Estimation of Phenothiazine and Some of Its Oxidation Products in Biological Material

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A method is described for the separation and estimation of phenothiazine and three of its oxidation derivatives, phenothiazone, thionol, and phenothiazine-5-oxide. The fractions were separated by paper chromatography, eluted, and estimated colorimetrically. The method has been used successfully in feed and animal tissues.

METHODS are available for the determination of phenothiazine in medicinal preparations, orchard sprays, and biological materials but none of them distinguishes selectively and quantitatively between phenothiazine and some of its metabolic products. The methods described herein separate the compounds by paper chromatography. The fractions are then eluted and estimated colorimetrically either unchanged, as nitroso derivatives, or as a phenazothionium chloride derivative.

Materials and Methods

Preparation of Phenothiazine and Its Products. Phenothiazine was purified with charcoal followed by triple recrystallization from 95% ethyl alcohol. The product had a melting point range of 183.5–184.5 ° C.

Phenothiazine-5-oxide was prepared by the method of Pummerer and Gassner (*7*). The product had a melting point range of 251 — 252° C.

Phenothiazone was prepared by the method of Houston, Kester, and De Eds (*6*) and had a melting point range of 162–163° C.

Thionol was prepared by the method of Granick and Michaelis and the spectral characteristics were similar to those described by these authors (*4*). The melting point of the reddish-brown crystals was not established, but was above 300° C.

Extraction from Tissues. Homogenized aliquots of tissue to which known amounts of each compound had been added were weighed into a 50-ml. Erlenmeyer flask and an acetone - methanol - formaldehyde mixture (1 : 1 : 0.05) was added at the rate of 10 ml. of solution per gram of tissue aliquot. The flasks were stoppered and shaken for 30 minutes at room temperature. The extracts were filtered and chromatographed.

Chromatography. The drug and its fractions were separated by ascending paper chromatography in a developing solvent of acetic acid–benzene–water (1 : 2 : 3). The chromatographic chamber consisted of a borosilicate glass cylinder, 18 × 13 × 0.25 inches, fitted above and below with stainless steel plates. The upper plate was fitted with a Teflon stopcock for the evacuation of air and the addition of nitrogen gas. A polyethylene sheet fixed on the inside of the plates improved contact between the rims of the cylinder and the plates, as well as protected the steel plates from possible corrosion by solvent. Oxidation and photochemical changes during chromatography were inhibited by displacing air from the vessel with nitrogen and shielding the system from light. The chromatographic paper, 10 × 11 inches (Whatman No. 1), was equilibrated for 16 hours. Extracts were applied by micropipet, the lower edge of the paper was immersed in both phases of the solvent, and a further 16 hours were allowed for development of the chromatogram.

Elution. The chromatograms were removed from the chamber and dried in an oven at 50 ° C. for 30 minutes. The section of paper containing phenothiazine, phenothiazone, or thionol was cut into a 50-ml. Erlenmeyer flask and extracted with 10 ml. of acetone-water (3 to 1) solution by shaking the stoppered flask for 30 minutes at room temperature. Phenothiazine-5-oxide was eluted from the paper with 5 ml. of glacial acetic acid.

Color Development. Phenothiazine. Eight milliliters of the eluate were transferred to a 50-ml. Erlenmeyer flask and 3 ml. of 1 to 1 acetic acid–water solution and 0.5 ml. of sodium nitrite solution (200 mg. % aqueous) were added. The flask was stoppered and shaken for 30 minutes. The solution was filtered into a cuvette and read against a similarly prepared blank on a Beckman Model DU spectrophotometer at 485 mμ or on a Klett-Summerson colorimeter using a No. 47 filter.

Phenothiazone. Six milliliters of the eluate were transferred to a 50-ml. Erlenmeyer flask and 10 ml. of acetate buffer (97 ml. of 0.1*M* acetic acid and 3 ml. of 0.1*M* sodium acetate) and 0.5 ml. of thiosulfate solution (2.5% aqueous sodium thiosulfate pentahydrate) were added. The flask was shaken for 30 minutes. Half a milliliter of a nitrite solution (200 mg. % of sodium nitrite in water) was then added and the flask was shaken further for 15 minutes. The solution was filtered and read against a similarly prepared blank at 440 mμ on a Beckman DU spectrophotometer or on a Klett-Summerson colorimeter using a No. 44 filter.

Thionol. Five milliliters of the eluate were transferred to an Erlenmeyer flask and 10 ml. of glycine buffer (7 grams of glycine per liter adjusted to pH 9.7 with 5% NaOH) were added. The solutions were mixed and read immediately against a similarly prepared blank at 590 mμ on a Beckman DU spectrophotometer or on a Klett-Summerson colorimeter using a No. 60 filter.

Phenothiazine-5-oxide. Three milliliters of the eluate were transferred to a 50-ml. Erlenmeyer flask. Three milliliters of 8*N* hydrochloric acid and one drop of ferric chloride solution (1% $FeCl_3$ in 8*N* HCl) were added. The flask was stoppered and shaken for 2 hours. The solution was read against a similarly prepared blank on a Beckman Model DU spectrophotometer at 520 mμ or on a Klett-Summerson colorimeter using a No. 52 filter.

Results and Discussion

When solutions of phenothiazine and its derivatives were chromatographed by the method described here, the following mean R_f values were obtained: phenothiazine 0.09, thionol 0.6, phenothiazone 0.75, and phenothiazine-5-oxide 0.9. These R_f values were similar to those obtained for phenothiazine, phenothiazone, and thionol recovered from the tissues of poultry which had received phenothiazine orally.

Known amounts of various compounds were added to 5 grams of macerated liver and determined by the method described here. The results are pre-

Table I. Recovery Following Addition of Known Amounts of Various Compounds to Liver

Compound	No. of Tests	Range, P.P.M. Added	Range, P.P.M. Recovered	Range Recovery, %
Phenothiazine	5	8–20	8–18.6	97–101.5
Phenothiazone	5	7–18	6.5–17.6	87.2 –100
Thionol	5	2– 9	1.8– 7.5	83.5 – 93
Phenothiazine-5-sulfoxide	5	4–15	3.2–13.5	86.5 – 94

sented in Table I. Known amounts also were added as single tests to kidney, bile, intestinal contents, and two samples of commercial feed stuffs. In each case, the recovery of the individual compound was within the ranges reported for that compound in Table I, column 5.

The quantitative determination of phenothiazine and phenothiazone was based on their conversion to the amber-colored *N*-nitroso derivatives. This was carried out by treating the phenothiazine eluate directly with nitrous acid and in the case of phenothiazone, by first reducing the eluate and then treating it with nitrous acid. In both instances the colors were stable for at least 1 hour. In the case of thionol, advantage was taken of its intense blue color in alkaline solution which had a strong absorption band at 590 mμ, possibly the spectrum of the phenolate ion (*3*). The color was not stable at high concentrations and so was read as soon as possible after addition of the alkaline buffer.

Phenothiazine-5-oxide is converted to the sulfonium salt (*1, 2, 5*) when it is dissolved in strong acid. The resulting orange-pink solution had an absorption band at 445 mμ and was unstable; however, addition of one drop of ferric chloride solution produced a stable color with a maximum absorption at 520 mμ.

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PESTICIDES AND FOOD FLAVOR

Effect of Insecticides and Fungicides on the Flavor Quality of Fruits and Vegetables

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A NORTHEAST REGIONAL PROJECT (NE-15) was activated in July 1954 to determine the effects of pesticides on the quality of fruits and vegetables by means of organoleptic tests. Its primary concern was to uncover any association of poor flavor quality or off-flavor with pesticidal treatments.

The first task of the committee was to determine the extent of agreement on sample ratings among panels in different laboratories through exchange of samples. A second objective was the development of a scoring technique which would quickly, economically, and precisely evaluate the off-flavor hazard in the use of pesticides.

Preliminary Procedure

In 1954, a lack of uniform test material hampered the work of the committee because the project was activated too late to plant and treat crops specifically for the study. The use of crops designed for other purposes forcefully pointed to the necessity of an adequate supply of samples as uniform as is possible with biological material and controlled in every respect except for the variable under study.

Some of the first year's experiments were confounded by extraneous influences whereby significantly different results could erroneously be attributed to pesticides. Three examples illustrate this confounding.

Incomplete sealing of a few cans of snap beans resulted in flat-sour development, thereby nullifying an experiment.

The inclusion of some immature squash (to obtain sufficient control sample) resulted in overprocessing at the same time-temperatures that were optimum for mature squash. The resulting scorched flavor confounded the results of the study.

Apple juices from methoxychlor-treated trees were significantly lower-scored and in some cases rated as off-flavor by nearly all of the participating laboratories. It was known that the methoxychlor-treated trees were heavily