Table 2. PNEUMONIA AND TESTICULAR ATROPHY IN MALE SHERMAN STRAIN RATS CAUSED BY HMPA

Dosage level (mg/kg)	No. of doses Stomach tube	Diet	Days between last dose and pathological examination	Total examined	Number of animals: Killed	Died	With pneumonia	With partial or complete testicular atrophy
2,000	1		36–41	9	9	0	2	9
1,000	1		38	5	5	0	3	2
500	1		38	6	6	0	1	0
0	1		38	6	6	0	0	0
400	36–99		0	8	2	6	2	8
200	26–99		0	5	3	2	3	5
100	47		0	4	surgery*	0	–	4
	60–99		0	10	9	1	5	10
0	48		0	4	surgery*	0	–	0
	98		0	10	10	0	0	0
80–40†		45	0	5	surgery*	0	–	5
		61–103		11	8	3	8	11
0		45	0	3	surgery*	0	–	0
		103	0	8	8	0	0	0

* Left testis was removed under sodium pentobarbital anaesthesia. The same animals as well as others in the group that did not undergo surgery are included in the autopsy material.

† The dietary level of HMPA was 750 p.p.m. In the first few weeks the animals ate about 110 g/kg/day so that the intake of HMPA was about 80 mg/kg/day; later as the rats matured the average food intake dropped to 55 g/kg/day and the HMPA intake to about 40 mg/kg/day.

this time the spermatids are the cells of the seminiferous epithelium most affected by HMPA, but the effect is not uniform throughout the testes. Particularly after repeated dosing at or above the rate of 80–40 mg/kg/day, some tubules of some of the testes still contained normal or almost normal seminiferous epithelium while others were devoid of it or showed only a few atypical cells. We have called this condition 'partial atrophy', as in complete testicular atrophy (Table 2) all tubules showed loss of the seminiferous epithelium except for a few remaining spermatogonia. Similar pictures of partial or complete atrophy were also observed with metepa in an experiment conducted earlier[4].

A single dose of 2,500 mg/kg was given to six females. No effect on the female reproductive organs was observed when the animals were killed 36 days later.

HMPA was given to ten female rats by stomach tube at a rate of 200 mg/kg/day. The dosing began 7 days before mating to undosed males and continued until the twentieth day of pregnancy. The females were then killed and their uteri opened. The offsprings and placentae were weighed and examined grossly; the foetuses were fixed in Bouin's solution, and cross-sections were examined later under the dissecting microscope. Abnormalities were not detected in the offsprings of either the rats dosed with HMPA or eight female control rats that had been dosed daily for the same period with tap water. The weight of each foetus, the weight of each placenta, the number of animals per litter, and the number of resorption sites in the treated animals did not differ statistically from the controls.

A dosage of 100 mg/kg/day of HMPA is about 4 per cent of the acute oral LD_{50}. In earlier investigations in which metepa was administered by stomach tube, the lowest dosage level that caused partial testicular atrophy in some rats after 55 days was 10 mg/kg/day; this represents about 7 per cent of the acute oral LD_{50} dose. Metepa was found to be more toxic to rats when given daily by stomach tube in water than when given as a component in the diet—10 mg/kg/day by stomach tube had about the same effect as 76 mg/kg/day in the diet[4]. On the other hand, HMPA had about the same effect whether it was given in the diet or by stomach tube (see Table 2)—probably owing to its greater stability. Because of its stability, HMPA might present a greater problem as a residue than the ethyleneimines, which deteriorate rapidly, although both kinds of compound have a cumulative effect on the testes.

These data show that HMPA has a very specific effect on the testes of rats when fed at a dietary level of 750 p.p.m. which may be compared with a level of 2,500 p.p.m. in food necessary to cause 100 per cent sterility in male flies[1]. This comparison shows that the safety of any future use of HMPA must be based on its moderate toxicity to mammals and on limitation of their contact with it rather than on any important difference in the inherent susceptibility of mammals and insects to the compound.

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CONVERSION OF PHENOTHIAZINE DERIVATIVES TO THE CORRESPONDING SULPHOXIDES ON THIN-LAYER PLATES

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PREVIOUS experimental work[1] reported from this laboratory on the phenothiazine derivatives has dealt mainly with colour, crystal and spectrophotometric tests as a means of identification. In a preliminary report[2] at a meeting of the American Academy of Forensic Science, attention was directed to the value of the sulphoxides as a further means of identification of these drugs, and a method for preparing them quickly and accurately was described. The sulphoxides have been used only in a limited manner up to now, because only a few of them have been available commercially and their preparation in the laboratory was very tedious and time-consuming. The present article extends the data of the preliminary report and deals with a complete investigation of forty phenothiazine derivatives and their respective sulphoxides using ultra-violet spectrophotometry, thin-layer and gas chromatography. It also makes it possible now to investigate these phenothiazine derivatives and their sulphoxides using infra-red spectrophotometry.

Reference Compounds

A few sulphoxides were obtained from the various manufacturers. Others were prepared according to the

method of Schmalz and Burger[3]. 20 mg of the salt of the phenothiazine derivative were dissolved in 20 ml. of a solution prepared by mixing 20 ml. of 3 per cent hydrogen peroxide with 70 ml. of 85 per cent ethanol. The solution was heated in a water bath at 90° C for 3·5 h, after which time the *p*H was adjusted to 12 with ammonia. The sulphoxide formed was extracted with ether and the solvent evaporated. The identity of the finished product was established by means of its ultra-violet and infra-red spectra, colour tests, thin-layer and, where applicable, gas chromatography.

The ultra-violet spectrophotometric data were obtained on a Beckman '*DK*-2*A*' ratio recording spectrophotometer, the infra-red data on a Beckman '*IR*-4' infra-red spectrophotometer and the gas chromatographic work was done on a 'Microtek *GC*-2500*R*' gas chromatograph. A short-wave ultra-violet lamp, model '*SL*2537' (Ultra-violet Products, Inc., South Pasadena, California), was used to locate the spots on the thin-layer chromatography plates.

Each of the following drugs in the form of their salts was used in the experiments described here: chlorpromazine, thioridazine, trifluoperazine, thioproperazine, levopromazine, perphenazine, fluphenazine, triflupromazine, promazine and promethazine.

During routine work on the identification of phenothiazines it was observed that when a developed thin-layer plate was left in the open air for some days, the ultra-violet spectrum of an eluted spot no longer resembled that of the original product, but was close to that of its sulphoxide.

Gas–liquid and thin-layer chromatography confirmed that the sulphoxide had indeed been formed. Because of this certain conditions influencing the oxidation of phenothiazines on thin-layer plates were further investigated.

Plates were prepared for ten different compounds. Each plate contained eight spots of the drug assigned to it. The plates were developed in the solvent and were allowed to stand in open air in daylight at approximately 20° C. Each day one spot was eluted and the ultra-violet spectrum recorded. After an interval of 48–72 h, depending on the derivative under investigation, marked changes in the absorption spectrum had taken place; the spectrum began to resemble that of the corresponding sulphoxide. At the end of 8 days the spectrum was that of the pure sulphoxide (see Fig. 1). While a change in colour of the spot was noted a few minutes after the dry plate was exposed to the daylight, it was not accompanied by a change in the ultra-violet spectra. Gas–liquid and thin-layer chromatography confirmed that only the sulphoxide was formed.

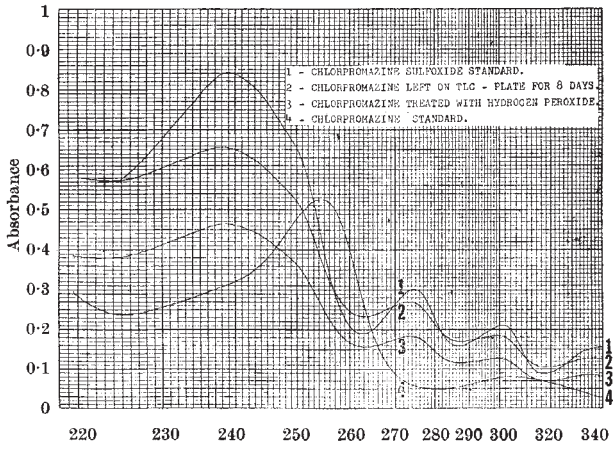

Fig. 1. Ultra-violet spectra of oxidized and unoxidized chlorpromazine. (1) Chlorpromazine sulphoxide standard; (2) chlorpromazine left on thin-layer chromatography plate for 8 days; (3) chlorpromazine treated with hydrogen peroxide; (4) chlorpromazine standard

Table 1. SPECTROPHOTOMETRIC DATA AT THE END OF 8 DAYS IN DAYLIGHT AND OPEN AIR (ABOUT 20° C) OBTAINED FROM THE ELUTED SPOTS

Generic names	Maxima of the standard drugs	Maxima of the sulphoxides
Chlorpromazine	254–305	238–274–298–340
Thioridazine	262–313	235–260–276–304
Trifluoperazine	257–308	244–275–304–336
Thioproperazine	234–264–315	249–275–304–342
Levopromazine	251–303	240–275–296–329
Perphenazine	255–307	233–275–300–340
Fluphenazine	258–308	233–255–274–302–340
Triflupromazine	257–308	233–255–274–300–340
Promazine	252–303	233–272–295–340
Promethazine	249–299	234–260–295–330

The oxidation was complete at the end of 8 days and was dependent mainly on the availability of oxygen. When the ten compounds, spotted and developed on plates, were placed in total darkness in an oven at 20° C over circulating air for 8 days, oxidation was complete. If similar experiments were carried out without circulating air in darkness or in daylight no oxidation occurred. The same was observed when the plate, spotted in the usual manner, was kept in a tightly closed vessel in an atmosphere of nitrogen for 2 weeks. This was confirmed by gas–liquid and thin-layer chromatography.

These experiments emphasize the importance of having sufficient air (oxygen) in order to obtain conversion of the phenothiazines to their respective sulphoxides. It was also evident that light was not essential in the oxidation process.

Numerous adsorbent gels are available on the market for thin-layer chromatography. Our experience with 'Silica Gel *G*', 'Adsorbosil *P*-2', 'Aluminium Oxide *G*' and 'Chromsorb-*G*' is that they do not by themselves have any effect on the formation of the sulphoxides in these experiments. It was further established that the thickness of the gels was not a decisive factor in the oxidation reaction since the ultra-violet spectra obtained from phenothiazine derivatives on plates of varying gel thickness were identical under standard conditions. The gels were not affected by the presence of hydrogen peroxide used to oxidize the phenothiazine derivatives to their sulphoxides. After spotting with 10, 20 and 30 per cent solutions of hydrogen peroxide, plates with silica gel were chromatographed, dried, eluted and examined in ultra-violet light. No absorption was detected.

The oxidation reaction appears to be associated with a relatively large increase in the surface area of the phenothiazine drugs on the plate. Such increase is brought about by the development of the plate after spotting with the drug.

Effect of Ultra-violet Irradiation

No change in the ultra-violet spectra was observed on the spotted plate up to and after 4 days of irradiation with ultra-violet light in a dark room without circulating air. It was observed, however, that deep brownish colours had developed in the spots. A control plate placed in the same room but excluded from ultra-violet irradiation had acquired a faint brown colour only, but no change in the ultra-violet spectra was noted. It is our opinion that ultra-violet irradiation does not have any appreciable effect on the oxidation of the phenothiazines by the method used. Further experiments may shed some light on the changes occurring under these conditions.

Effect of Temperature

When a series of plates, spotted with these drugs, is placed in an oven at 60° C with circulating air, conversion to the sulphoxides becomes measurable after about 2 h and is complete after 3–4 days. A peculiar phenomenon was encountered when a plate spotted with phenothiazine derivatives and developed in the usual manner was placed in an oven with circulating air at 120° C. Contrary to our expectations, only traces of oxidized material were detectable by ultra-violet measurements at the end of 3 days. Further experiments are in

progress to elucidate this phenomenon. Oxidative activity was practically absent at low temperature; no oxidation took place on a plate left at 0° C for 5 weeks.

Formation of Colour

Our experiments were carried out on plates, some of which were exposed to daylight while others were kept in darkness or exposed to ultra-violet irradiation. Other plates, in addition, were maintained in ovens where air was not circulated, in jars where there was no circulation of air, or in an atmosphere of nitrogen. The temperature of the atmosphere in which the plates were kept was also varied. In each case a colour developed in the spotted areas and varied as described later.

When plates were spotted with these drugs and maintained in daylight at approximately 20° C with or without oxygen, colour developed in the spots within 15 min. In darkness, the development of colour was delayed. Under these conditions the colours varied widely, depending on the concentration of drugs used. Also, the colour of any individual spot was not uniform, but varied from light shades in the central areas to deeper and darker shades in the periphery, or vice versa. When ultra-violet irradiation was applied to spots at 20° C in a dark room without circulating air, colour developed about as rapidly as it did in daylight. The shades were pastel at first and resembled those on the plates exposed to daylight, but with longer irradiation they changed colour. In those developed in daylight, on the other hand, the shade of the colour increased. The temperature at which the plates were stored was important in the development of the colour. After 6 weeks at 0° C only faint spots were evident, whereas with temperatures well above 20° C the spots appeared very rapidly and were more intense.

Hydrogen Peroxide as Oxidizing Agent

In our preliminary report[2] it was shown that spots of phenothiazine developed on thin-layer plates could be oxidized rapidly to the sulphoxide, using 3 per cent solutions of hydrogen peroxide (see Fig. 1). At that time the optimum concentration of peroxide had not been determined. Further investigations have shown that the concentration of hydrogen peroxide is not critical, but that best results are obtained when the concentration is between 10 and 20 per cent. The oxidation product obtained in this manner on the plate was confined to one spot only, as can be seen in Fig. 1 above the spot treated with 10–20 per cent hydrogen peroxide. The R_F value corresponds to that of the pure sulphoxide. The optimum of hydrogen peroxide concentration was determined as follows.

In Fig. 2 the original compound chlorpromazine HCl was spotted in such a manner as to obtain a large spot without tailing and the chromatogram was allowed to develop in direction 1. The spot was outlined under ultra-violet light and one strength of peroxide was applied to the upper part and another concentration to the lower part of the spot. Two reference spots were applied on line with and next to the spot, one with chlorpromazine and one with chlorpromazine sulphoxide. The plate was

Table 2. Spectrophotometric Data on Phenothiazine Derivatives kept in Circulating and Non-circulating Air (about 20° C) under Oxidizing and Non-oxidizing Conditions

Generic names	Maxima standard drugs	Maxima non-circulating air or nitrogen atmosphere	Maxima circulating air (sulphoxide)
Chlorpromazine	254–305	253–302	240–251*S*–274–300–340
Thioridazine	262–313	262–312	260–276*S*–304–338
Trifluoperazine	257–308	257–308	235–260–276–306–344
Thioproperazine	234–264–315	234–265–314	238–264–310–340
Levopromazine	251–303	250–502	250–275–298–332
Perphenazine	255–307	255–307	239–251*S*–275–306–344
Fluphenazine	258–308	256–307	234–260–276–306–348
Triflupromazine	257–308	255–307	238–258–281–310–342
Promazine	252–303	251–301	233–254*S*–270–300–340
Promethazine	249–229	249–300	232–250*S*–270–298–334

S, shoulder.

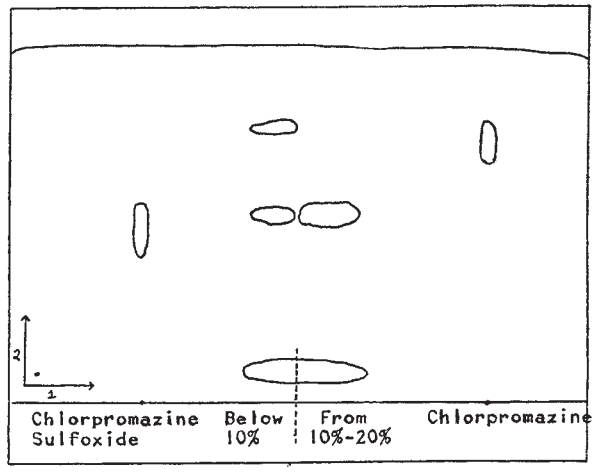

Fig. 2. Effect of concentration of hydrogen peroxide on complete oxidation of chlorpromazine

now allowed to develop in direction 2 perpendicular to the first one. This experiment indicated that in order to obtain a complete conversion to the sulphoxides for the quantity used, a concentration of at least 10–20 per cent hydrogen peroxide was essential. Below that concentration, only portions of the drug were oxidized and two spots were obtained on re-chromatographing. There was some indication that a second compound was formed when 30 per cent hydrogen peroxide was used together with hot air to dry the spot. This compound travelled behind the sulphoxide on the plate and might be the corresponding sulphone. The sulphoxides were identified by their ultra-violet spectra, colour tests, thin-layer chromatography and, where applicable, by gas–liquid chromatography.

Gas Chromatography

Gas chromatography is at present the most satisfactory method of separating and identifying the phenothiazines and their respective sulphoxides. Excellent separations were achieved using a 2-ft. 3 per cent '*SE*-30' column at different temperatures (Tables 3 and 4). It should be noted, however, that only very broad peaks could be obtained for the sulphoxides of the four phenothiazines with the highest molecular weights.

The retention times for these four compounds varied from 40 to 60 min. All compounds were injected in the free state as well as in salt form without any differences appearing in the chromatogram.

Table 3. Retention Times for Phenothiazine Derivatives

Compound	Temperature: Flow rate (ml./min):	200° C 80	Retention time 210° C 90	250° C 120
Triflupromazine		6·8	2·4	—
Promethazine		7·8	2·8	—
Promazine		9·1	3·1	—
Chlorpromazine		—	4·5	—
Levopromazine		—	5·7	—
Trifluoperazine		—	8·6	—
Fluphenazine		—	28·7	4·3
Thioridazine		—	29·8	5·8
Perphenazine		—	—	9·6
Thioproperazine		—	—	17·6

Table 4. Retention Times for the Sulphoxides

Sulphoxide of	Temperature: Flow rate (ml./min):	Retention time 210° C 90	250° C 120
Triflupromazine		4·2	—
Promethazine		6·6	—
Promazine		7·8	—
Chlorpromazine		10·2	2·5
Levopromazine		11·8	3·1
Trifluoperazine		16·8	3·6
Fluphenazine		—	—
Thioridazine		—	—
Perphenazine		—	—
Thioproperazine		—	—

Table 5

No.	Generic name	R_F of original drug	R_F of sulphoxide	Spectrophotometric data of sulphoxides obtained in the manner described
1	Acetophenazine	0·69	0·16	251–274*S*–310
2	Acetopromazine or acetylpromazine	0·58	0·39	251–272*S*–310–343
3	Aminopromazine or proquamazine	0·60	0·28	232–266–295–333
4	Carphenazine	0·71	0·16	246–277*S*–310
5	Chlorpromazine	0·62	0·48	238–273–298–340
6	Chlorprocthazine	0·69	0·49	238–250*S*–273–298–340
7	Chlorprothixene	0·66	0·45	255–302
8	Cyamepromazine	0·61	0·39	243–274*S*–304–340
9	Diethazine	0·69	0·44	233–268–293–338
10	Dimethoxanate	0·56	0·41	240–274–295
11	Ethopropazine or prophenamine	0·70	0·47	233–267–292–336
12	Fluphenazine	0·75	0·59	232–273–304–343
13	Isopromethazine	0·63	0·39	233–267–291–336
14	Isothipendyl	0·64	0·41	238–273–336
15	Levopromazine	0·87	0·59	250–276*S*–296–333
16	Mepazine	0·58	0·39	231–272–299–342
17	Methdilazine	0·64	0·43	232–272–298–342
18	Methopromazine or methoxypromazine	0·67	0·35	244–274*S*–294–330
19	Methylpromazine	0·62	0·40	238–272–299–340
20	Perphenazine or chlorpiprozine	0·65	0·45	240–250*S*–274–342
21	Phenothiazine or penethazine	0·69	0·37	232–266–294–334
22	Pipamazine	0·83	0·53	239–274–300–342
23	Prochlorperazine	0·55	0·15	238–274–300–340
24	Promazine	0·51	0·37	231–271–299–342
25	Promethazine	0·66	0·38	232–270–297–340
26	Pro iomazine	0·77	0·54	246–265*S*–304–360
27	Pro hipendyl	0·69	0·48	238–276–340
28	Pyrathiazine or pyrrolazate	0·64	0·43	232–269–295–336
29	Thiazinamium	0·53	0·33	232–269–294–336
30	Thiethylperazine	0·47	0·28	238–272–301–350
31	Thiopropazate	0·77	0·25	238–274–300–340
32	Thioproperazine	0·43	0·26	245–262*S*–275–304–342
33	Thioridazine	0·71	0·46	237–273–302–340
34	Transergan	0·53	0·33	225–266–291–330
35	Trifluoperazine	0·63	0·41	233–273–302–343
36	Triflupromazine	0·69	0·50	233–274–301–343
37	Trimeprazine or alimemazine	0·71	0·50	232–297–340
38	No. 6710 Rhône-Poulenc	0·63	0·46	251–273–298–332
39	No. 9269 ,,	0·85	0·59	240–274–305
40	No. 7261 Smith Kline and French	0·78	0·27	233–272–302–340

Proposed Method of identifying Phenothiazine Derivatives

The phenothiazine derivative is spotted in the lower two corners of a thin-layer plate (20 cm × 20 cm) approximately 3 cm from the edges. The plate is placed in the developing tank, with the spots at the bottom, until the solvent has travelled to about 3–4 cm from the top, after which time it is taken out and allowed to dry. The spots are now outlined under the short-wave ultra-violet light (cited previously) and the right-hand one is just wetted with small drops of 10–20 per cent hydrogen peroxide solution added drop by drop. The spot is dried in a hot air stream (60° C), after which the plate is placed in the tank again with the peroxide-treated spot at the bottom and allowed to develop in a direction perpendicular to the initial one. In this way, two R_F values can be obtained, one for phenothiazine derivative and one for the sulphoxide. The spots can now be removed from the plate and eluted with distilled water. After the ultra-violet spectra are obtained from the supernatant, the original phenothiazine and its sulphoxide can be compared with reference compounds with the use of thin-layer and gas–liquid chromatography. It should be noted that even after the most careful elution the gel remaining in the centrifuge tube still contains some sulphoxide which can be demonstrated by colour tests.

Table 5 shows the ultra-violet spectrophotometric data on the forty phenothiazine sulphoxides investigated, together with the R_F values and those of the parent compound.

We thank Smith Kline and French Laboratories and Dr. I. S. Forrest, Biochemical Research Laboratory, *T*-47 Veterans Administration Hospital, Palo Alto, California, for supplying samples of sulphoxides.

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MECHANISM OF THE CYTOTOXIC ACTION OF ALKYLATING AGENTS IN MAMMALIAN CELLS AND EVIDENCE FOR THE REMOVAL OF ALKYLATED GROUPS FROM DEOXYRIBONUCLEIC ACID

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THE reaction of alkylating agents of the mustard type with cellular deoxyribonucleic acid has been proposed as the basis for their cytotoxic action[1,2]. In an earlier report[3] results were presented which supported this view in the case of the reaction of mustard gas di-(2-chloroethyl)sulphide with mouse lymphoma cells (Fisher *L*5178*Y*) growing in culture. In this work two cell lines differing by a factor of about 2·5 in their sensitivity to the agents were investigated. With the more sensitive line, it was found that a dose which killed 90 per cent of the cells resulted in a depression of about 50 per cent in the rate of DNA synthesis within the first 2 h. This was the only rapid biochemical effect which was observed, protein and ribonucleic acid synthesis not being affected. It was also observed, however, that administration of a similar dose of sulphur-35-labelled mustard gas to the resistant line, which had very little biological effect, resulted in a similar extent of reaction of alkylating agent with the DNA of the cells. Walker has also recently reported[4] that the DNA of cells in the *S* or G_2 phases of