species, we know quite well. The only concern for present purposes is whether during the experiments or during the isolation procedure we have changed the physicochemical properties of the granule, so that its reactivity toward the phenothiazines and other chemicals is drastically different from that shown by it in the cell. On the basis of the best evidence at hand there is no significant change. Although isolation in distilled water does change some metabolic properties of melanin granules these changes are attributable to leaching out of essential small molecular weight metabolites such as cytochrome-C and DPN,[25,26] and the properties are restored with replacement of the needed cofactor. On the basis of electron microscopy much more drastic treatment than we have ever used leaves the granules unaffected. Treatment in 3N HCl in a sealed tube at 100 degrees centigrade for 18 to 24 hours shows no demonstrable morphological change under the electron microscope.[28]

A second assumption is that the reactions described with the melanin granule are truly a function of the melanin content and not of other factors. A very potent argument that this is so lies in the difference

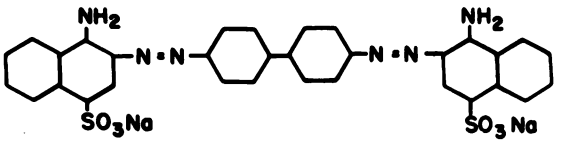
TABLE 10. REACTION OF SELECTED COMPOUNDS WITH CHOROIDAL PIGMENT GRANULE SUSPENSION *in vitro*

Pigment = 10 mg. dry weight Reagent = 2.5 μmole
Total volume = 7.0 ml.

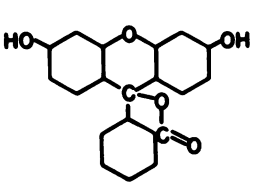
	Substance	Uptake	
		%	μg
(1)	Congo red	100	1741
(2)	Fluorescein	98	911
(3)	Acridine orange	96	728
(4)	Biebrich scarlet	92	1288
(5)	Alizarin red S	88	768
(6)	Primulin	88	1038
(7)	Bismarck brown Y	88	915
(8)	Methylene-blue	87	814
(9)	Thionin	80	520
(10)	Malachite green	80	728
(11)	Fast green FCF	74	1495
(12)	Methyl orange	61	498
(13)	Neutral violet	61	622
(14)	Chlorpromazine	59	511
(15)	Trifluoperazine	55	546
(16)	Safranin O	52	463
(17)	Prochlorperazine	50	591
(18)	Neutral red	50	360
(19)	Brom phenol blue	40	269
(20)	Quinine DiHCl	31	310
(21)	Buffalo black NBR	30	462
(22)	Aniline	26	60
(23)	Nile blue A	26	470
(24)	Janus green B	18	229
(25)	Pyridine	0	0
(26)	Hydroquinone	0	0

observed between storage of phenothiazines in the pigmented as compared to the albino animal. The Birbeck and Barnicot[28] studies of the albino hair follicle show that, in the albino "melanocyte," the protein framework of the melanin granule exists apparently intact. The only difference is that no melanin is hung on the framework in the albino. Thus it is reasonable to assume that the only difference between storage of drug in the pigmented animal and non-storage in the albino is the melanin itself.

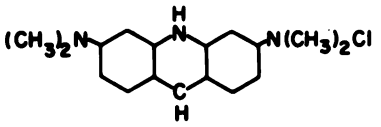
One further possibility must be considered, namely, that melanin as such is not enough but that the storage phenomenon requires melanin in the special lamellar organization that it seems to possess in the melanin granule. Techniques are available for checking out this hypothesis and experiments are now under way. Measurement of uptake by synthetic melanin made in the test tube from dihydroxyphenylalanine is one approach being used. The other is measurement



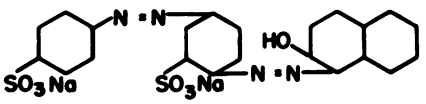
1. Congo Red (100)



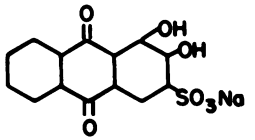
2. Fluorescein (98)



3. Acridine Orange (96)

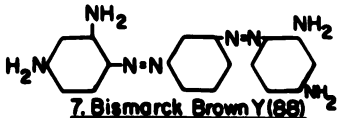


4. Biebrich Scarlet (92)

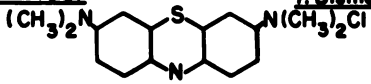


5. Alizarin Red S (88)

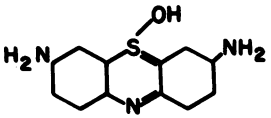
6. Primulin (88)



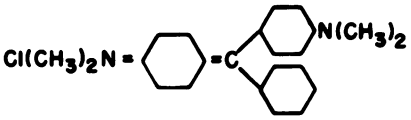
7. Bismarck Brown Y (88)



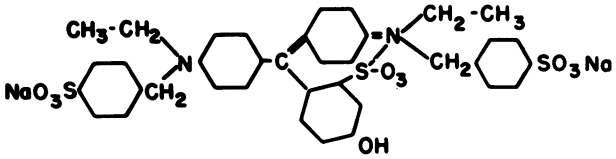
8. Methylene Blue (87)



9. Thionin (80)

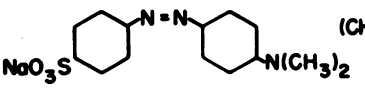


10. Malachite Green (80)

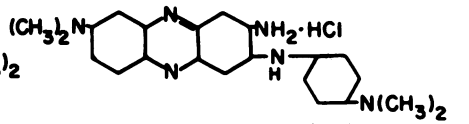


11. Fast Green F.C.F. (74)

FIGURE 13. COMPOUNDS USED *in vitro* WITH CHOROIDAL PIGMENT



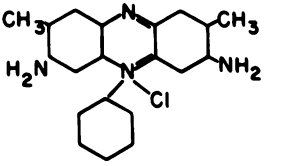
12. Methyl Orange (61)



13. Neutral Violet (61)

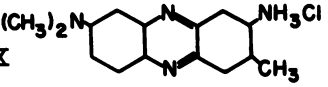
See Figure 1 V

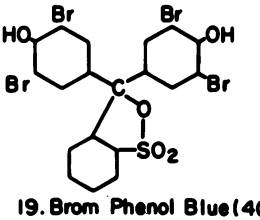
See Figure 1 XI



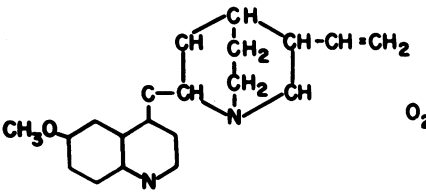
14. Chlorpromazine (59) 15. Trifluoperazine (55) 16. Safranin O (52)

See Figure 1 IX

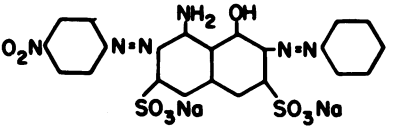




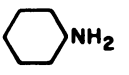
17. Prochlorperazine (50) 18. Neutral Red (50) 19. Brom Phenol Blue (40)



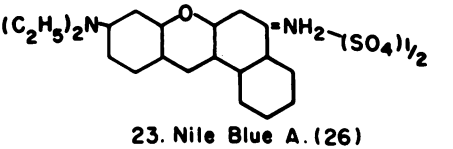
20. Quinine di Hydrochloride (31)



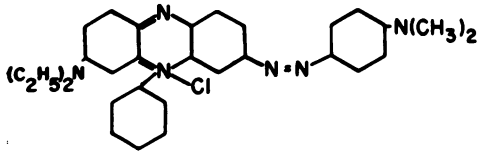
21. Buffalo Black NBR (30)

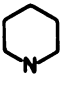


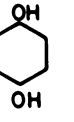
22. Aniline (26)



23. Nile Blue A. (26)







24. Janus Green B. (18) 25. Pyridine (0) 26. Hydroquinone (0)

Numbers in parentheses = % uptake by 10 mg. pigment, stand, and conditions.

on natural melanin prepared by disrupting the protein framework of the granule with concentrated bile salts, with enzymes, or with both.

The other important subject for discussion is the nature of the combination of phenothiazine compounds and melanin. This combination is reversible, albeit with difficulty, so that formation of an ordinary organic covalent bond seems to be eliminated. Similarly, an ionic bond or ion exchange seems to be out of the question. The broad range of pH over which the phenothiazine drugs combine (Table 9) and the fact that both anions and cations combine with vigor (Table 10) seem to eliminate ionic binding as such. This appears to leave the effect to such physical forces, as are grouped under the heading of "adsorption." A résumé of these forces is given briefly by Cassidy.[33] At the present state of our knowledge one cannot specify whether hydrogen bonding, or induction interaction, or dipole-dipole interaction play a significant role.

In addition to these there is one binding force which has not been mentioned, namely, the charge transfer reaction. An account of this reaction for biologists may be read in the recent book of Szent-Györgyi.[34] Suffice it to say that this reaction involves donation of a single electron from a molecule whose structure predisposes to such donation, and acceptance of an electron by a molecule whose structure is suited for this role. The result is two free radicals which may or may not remain closely associated with one another. If one were considering such a reaction between two soluble molecules, this type of reaction would be unlikely to be significant, for the high dielectric constant of water would make permanent association of the charged particles virtually impossible. However, in our system we are dealing with a solid "adsorbent" and a solute in aqueous solution. Conditions at the surface of the solid may be far different from those in the solution itself. Of additional impact are studies already done on melanin, both natural and synthetic, showing it to possess free radical properties and suggesting that it acts as an "electron trap."[35–37] To exactly complement these findings is the report by Szent-Györgyi and co-workers[38] that by their calculations chlorpromazine is one of the best electron donors known. Thus it is extremely tempting to assume that the charge transfer reaction specially modified by the surface properties of melanin is responsible for the phenomena reported in this paper. Studies are now in progress to determine whether the electron spin resonance of choroidal melanin granules—a measure of free radical properties—is modified by saturation with chlorpromazine and other compounds.

In chlorpromazine it is the configuration of three coplanar aromatic rings which is largely responsible for the excellent electron donor properties. Hence when dyes were chosen for the experiment of Table 10 and Figure 13, a number of substances possessing similar fused rings were selected. Some of these such as fluorescein, acridine orange, alizarin red, methylene-blue, and thionin were, in fact, good reactors. However, compounds such as nile blue A and janus green B which possess additional aromatic rings besides the three fused rings showed measurable but much lower reactivity. Furthermore, compounds with no ring systems such as the azo dyes congo red, biebrich scarlet, bismarck brown, and methyl orange, and the triphenylmethane dyes malachite green and fast green F.C.F. were also good reactors. However, most of these compounds are known to be good electron donors on other grounds.

As can be seen from Figure 11, expressing results as per cent uptake would be a highly unsatisfactory method of presentation if in all cases the same number of micromoles had not been offered to the pigment. The per cent uptake curve passes through a maximum and there is no way to tell from this figure alone on which side of the maximum a single point lies. For complete presentation the whole "adsorption isotherm" would have to be plotted and the constants of slope and intercept obtained from a log-log plot as in Figure 12. Such a procedure would be time-consuming and far out of proportion to the knowledge to be obtained. For this reason the single point method with a constant molarity of compound was chosen.

V. SUMMARY

(1) The tissues of the uveal tract of pigmented animals concentrate N-substituted phenothiazine derivatives. Uveal tissue levels may reach 50 times the mean distribution value even 48 hours after a single dose of 5 mg./kg.

(2) All other tissues have lower concentrations than the mean distribution value. Only samples involved in excretion of the compounds (urine, kidney, liver) approach the mean distribution value.

(3) Albino rabbits do not show the uveal concentration phenomenon.

(4) Species and compound differences in the concentration phenomenon are noted. Chlorpromazine reaches higher levels in the rabbit uvea than does prochlorperazine. The reverse is true in hamsters. Ciliary body is higher than iris for prochlorperazine in rabbits. Iris is higher than ciliary body for chlorpromazine.

(5) Phenothiazine itself does not concentrate in the uveal tract of pigmented rabbits or hamsters.

(6) The time course of disappearance of chlorpromazine from the rabbit uvea is biphasic. The first phase has a half time of 2.8 to 4.0 days. After the first 10 to 12 days a very slow phase becomes dominant, so that even 30 days after a single dose all parts of the uvea are still above the mean distribution value.

(7) There is no significant concentration of phenothiazine derivatives in the pigmented Greene melanoma of the hamster when the tumor is carried in the abdominal skin. When the tumor is carried in the eye, chlorpromazine *concentrations* are above the mean distribution value but below those in the control eye. However, *absolute* amounts are above those in the control eye. Hence phenothiazines may be useful for the detection of ocular melanomas.

(8) When solutions of phenothiazines are mixed with suspensions of beef uveal pigment granules, the compound is removed from solution by the pigment. Concentrations on the pigment exceed even the highest concentrations observed *in vivo*. It is concluded that the uveal pigment granules are the site of *in vivo* storage of phenothiazines.

(9) The storage on separated pigment granules takes place over a wide range of pH and salt concentration.

(10) The storage of phenothiazine derivatives on uveal pigment, when plotted as amount stored *vs.* equilibrium concentration, gives a curve resembling an adsorption isotherm. A log-log plot shows this to represent a biphasic process.

(11) Many other compounds which possess resonating aromatic structures show storage on uveal pigment granules.

ACKNOWLEDGMENTS

I am indebted to many persons for technical and material assistance and for helpful suggestions regarding the work reported here. If any name has been omitted below, it has been done inadvertently.

I wish to acknowledge the help of Dr. A. Cerletti, Sandoz A. G., Basel, Switzerland, and Dr. R. Bircher, Sandoz, Inc., Hanover, New Jersey, for making possible my visit to the Basel laboratory and for supplies of NP-207, thioridazine, and KS-24.

Dr. Harry Green and Dr. Alfred R. Mass of Smith, Kline and French Laboratories were kind enough to supply me with a quantity of S^{35} labelled prochlorperazine.

Mr. Ronald Lizik, Mr. Cornelius Young, Mrs. Charlotte Tanner, Mrs. Mary R. Rowlands, and Mrs. Marion Shor rendered technical assistance at some time during this project.

I am grateful to Dr. R. Burns for hamsters bearing the Greene melanoma, and to Dr. F. Fraunfelder for demonstrating the technique of tumor propagation and eye inoculation.

Dr. F. W. Newell and Dr. T. E. Sanders kindly read an early draft of the manuscript and made suggestions on revision.

I am most grateful indeed to Mrs. Edith G. Goldman who typed the several revisions of the manuscript, and who was helpful in innumerable other ways during the writing and preparation of this material.

REFERENCES

1. Ehrlich, P., and A. Leppmann, Ueber schmerzstillende Wirkung des Methylenblau, Deutsche med. Wchnschr., 16: (23) 493–4, 1890.
2. DeEds, F., A. B. Stockton, and J. O. Thomas, Studies on phenothiazine, VIII, Antiseptic value of phenothiazine in urinary tract infections, J. Pharmacol. & Exper. Therap., 65:353–71, 1939.
3. Manson-Bahr, P., Phenothiazine as an anthelminthic in threadworm and round worm infections, Lancet, 239:808–9, 1940.
4. Halpern, B. N., and R. Ducrot, Recherches expérimentales sur une nouvelle série chimique de corps doués de propriétés antihistaminiques puissantes: Les dérivés de la thiodiphenylamine (TDA), Compt. rend. Soc. de biol., 140: 361–3, 1946.
5. Sigwald, J., D. Bovet, and G. Dumont, Le traitement de la maladie de Parkinson par le chlorhydrate de diethylaminoethyl N-thiodiphenylamine (2987 RP): Premiers résultats, Rev. neurol., 78:581–4, 1946.
6. Laborit, H., and P. Huguenard, L'hibernation artificielle par moyens pharmacodynamiques et physiques, Presse méd., 59:1329, 1951.
7. Delay, J., P. Deniker, and J. M. Harl, Traitement des états d'excitation et d'agitation par une méthode médicamenteuse dérivée de l'hiberno thérapie, Ann. méd.-psychol., 110:267–73, 1952.
8. Kinross-Wright, V., Clinical trial of a new phenothiazine compound: NP-207, Psychiatric Research Reports, 4:89–94, 1956.
9. Goar, E. L., and M. C. Fletcher, Toxic chorioretinopathy following the use of NP-207, Tr. Am. Ophth. Soc., 54:129–39, 1956.
10. Burian, H. M., and M. C. Fletcher, Visual functions in patients with retinal pigmentary degeneration following the use of NP-207, Arch. Ophth., 60:612–29, 1958.
11. Verrey, F., Dégénérescence pigmentaire de la rétine d'origine médicamenteuse, Ophthalmologica, 131:296–303, 1956.
12. Rintelen, F., G. Hotz, and P. Wagner, Zur Klinik und experimentellen Pathologie der Pigmentepithelerkrankung nach Medikation mit einem Piperidinphenothiazin, Ophthalmologica, 133:277–83, 1957.
13. May, R. H., *et al.*, Thioridazine therapy: Results and complications, J. Nerv. & Ment. Dis., 130:331–9, 1960.
14. Weekley, R. D., *et al.*, Pigmentary retinopathy in patients receiving high doses of a new phenothiazine, Arch. Ophth., 64:65–76, 1960.
15. Harris, J., Personal communication.

16. Apt, L., Personal communication.
17. Cerletti, A., Personal communication, 1959.
18. Cerletti, A., Personal communication, 1960.
19. DeEds, F., C. W. Eddy, and J. O. Thomas, Studies on phenothiazine, V, Fate of phenothiazine in the body, J. Pharmacol. & Exper. Therap., 64:250–62, 1938.
20. Thomas, C. I., J. S. Krohmer, and J. P. Storaasli, Detection of intraocular tumors with radioactive phosphorus, Arch. Ophth., 47:276–86, 1952.
21. Greene, H. S. N., A spontaneous melanoma in the hamster with a propensity for amelanotic alteration and sarcomatous transformation during transplantation, Cancer Research, 18:422–5, 1958.
22. Fortner, J. G., and A. C. Allen, Hitherto unreported malignant melanomas in the Syrian hamster: An experimental counterpart of the human malignant melanoma, Cancer Research, 18:98–104, 1958.
23. Burns, R. P., and F. T. Fraunfelder, Experimental intraocular malignant melanoma in the Syrian golden hamster, Am. J. Ophth., 51:977–92, 1961.
24. Callender, G. R., H. C. Wilder, and J. E. Ash, Five hundred melanomas of the choroid and ciliary body followed five years or longer, Am. J. Ophth., 25:962–7, 1942.
25. Riley, V., G. Hobby, and D. Burk, Oxidizing enzymes of mouse melanomas: Their inhibition, enhancement, and chromatographic separation, in M. Gordon, Pigment Cell Growth. New York, Academic Press, Inc., 1953, pp. 231–66.
26. Dorner, M., and E. Reich, Oxidative phosphorylation and some related phenomena in pigment granules of mouse melanomas, Biochim. et biophys. acta, 48:534–46, 1961.
27. Herrmann, H., and M. B. Boss, Dopa oxidase activity in extracts from ciliary body and in isolated pigment granules, J. Cell. & Comp. Physiol., 26:131–8, 1945.
28. Birbeck, M. S. C., and N. A. Barnicot, Electron microscope studies on pigment formation in human hair follicles, in M. Gordon, Pigment Cell Biology. New York, Academic Press, Inc., 1959, pp. 549–61.
29. Drochmans, P., Electron microscope studies of epidermal melanocytes, and the fine structure of melanin granules, J. Biophys. & Biochem. Cytol., 8:165–80, 1960.
30. Feeney, L., and M. J. Hogan, Electron microscopy of the human choroid, I, Cells and supporting structures, Am. J. Ophth., 51:1057–72, 1961.
31. Dalton, A. J., and M. D. Felix, Phase contrast and electron micrography of the Cloudman S 91 mouse melanoma, in M. Gordon, Pigment Cell Growth. New York, Academic Press, Inc., 1953, pp. 267–76.
32. Woods, M. W., *et al.*, Cytological studies on the nature of the cytoplasmic particulates in the Cloudman S 91 mouse melanoma, the derived Algire S 91 A partially amelanotic melanoma and the Harding Passey mouse melanoma, J. Nat. Cancer Inst., 9:311–23, 1948.
33. Cassidy, H. G., Fundamentals of Chromatography. New York, Interscience Publishers, Inc., 1957, chap. 3.
34. Szent-Györgyi, A., Introduction to a Sub-Molecular Biology. New York, Academic Press, Inc., 1960.
35. Commoner, B., J. Townsend, and G. E. Pake, Free radicals in biological materials, Nature, 174:689–91, 1954.
36. Mason, H. S., D. J. E. Ingram, and B. Allen, The free radical property of melanins, Arch. Biochem., 86:225–30, 1960.
37. Longuet-Higgins, H. C., On the origin of the free radical property of melanins, Arch. Biochem., 86:231–2, 1960.
38. Karreman, G., I. Isenberg, and A. Szent-Györgyi, On the mechanism of action of chlorpromazine, Science, 130:1191–2, 1959.