THE MODE OF ACTION OF PHENOTHIAZINE AS AN ANTHELMINTIC

II. PHENOTHIAZINE IN THE INTESTINAL FLUID AND NEMATODE PARASITES OF TREATED ANIMALS

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Summary

Methods are described for the identification of phenothiazine and its oxidation products by paper chromatography and by microionophoresis with the use of ^{35}S-labelled phenothiazine.

Extracts of the intestinal fluid from rats and chickens dosed with the drug were analysed for the presence of phenothiazine and its derivatives by these techniques. Only phenothiazine was detected in the intestinal fluid of either host but it appeared to be attached to a fatty substance from which it could not be separated by acid or alkaline hydrolysis. This phenothiazine-fat complex was not formed under anaerobic conditions *in vitro*; again, no oxidation products of the drug were detected but only phenothiazine was identified.

When *Ascaridia galli* was exposed to radioactive phenothiazine *in vivo*, 10 per cent. of the ^{35}S recovered was found in the acid-soluble fraction of the tissues and 68 per cent. in the alcohol-soluble fraction.

The parasites also appeared to contain the fatty complex of phenothiazine, and oxidation products of the drug could not be detected. The phenothiazine complex found in extracts of the gut fluid of the chicken penetrated *in vitro* the tissues of *Ascaridia galli* at a slower rate than did a suspension of the pure drug.

The results of the investigation indicate that phenothiazine itself, not its oxidation derivatives, is the anthelmintic agent.

I. INTRODUCTION

When phenothiazine was given to rats infested with *Nippostrongylus muris* and to chickens infested with *Ascaridia galli*, phenothiazine and its sulphur-containing derivatives, if such compounds were formed, appeared in the tissues of both parasites in similar amounts and remained in the parasites for long periods (Lazarus and Rogers 1950, 1951). However, as the result of the treatment *Ascaridia galli* was expelled from the host whereas *Nippostrongylus muris* appeared to be unaffected by the drug. The difference in action of the drug on the two parasites might be explained if toxic derivatives of phenothiazine were formed in the gut contents of the chicken or in the tissues of *Ascaridia galli* but not in the intestinal contents of the rat or its parasite. Another explanation of the differential toxicity of the drug to the two parasites might be attributed to differences in the metabolism of the para-

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sites. It is known, for instance, that the metabolism of *Nippostrongylus muris* is predominantly aerobic *in vivo* whereas that of *Ascaridia galli* is probably anaerobic (Rogers 1949).

The investigation described in this paper was carried out to examine the first hypothesis. Attempts have therefore been made to discover whether appreciable amounts of phenothiazine derivatives are formed in the gut contents of host animals treated with the drug and in their parasites, and whether the nature of the derivatives formed differs in different animals.

II. Methods

(*a*) *Biological Materials*

Ascaridia galli was obtained from experimentally infested chickens. After collection, the parasites were washed repeatedly in normal saline until free from debris and contaminating radioactive material.

Chickens that had been fasted overnight were dosed with phenothiazine directly into the crop. Rats were also fasted overnight and lightly anaesthetized with ether before phenothiazine was administered through soft catheter tubing directly into the stomach.

Phenothiazine labelled with ^{35}S was always diluted with suitable amounts of carrier before use and traces of wetting agent were added to facilitate suspension in water.

The intestinal fluid, referred to later, was obtained immediately after the animal was killed; the gut was ligated, the contents were collected and centrifuged. The supernatant fluid was used.

(*b*) *Preparation of Phenothiazine and its Derivatives*

Radioactive phenothiazine was synthesized from elemental ^{35}S and purified according to Lazarus and Rogers (1951). Large quantities of the unlabelled drug were purified from the commercial product by the method of Baker and Brickman (1945). The melting point of the pure phenothiazine obtained from both methods was 181-183°C.

Phenothiazone-3 was prepared by the method of Pummerer and Gassner (1913) and further purified by recrystallization, once from alcohol and once from water, m.p. 160-161°C.

The thionol and phenothiazine-5-oxide were gifts from Dr. Floyd De Eds, U.S. Department of Agriculture.

(*c*) *Separation of Phenothiazine and its Derivatives by Paper Chromatography*

A mixture containing 50 μg. each of phenothiazine and the derivatives, dissolved in 10 μl. of hot methanol, was applied to Whatman No. 1 special chromatography paper and the action of solvents was examined by the 1-dimensional ascending chromatographic technique of Williams and Kirby (1948). The R_F values of the substances in some of the many solvents tried are given in Table 1. It was found that a minimum of 10-20 μg. of phenothiazine, thionol, and phenothiazone-3 and 20-30 μg. of phenothiazine-5-oxide could be detected by the chromatographic method.

A good separation was effected by means of a 2-dimensional chromatogram with an acetone and water mixture in the proportions of 3 to 1, followed by absolute methanol. In each case the developing solvent was also used to saturate the atmosphere. A diagram of the chromatogram is given in Figure 1.

A 1 per cent. alcoholic solution of iodine sprayed on to the paper showed up the phenothiazine-5-oxide as a yellowish-brown spot which faded quickly, and the green, red, and blue colours of phenothiazine, phenothiazone-3, and thionol respectively were intensified by this reagent. However, the positions of the spots on the chromatogram could usually be detected without spraying.

TABLE 1

R_F VALUES OF PHENOTHIAZINE DERIVATIVES IN SEVERAL SOLVENTS

Compound	Acetone +Water 3 : 1	Absolute Methanol	Acetone +Methanol +Water 3 : 2 : 1	Dioxane +Water 2 : 1	Benzene +Methanol 3 : 1	Methyl Ethyl Ketone+Water 1 : 1	Butanol +Water 1 : 1
Phenothiazine	0·97	0·69	0·94	0·98	0·98	0·99	0·89
Phenothiazone-3	0·83	0·59	0·80	0·90	0·97	0·96	0·88
Thionol	0·66	0·45	0·58	0·82	0·43	0·94	0·70
Phenothiazine-5-oxide	0·85	0·68	0·83	0·91	0·75	0·94	0·89

(*d*) *Identification of Phenothiazine and its Derivatives Associated with Biological Materials*

Chromatograms of extracts of parasites and intestinal fluids of the host animals were prepared. The spots, cut from the chromatograms that had not been sprayed with iodine, were extracted with methanol and the extracts examined in a Beckman spectrophotometer, model D.U.

When the drug labelled with ^{35}S was used, radio-autographs were made of the chromatograms, or the distribution of activity in the chromatographed material was measured by the method of Taurog, Tong, and Chaikoff (1949) of passing the filter paper underneath the tube of a Geiger-Müller counter and determining the counts per minute at regular intervals along the strip.

Chromatograms were examined for the presence of proteins by the method of Wynn and Rogers (1950), tests for carbohydrates were made according to Trevelyan, Procter, and Harrison (1950) and a 2 per cent. aqueous solution of osmic acid was applied to detect the presence of fats.

(*e*) *Spectrophotometric Analysis*

The pure substances phenothiazine, thionol, phenothiazone-3, and phenothiazine-5-oxide, dissolved in methanol, were examined by spectrophotometry; their spectra agreed with those obtained by Houston, Kester, and De Eds

(1949*a*, 1949*b*). However, this method of analysis was found to be too insensitive for the identification of the oxidation products of phenothiazine in the amounts likely to be present in the intestine.

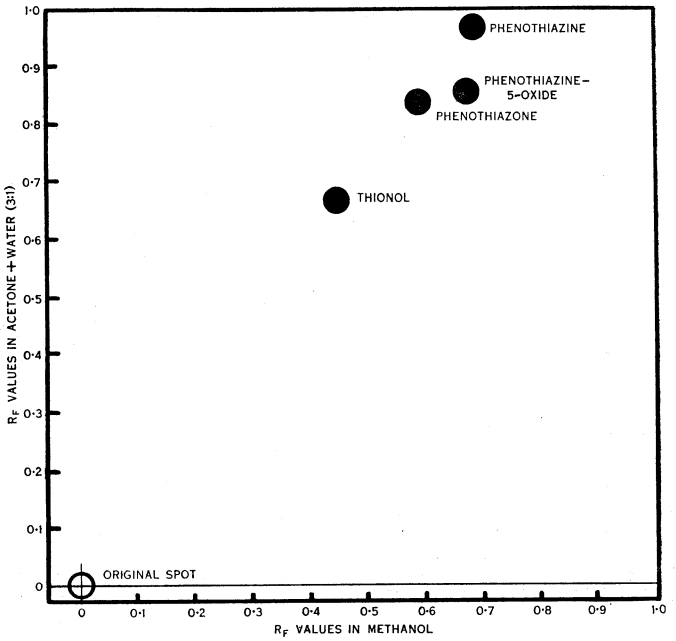

Fig. 1.—Two-dimensional chromatogram of phenothiazine and derivatives, using acetone plus water (3 : 1) followed by absolute methanol as solvents.

(*f*) *Microionophoresis*

Phenothiazine and its derivatives, in the pure state and in association with biological materials, were subjected to treatment by the microionophoretic technique of Durrum (1950), with a slightly modified apparatus. The solution to be analysed, 0.1 ml. containing 50 μg. each of phenothiazine and the derivatives, was applied as a circle of about 6 mm. diameter at the centre of a strip of Whatman No. 1 filter paper, 37 by 1 cm. When dry, the strip was saturated with the electrolyte, 0.2M phosphate buffer, pH 6.0, by Durrum's technique of hanging the paper over a glass rod and applying the electrolyte on either side of the test spot with Pasteur pipettes. After it had drained well, the strip was placed between two slabs of plate glass and set up as shown in Figure 2. A potential, usually of about 600 V., obtained from dry cells, was applied across the carbon electrodes in series with a milliammeter and a rheostat. The current usually varied from 2 to 6 mA. At the end of 3-6 hours the strip was removed, dried, and sprayed with a colour reagent, either iodine or dilute hydrochloric acid; the latter converts phenothiazine compounds into pink thionium derivatives.

It was found that thionol migrated towards the anode, phenothiazone-3 and phenothiazine-5-oxide moved towards the cathode, and phenothiazine itself was electrically neutral.

The migration of radioactive compounds was followed by measuring the activity of strips 2 mm. wide under the tube of a Geiger-Müller counter.

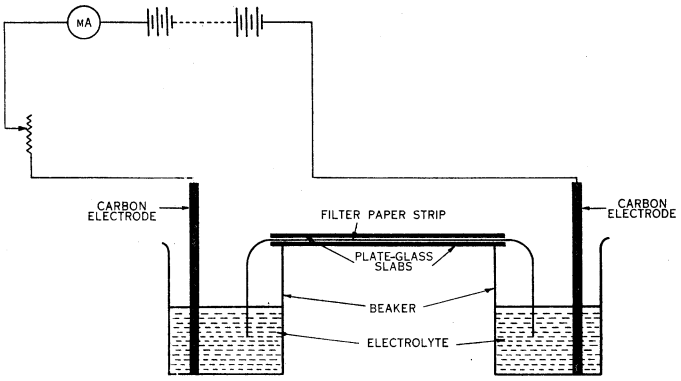

Fig. 2.—Apparatus used for separation of phenothiazine derivatives by microionophoresis. For explanation see text.

III. Procedure and Results

(*a*) *Phenothiazine in the Intestinal Fluid of the Host*

Groups of four normal rats, weighing approximately 100 g. each, were fasted overnight and then dosed, 1 g./kg., with phenothiazine. Each dose was given in 2 ml. of water, containing small amounts of an anionic detergent. In tracer experiments, 1-2 μc. of ^{35}S-labelled phenothiazine was included in the dose.

After 1½ hr. the rats were killed, the small intestines ligated, and the contents of the small intestines collected, pooled, and centrifuged; the supernatant fluid was extracted with 30 ml. of oxygen-free hot methanol or benzene and the extract was then concentrated *in vacuo*. All operations were carried out in an atmosphere of nitrogen.

Chickens were also fasted overnight and dosed with appropriate amounts of the drug, either in the non-radioactive form or containing 2-4 μc. of ^{35}S-labelled material. The birds were killed after 6 hr. and the intestinal fluid was collected and extracted in the same way as from the rats. The fluid from one chicken was sufficient for one experiment.

Intestinal fluid extracts from both rats and chickens behaved in a similar manner on chromatograms. When developed with the acetone plus water solvent, the material on the chromatogram showed the green colour of phenothiazine but trailed down the paper as if some other substance were interfering. When developed further with absolute methanol, the substance that trailed in the acetone plus water solvent gave R_F values to be expected from phenothiazine.

It should be stated here that sometimes the presence of phenothiazone also was noted on the chromatograms. However, at no time was phenothiazone observed in ionophoresis experiments, so its occasional appearance on chromatograms might have been due to unavoidable aerial oxidation.

Extracts of blood and bile from dosed chickens contained phenothiazine, which trailed in the acetone plus water solvent. In some experiments phenothiazone and thionol were identified in bile extracts.

The material associated with the phenothiazine on the chromatogram was negative to tests for proteins and carbohydrates but positive to tests for fats. No trailing was observed in chromatograms of extracts of intestinal fluid that had been incubated anaerobically *in vitro* with phenothiazine. Thus it appeared that the phenothiazine complex formed was peculiar to conditions *in vivo*. A comparison of chromatograms of *in vivo* and *in vitro* extracts is shown in Figure 3.

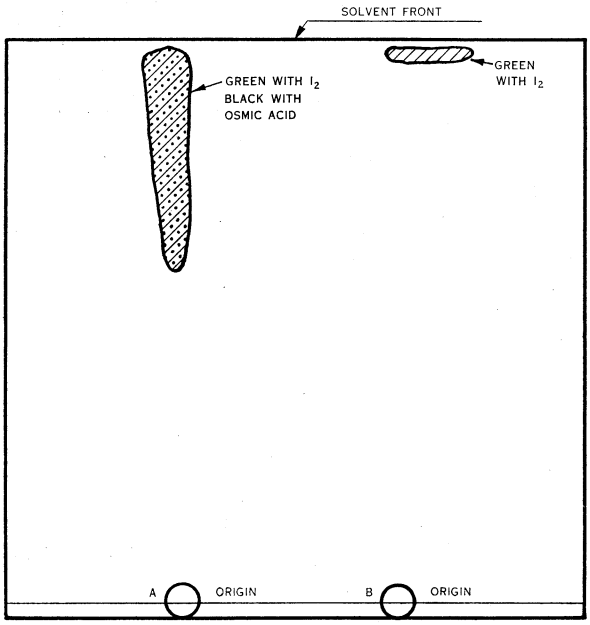

Fig. 3.—Typical chromatogram of an extract of intestinal fluid plus phenothiazine *in vivo*, *A*, and *in vitro*, *B*.

Attempts to hydrolyse the phenothiazine-fat complex with boiling 1N HCl for 2-3 hr. and with boiling 5N NaOH for 4 hr. failed.

Figure 4 gives a typical curve for the distribution of radioactivity in the chromatograms of intestinal fluid extracts from chickens dosed with the ^{35}S-labelled drug. A previous dose of peanut oil given to chickens to increase the fat content in the small intestine did not increase the trailing effect.

Gut fluid extracts were examined by microionophoresis and the results were compared with the ionophoretic behaviour of pure phenothiazine compounds. No migration to anode or cathode by extracts of intestinal fluid was observed, so it appeared that phenothiazine was, indeed, the only compound present.

(*b*) *Phenothiazine in the Parasites*

Unfortunately the infestations of *Nippostrongylus muris* in the rats gave insufficient material for the examination of phenothiazine compounds in the parasites, therefore these experiments were confined to *Ascaridia galli* from chickens.

The worms were collected 6-7 hr. after the host had been dosed with ^{35}S-labelled phenothiazine. The parasites were extracted with alcohol, which had been boiled to remove oxygen, for a period of 2-4 hr. in a Soxhlet apparatus. The alcoholic extract, concentrated *in vacuo,* was then chromatographed in acetone and water. It trailed on the paper in a similar manner to the extract from the host's intestinal fluid. However, the distribution of radioactivity in the chromatographed material was reversed (Fig. 5). This may have been due to the fact that the intestinal fluid extract was prepared in a different manner from the extract of the parasites, but in fact no adequate explanation has yet been found.

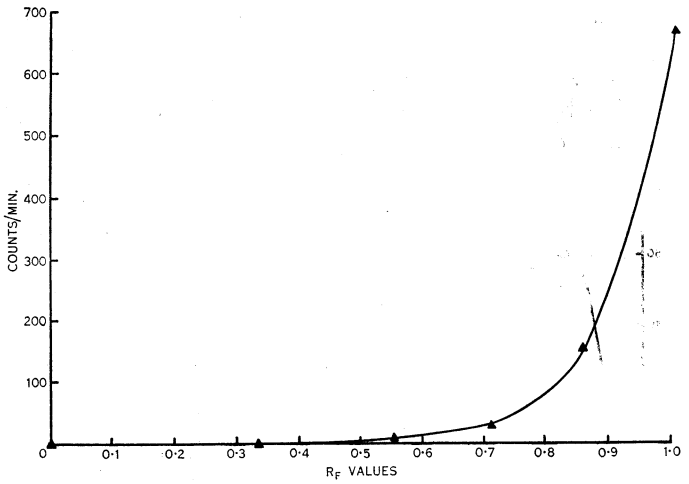

Fig. 4.—Distribution of radioactivity in chromatograms of extracts of intestinal fluid taken from chickens dosed with ^{35}S-labelled phenothiazine. The developing solvent was acetone plus water (3 : 1).

Ionophoresis of alcoholic extracts of the worms again indicated the presence of phenothiazine only.

A comparison was made of the rates of penetration *in vitro* into *Ascaridia galli* of the phenothiazine complex found in intestinal fluid extracts and of pure phenothiazine. Worms were collected from an undosed chicken and thoroughly washed in saline. The intestinal fluid of a chicken dosed with the ^{35}S-labelled drug, containing wetting agent, was extracted with benzene, concentrated *in vacuo* to 0.5 ml., and made up to 4 ml. with buffered saline (flask *A*). A suspension of radioactive phenothiazine in saline, containing 0.05M phosphate buffer at pH 6.0 and a trace of wetting agent, was made up so that 4 ml. of the suspension (flask *B*) was equal in radioactivity to the 4 ml. of the intestinal

fluid extract. One group of parasites was incubated in flask *A* and one in flask *B* for 2 hr. at 37°C. Then the parasites were washed repeatedly in saline, dried between filter papers, and prepared for assay of radioactivity by the method of Lazarus and Rogers (1951). The phenothiazine in the intestinal fluid extract was taken up at a slower rate than the phenothiazine in the suspension (Table 2).

TABLE 2

UPTAKE OF PHENOTHIAZINE BY *ASCARIDIA GALLI* AFTER EXPOSURE TO THE DRUG *IN VITRO* FOR 2 HR.

Medium	Phenothiazine Uptake (μg./g. wet wt.)
Buffered saline plus intestinal fluid extract	19, 13, 19, 17 (17)
Buffered saline plus wetting agent	19, 31, 26, 24 (25)

The figures in brackets show the mean value of the phenothiazine uptake; four experiments were carried out with each medium.

The tissues of *Ascaridia galli,* exposed to ^{35}S-labelled phenothiazine *in vivo,* were extracted by the following procedure in order to determine in what fraction the ^{35}S was concentrated.

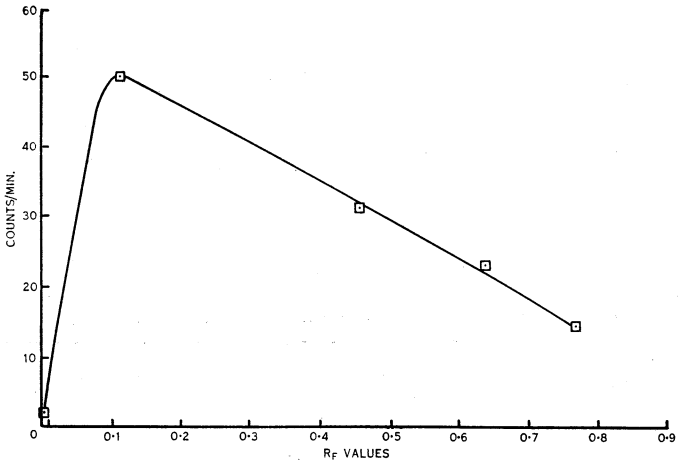

Fig. 5.—Distribution of radioactivity in chromatograms of alcoholic extracts of *Ascaridia galli* dosed *in vivo* with ^{35}S-labelled phenothiazine. The developing solvent was acetone plus water (3 : 1).

The worms were collected, washed well in saline and ground with cold 7.5 per cent. trichloracetic acid. Aliquots of the brei were assayed for total ^{35}S content (Lazarus and Rogers 1951). The remainder was centrifuged at 3,000 r.p.m. (*c.* 1300g) and aliquots of the supernatant were used to determine the acid-soluble ^{35}S. The residue was washed once with cold 8.5 per cent. trichloracetic acid, dried in a desiccator, and the supernatant discarded. The dried

residue was extracted with alcohol and alcohol plus ether (3 to 1), the extracts were combined, and the alcohol-soluble ^{35}S estimated. The amount of ^{35}S in the final residue was also estimated. From Table 3 it can be seen that most of the ^{35}S was contained in the alcohol-soluble fraction of the tissues of *Ascaridia galli.*

TABLE 3

DISTRIBUTION OF ^{35}S IN THE TISSUES OF *ASCARIDIA GALLI* DOSED WITH RADIOACTIVE PHENOTHIAZINE *IN VIVO*

Fraction	Percentage ^{35}S Recovered
Acid-soluble	10
Alcohol-soluble	68
Residue	22

IV. DISCUSSION

The immediate problem involved in the study of the mode of action of phenothiazine as an anthelmintic is the determination of the form in which the drug is active. Very large doses of the drug are required to obtain efficiency; the drug, though it enters the tissues of *Nippostrongylus muris*, has no anthelmintic action on this species (Lazarus and Rogers 1951). Further, the efficiency of phenothiazine against a species normally affected by the drug is variable and no satisfactory explanation of this variability has been given. It is possible, therefore, that phenothiazine itself is not the active agent and that the activity is due to other substances, either present in the drug as contaminants or formed as derivatives of phenothiazine within the host. The first suggestion is evidently incorrect because highly purified phenothiazine given with a suitable detergent is as active as the ordinary commercial product. The alternative explanation, however, requires examination.

The biological activity of phenothiazine and its derivatives against enzyme systems of host animals has been studied by Collier (1940) who found that mammalian catalase and cytochrome oxidase were not affected by phenothiazine but were strongly inhibited by leucophenothiazone, leucothionol, and thionol. Collier and Allen (1942) also reported the inhibition of cholinesterase by phenothiazone.

Field tests by Gordon and Lipson (1940) showed that phenothiazone had no anthelmintic effect on parasites in sheep and that thionol was ineffective against *Haemonchus contortus* and *Trichostrongylus* spp. Guilhon (1947), who worked with pigeons, also found that thionol was inactive *in vivo* against *Ascaridia colombae.* However, De Eds and Thomas (1941) examined the effect of thionol *in vitro* on *Ascaris lumbricoides* and claimed that thionol was a more effective poison than phenothiazine.

Whitten (1948) found that the activity of phenothiazine-5-oxide against the parasites of sheep was comparable with that of phenothiazine. However, as Whitten pointed out, only a small proportion of phenothiazine administered

to an animal is converted to the sulphoxide. Therefore, if the latter were responsible for the anthelmintic effect, a higher efficiency would be expected when the pure substance was given. No information is available concerning the stability of phenothiazine-5-oxide in the alimentary tract, especially as regards the possibility of its reduction to phenothiazine. Guilhon (1948) found the sulphoxide to be ineffective against *Ascaridia colombae* in pigeons.

Harpur, Swales, and Denstedt (1950) and Harpur, Denstedt, and Swales (1950) showed that after oral administration, the absorption of phenothiazine from the intestine was not dependent on oxidation to phenothiazone in the rumen. Indeed, the presence of phenothiazine in blood draining the rumen indicated that the drug was absorbed from it in the unoxidized state. The presence of leucophenothiazone was also observed in the peripheral circulation of lambs dosed with phenothiazine. No direct information on the anthelmintic activity of phenothiazine was brought forward by these authors but they are of the opinion that it is "attributable to the unabsorbed, but not necessarily unaltered fraction of the drug."

The results of the present investigation suggest that phenothiazine is the major component found in the intestinal contents and in the parasites treated with the drug. Other derivatives, especially those containing sulphur, were not detected and could not have been present in appreciable amounts. The conditions prevailing in the intestine of the host are unlikely to favour the formation of oxidation products of phenothiazine and these conditions were duplicated as far as possible during *in vitro* experiments.

Although it appears that conditions in the gut contents are such that the "chemical" nature of phenothiazine is not markedly affected, it is still possible that the physical state of the compound is important in relation to its anthelmintic activity. So far, little information concerning this matter is available. Phenothiazine, under some circumstances, can exist as resonance-stabilized free radicals (Murphy, Ravner, and Smith 1950). The significance of this mechanism in relation to biological activity is unknown.

The present work has shown that phenothiazine, both in the host's intestine and in the worms, appears to be attached to a fatty substance. It was thought that the nematode cuticle, which is largely albuminoid (Chitwood 1936), probably with a fatty surface layer (Trim 1949), might be more rapidly penetrated by such a fatty complex. However, experiments indicate that the presence of the fat does not increase the rate of uptake of the drug by the parasites.

It is clear from this investigation that further study is required concerning the biological action of phenothiazine itself in the tissues of parasites. Nematodes poisoned with phenothiazine are not unduly affected by it in aerobic environments (Lazarus and Rogers 1951); therefore the biological lesion caused by phenothiazine may be found among the mechanisms by which parasites obtain energy from anaerobic sources.

V. Acknowledgments

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VI. References

Baker, B. E., and Brickman, L. (1945).—*J. Amer. Chem. Soc.* **67**: 1223.
Chitwood, B. G. (1936).—*Proc. Helminth. Soc. Wash.* **3**: 1.
Collier, H. B. (1940).—*Canad. J. Res.* B **18**: 345.
Collier, H. B., and Allen, D. E. (1942).—*Canad. J. Res.* B **20**: 189.
De Eds, F., and Thomas, J. O. (1941).—*J. Parasit.* **27**: 143.
Durrum, E. L. (1950).—*J. Amer. Chem. Soc.* **72**: 2943.
Gordon, H. Mc.L., and Lipson, M. (1940).—*J. Coun. Sci. Industr. Res. Aust.* **13**: 173.
Guilhon, J. (1947).—*Bull. Acad. Vet. Fr.* **10**: 263.
Guilhon, J. (1948).—*Bull. Acad. Vet. Fr.* **11**: 227.
Harpur, R. P., Denstedt, O. F., and Swales, W. E. (1950).—*Canad. J. Res.* D **28**: 162.
Harpur, R. P., Swales, W. E., and Denstedt, O. F. (1950).—*Canad. J. Res.* D **28**: 143.
Houston, D. F., Kester, E. B., and De Eds, F. (1949*a*).—*J. Amer. Chem. Soc.* **71**: 3816.
Houston, D. F., Kester, E. B., and De Eds, F. (1949*b*).—*J. Amer. Chem. Soc.* **71**: 3819.
Lazarus, Marian, and Rogers, W. P. (1950).—*Nature* **166**: 647.
Lazarus, Marian, and Rogers, W. P. (1951).—*Aust. J. Sci. Res.* B **4**: 163.
Murphy, C. M., Ravner, H., and Smith, N. L. (1950).—*Industr. Engng. Chem.* **42**: 2479.
Pummerer, R., and Gassner, S. (1913).—*Ber. dtsch. chem. Ges.* **46**: 2324.
Rogers, W. P. (1949).—*Aust. J. Sci. Res.* B **2**: 166.
Taurog, A., Tong, W., and Chaikoff, I. L. (1949).—*Nature* **164**: 181.
Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950).—*Nature* **166**: 444.
Trim, A. R. (1949).—*Parasitology* **39**: 281.
Whitten, L. K. (1948).—*Aust. Vet. J.* **24**: 114.
Williams, R. J., and Kirby, H. (1948).—*Science* **107**: 481.
Wynn, V., and Rogers, G. (1950).—*Aust. J. Sci. Res.* B **3**: 124.