A STUDY OF THE SYNTHESIS, ANTIMYCOTIC ACTION, AND TOXICITY OF DIIODO-PARA-AMINOSALICYLIC ACID

by

William M. Bethmann

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A STUDY OF THE SYNTHESIS, ANTIMYCOtic ACTION, AND TOXICITY OF DIODO-Para-AMINOSALICYLIC ACID

INTRODUCTION

The first microorganism to be identified as the etiological agent of a disease was the fungus Achorion schoenleini, reported by Schoenlein in 1839 as the cause of favus ringworm. Most of the fungi which cause disease in man and animals were isolated and identified before 1900 (1). However, despite the ubiquitous nature of fungal diseases, the control of fungus infections has been a problem met with only limited and inconsistent success.

In 1942 it became apparent that the high percentage of dermatophytosis occurring in the armed forces, and the seriousness of the infections incurred in the tropics necessitated better methods of therapy. The Council on Pharmacy and Chemistry of the American Medical Association proposed that several authorities on the subject of pathogenic fungi prepare a report on the status of fungicidal agents and offer criteria to aid in the evaluation of such preparations.

The report (2), when completed, revealed there was a need for standardized procedures for testing fungistatic and fungicidal materials, and that many of the methods used gave incomparable results, or results that could not be reproduced under different conditions.

The committee, in recognizing the weakness of the testing procedures, stated that no compromise tests could be proposed which would satisfactorily evaluate all compounds from the standpoint of fungicidal
(or fungistatic) activity, toxicity or irritant properties, and effectiveness in the presence of body tissue.

The characteristics of the ideal disinfectant listed in the New and Nonofficial Remedies (3) can be extended to include the antimycotics. These properties are: high coefficient of disinfection, stability, solubility, penetrability, nontoxicity, noncorroding and nonbleaching action, non-specific action on microorganisms.

As a guide in testing antimycotic substances the Council on Pharmacy and Chemistry divided the tests into three divisions: laboratory tests of fungicidal action, toxicity tests, and clinical tests.

The laboratory tests consist of the in vitro evaluation of the ability of the preparation in question to destroy or inhibit the growth of fungi.

The toxicity studies are to determine the untoward effects, and the toxic level of the antimycotic upon the host animal.

The clinical tests are those performed upon persons exhibiting suitable pathological conditions, and are reserved for the compounds that have passed the most stringent screening procedures possible.

The combination of these tests provides the data necessary for choosing an antimycotic whose toxic levels are recognized and which does not have excessive irritating properties.

Much of the therapy used in the past was heroic in nature and left much to be desired. Iodine, silver nitrate, sulfurated lime, or potassium permanganate solutions, often used, are both destructive to tissue and unsightly.

The organic compounds have been widely investigated to find a substance that is effective against fungi without harming the host. Much
interest has been shown in the combination of the phenols and organic acids with the effective but corrosive halogens.

Klarman et al. (4,5,6,7) have reported on the systematic investigation of the germicidal and fungicidal action of monohalogenated phenol homologs. They found that substituting halogen atoms into the nucleus intensified the potency of the phenols studied.

Woodward, Kingery and Williams (8), and Walker, Porter and DeKay (9) in their investigations of miscellaneous alkyl and halogenated phenols found that halogen atoms increased the fungicidal power of the compounds from four to ten times, the fungicidal strength increasing from chlorine, through bromine to iodine.

Benzoic and salicylic acids have been used for the treatment of dermatophytosis for many years. In comparative tests against other marketed fungicides these aromatic acids have shown a relatively high degree of efficiency (10,11,12).

Delauney (13) reported the graded superiority of halogenated compounds over phenol as bacteriostats, again showing the ascending potency of chlorine, bromine and iodine. In the search for antituberculin drugs the halogen substituted aromatic acids have been studied. Saz and Bernheim (14,15,16) reported that 2,3,5 triiodo—benzoic acid and 3,5 diiodo—salicylic acid were effective in inhibiting the growth of Bacillus tuberculosis, in low concentrations. The substitution of a hydroxyl group in the ortho position made the diiodo benzoate as effective as the triiodo compound.

During the further investigation of salicylic acid derivatives for the treatment of tuberculosis, Lehman (17) found that p-aminosalicylic acid (PAS) was particularly toxic to Bacillus tuberculosis.
Drain, Goodacre and Seymour (18) prepared a series of PAS derivatives, hoping to enhance its antituberculin characteristics. Among their compounds was 3,5 diiodo-\(\beta\)-aminosalicylic acid, which was found to be active against the tubercule bacilli. However, they found that a high degree of toxicity to the mouse would limit its usefulness in tuberculosis therapy.

It was hoped at the beginning of this problem that satisfactory antimycotic properties for diiodo-para-aminosalicylic acid could be demonstrated.
I. EXPERIMENTAL PART

Iodination of Para-Aminosalicylic Acid

Analytical Methods

The analysis of the iodine content of the compound prepared was done by the potentiometric titration of the iodide ion, formed by the fusion of the iodinated compound with sodium carbonate.

Samples of the iodinated acid, weighing from 0.1 to 0.3 Gm., were fused with anhydrous reagent sodium carbonate in the manner described in the U. S. Pharmacopeia XIV (19). The crucibles were heated over Meker burners for three-quarters of an hour to one hour to insure complete fusion of the samples.

The cooled fusion mixture was transferred to a 400 cc. beaker. The larger crucible was put in the beaker without any attempt to remove the contents adhering to it. The smaller crucible was washed, and the washings added to the titration beaker. Ten per cent sulfuric acid was added to the fusion mass, care was taken to cover the beaker with a watch glass to prevent the loss of material from spattering due to effervescence. Sulfuric acid was added until effervescence ceased, and the beaker contained enough liquid to cover the crucible and allow immersion of the electrodes. The watch glass cover and the sides of the beaker were washed with distilled water. The solution was titrated without being filtered or transferred to another beaker.

In these determinations, tenth normal silver nitrate was used as the standard solution. In the first determinations, the potential
difference was measured between a pure silver wire electrode and a mercurous sulfate reference half cell by means of a Leeds and Northrup K-1 type potentiometer.

It was found to be faster and more convenient to make the titrations by using a line operated A.C. Dual Titrometer (Precision Scientific Company, Chicago, Illinois) and by measuring the potential difference between a silver electrode and a glass reference electrode. This method was used for the majority of the determinations.

The iodine was calculated from the volume of silver nitrate solution used between the initial reading of the burette and the first break in potential, and was calculated as per cent of iodine found in the compound.

Preparation of diiodo-\(p\)-aminosalicylic acid with iodine monochloride

Iodinated \(p\)-aminosalicylic acid was first prepared using iodine monochloride by the method of Evertsbusch and Bang (20).

Five grams of \(p\)-aminosalicylic acid (PAS) was dissolved in 100 cc. of ether and stirred constantly, as 10.3 Gm. of iodine monochloride was slowly added. The temperature of the reaction was kept below 30°C. The solution became a dark red color and a precipitate formed immediately. The reaction mixture was tightly stoppered and allowed to stand for twelve hours.

The light tan colored precipitate was removed by filtration. The precipitate was washed with ether and dried, and the melting point determined (219°C, uncorrected). The solubility characteristic and the melting point which corresponded to 220–222°C found in the literature (21) indicated that the precipitate was \(p\)-aminosalicylic acid.
hydrochloride.

The ether filtrate from the reaction mixture was evaporated without the use of heat, and the mass remaining was triturated with sodium carbonate to neutralize the hydriodic and hydrochloric acids present. The entire mixture was washed with water, and filtered to obtain the insoluble iodinated p-aminosalicylic acid.

Purification of the iodinated acid was accomplished by dissolving it without heat in a minimum of alcohol, and precipitating the acid by the addition of water. It was found that a colloidal suspension was formed with a gelatinous precipitate, if the pH of the solution was nearly neutral. However, if the acidity was increased to pH 3.8, the precipitate was heavy and flocculent in nature.

The acid was found to be instable if left in solution for prolonged periods. The dry crystals decomposed when heated to 90°C for twelve hours.

A yield of 23.45% of the theoretical was obtained.

Analysis: Calculated for C7H5O3N2I: I, 62.7% Found: I, 62.0%

Preparation of diiodo-p-aminosalicylic acid with iodine monochloride in the presence of pyridine

The formation of a precipitate which was assumed to be p-aminosalicylic acid hydrochloride, because of its melting point and solubility, suggests that instead of reaction I (below), reaction II occurs.

\[
\text{COOH} + 2 \text{I}Cl \rightarrow \text{COOH} + 2 \text{HCl}
\]
In an attempt to increase the yield of diiodo-\(p\)-aminosalicylic acid, pyridine, in excess, was added to the reaction solution to remove the hydrogen chloride from the reaction as it was evolved.

Five grams of PAS was dissolved in 200 cc. of ether and 40 cc. of pyridine. The reaction was carried out in a flask to minimize spattering as the iodine monochloride (10.6 Gm.) was slowly added.

The mixture was allowed to stand for twelve hours, after which time the solution was filtered to remove the red and yellow precipitate that had formed.

The dried precipitate was washed repeatedly with ether and the washings added to the clear, yellow ether filtrate. Addition of 25 cc. of concentrated sodium carbonate solution and agitation in a separatory funnel resulted in a heavy white precipitate, but produced no evolution of gas, or odor of pyridine. The precipitate and aqueous portion were removed from the separatory funnel, and the ether washed with several portions of dilute sodium hydroxide.

The combined aqueous portions were made acidic and the resulting precipitate dried and recrystallized from alcohol. The yield was 64.45% of the theoretical.

Analysis: Calculated for \(C_7H_5O_3NI_2\): I\(_2\) 62.7% Found: I\(_2\) 62.4%

The colored precipitate from the reaction solution was placed in water, where the yellow portion dissolved. When sodium carbonate was
added to this solution, a gas was evolved and the odor of pyridine released.

**Preparation of diiodo-p-aminosalicylic acid with iodine and hydrogen peroxide**

The method of preparation proposed by Seymour et al (22) was used.

Five grams of PAS were suspended in 27 cc. of alcohol, and 3 cc. of concentrated sulfuric acid slowly added. The temperature was kept below 60°C and the mixture was constantly stirred. Iodine crystals (8.2 Gm.) were added to the heavy, viscous mixture and the stirring continued. Five cubic centimeters of 30% hydrogen peroxide were added over a period of thirty minutes by slowly dropping it into the agitated mixture.

Stirring was continued for five minutes after the addition of hydrogen peroxide had been completed. The thick gray paste obtained was dissolved in alcohol and precipitated by the addition of distilled water. The compound was recrystallized from alcohol with a yield of 84.7% of the theoretical.

**Analysis**: Calculated for C₇H₅₀₃N₂I₂: I₂ 62.7% Found: I₂ 62.0%

**Preparation of sodium diiodo-p-aminosalicylate**

The diiodo acid was found to decompose if left in water or the organic solvents; however, the alkaline salt of the compound showed stability in water even at temperatures approaching 100°C.

To prepare the sodium salt, the iodinated acid was dissolved in a minimum of 2% sodium hydroxide solution by heating the mixture on a water bath. The aqueous solution became a clear, dark red color as the acid dissolved. Upon cooling, lustrous white platelets formed, and the addition of 1-2% sodium chloride completely precipitated the remaining
sodium salt, leaving a clear, colorless supernatant liquid.

The crystals of sodium diiodo-p-aminosalicylate were colorless, fine needles. The solubility of the sodium salt was approximately two percent with warming.

Analysis: Calculated for C₇H₄O₃NI₂Na: I, 59.45%  Found: I, 58.3%
Determination of the Antifungal Properties
of Diiido-p-Aminosalicylic Acid

The committee appointed by the Council on Pharmacy and Chemistry of
the American Medical Association indicated that the methods of testing
antimycotics were unsatisfactory (2). Much of the confusion arising
from many of the proposed procedures is due to the failure to differenti—
tate between the terms "fungistatic" and "fungicidal".

"Fungistatic" is understood to mean the ability to prevent growth
of fungi as long as the organisms are in contact with the antimycotic.
"Fungicidal" refers to the ability of a compound to prevent fungal
growth after having had contact with the organism for only a limited
time.

To differentiate between the fungistatic and fungicidal properties
of a substance, completely different testing procedures must be
employed. Several methods which incorporate the conditions necessary
for evaluating the worth of antifungal materials have been suggested
(11,12,23). These tests not only include the recommendations of the
Council on Pharmacy and Chemistry (2) but are more stringent and indica—
tive of the clinical worth of the antimycotic.

The fungal culture used in this work is Trichophyton mentagro—
phytes¹ (T. interdigitale, T. gypseum, Kauffman-Wolff fungus), which has
been demonstrated as representative of the pathogenic dermatophytes (11)
and is recommended by the Council. The stock cultures were stored at
2-5°C on agar slants. Transfers of the culture were made every thirty

¹Obtained from the Carolina Biological Supply Company, Elon
College, North Carolina.
days, allowed to grow for ten days, and then stored at 2-5°C until needed for seeding.

The horse serum used was drawn under as closely aseptic conditions as possible, stored for forty-eight hours, and the serum decanted from the clot that had formed. No contamination of the test plates due to the serum was noted.

The solubility of the diiodo-acid and its sodium salt prevented the testing of concentrations greater than two per cent.

Tests for fungicidal properties

For the testing of the acids in 95% alcohol the modification of Burlingame and Reddish's procedure (23) used by Oster and Golden (12) was employed. The time limit of five minutes of contact of the fungus with the fungicide has been demonstrated by Oster and Golden as sufficient to destroy fungal life with many substances and that one minute of contact was a criteria yielding more significant results.

One-Minute Contacts With

Sodium Diiodo-β-Aminosalicylate Solution

Petri dishes of Sabouraud's agar were streaked with a culture of T. mentagrophytes and permitted to grow at 37°C for ten days. The ten-day-old cultures were cut into disks of 1 cm. diameter with a sterile cork borer and transferred with aseptic precautions to seeding tubes containing 10 cc. of the fungicide in water. After a one-minute contact with the fungicide the disk was transferred to 10 cc. of sterile broth and shaken lightly for three minutes to free the matted culture of the water soluble fungicide. The culture block was then removed from the broth and washed in 10 cc. of sterile water for five minutes. Following
the water wash step, the disk was once more immersed in sterile broth for two minutes and then spread, culture side down, over the surface of a sterile slant of Sabouraud's agar. The slants were then incubated at room temperature.

This procedure was repeated for each of the 2%, 1%, 0.5%, 0.25%, and 0.1% concentrations of sodium PAS and sodium diiodo-p-aminosalicylate. Fresh, sterile tubes of broth and water were used for each concentration, and five slants of each concentration tested were prepared.

At the end of five days, a luxuriant growth was observed on each slant. No fungicidal effect was produced at any concentration.

Five-Minute Contacts With
Sodium Diiodo-p-Aminosalicylate Solution

The procedure was repeated using five minutes of exposure to the test solutions. At the end of five days, growth was seen on all of the agar slants. No fungicidal effect was produced at any concentration.

One-Minute Contacts With
Alcoholic Diiodo-p-Aminosalicylic Acid Solution

Petri dishes of Sabouraud's agar were streaked with a culture of T. mentagrophytes and permitted to grow at 37°C for ten days. The cultures were cut into disks of 1 cm. diameter with a sterile cork borer and transferred with aseptic conditions to seeding tubes containing 10 cc. of the various concentrations of the test compound in 95% ethyl alcohol. After a one-minute contact with the fungicide the disk was transferred to 10 cc. of sterile broth and shaken lightly for three minutes to free the matted culture of any water soluble or miscible
material. The culture block was then removed from the broth and washed in 10 cc. of 95% alcohol for five minutes, thereby removing the fungicide adhering to the mycelium. Following the alcohol washing step, the disk was once more immersed in sterile broth for two minutes to remove possible traces of alcohol and then spread, culture side down, over the surface of a sterile slant of Sabouraud's agar. This procedure was repeated for each of the 2%, 1%, 0.5%, 0.25%, 0.1% concentrations of PAS and diiodo-p-aminosalicylic acid in alcohol. Five test slants were prepared for each concentration and fresh sterile tubes of broth and alcohol were used for each concentration.

At the end of five days of incubation at room temperature, all of the test slants showed growth. No fungicidal action was shown.

Five-Minute Contacts With

Alcoholic Diiodo-p-Aminosalicylic Acid Solution

The procedure was repeated using five minutes of exposure to the alcoholic solutions of acids.

At the end of five days of incubation, only two slants showed no growth. One out of five disks exposed to the 2% concentration, and one out of five disks exposed to the 1% concentration of the diiodo-p-aminosalicylic acid in alcohol failed to produce evidence of growth.

Tests for fungistatic properties

The method used is designed to show the concentration at which fungistasis may be produced. The zone of inhibited growth about the cup containing the test substance would have given a basis for comparison, had both the PAS and diiodo-p-aminosalicylic acid shown this action.
Agar-Cup-Plate Method Employing Normal Horse Serum

Sabouraud's agar media was prepared and sterilized under 15 pounds of pressure for thirty minutes. The media was cooled to 40°C and enough normal horse serum added to equal ten per cent of the total volume. Twenty cubic centimeters of the media was poured into sterilized Petri dishes 9 cm. in diameter. A plate streaked with a broth suspension of fungi gave an uneven distribution of growth, therefore the following method of inoculation was used. To a 500 cc. portion of the media containing serum a small piece of the mycelial mat of a ten-day-old culture of T. mentagrophytes was introduced with a flamed wire loop. The media was shaken until the fungal matter could no longer be seen and 10 cc. of the culture media was added to each Petri dish. The agar media was allowed to harden and a disk 1 1/2 cm. in diameter was removed from the center of the plate with a flamed cork borer. Any cracks or crevices produced by the removal of the disk were sealed with a few drops of sterile agar media.

The sodium salt solution of both PAS and diiodo-p-aminosalicylic acid in concentrations of 2%, 1%, 0.5%, 0.25%, 0.1%, 0.05% in water (pH 6.9) were used. One-half cubic centimeter of each concentration was placed in the cup of three inoculated dishes by means of a hypodermic syringe. Care was taken to avoid dropping the solution onto the surface of the media, or spilling the liquid from the filled cavity.

The test plates were incubated at 37°C for five days, after which all the plates were overgrown with fungal growth, with no indication of inhibition.
Agar-Cup-Plate Method Omitting Normal Horse Serum

The presence of protein matter is known to reduce the antifungal action of many agents. It was for this reason that horse serum was included in the tests of Burlingame and Reddish (23) and Oster and Golden (11). To reduce the stringency of the test the horse serum was omitted from the media and the test repeated exactly as before. Three culture plates showed zones of inhibited growth at the end of four days but were overgrown after eight days of incubation at 37°C. The results are shown in Table I.

The clear zones of inhibition about the cups were all symmetrical and of the same width within each set. The zones were measured from the edge of the hole to the perimeter of the cleared area.

Table I. Response of T. mentagrophytes to Sodium Diiodo-p-Amino-salicylate in the Agar-Cup-Plate Fungistasis Test Without Normal Horse Serum in the Media

<table>
<thead>
<tr>
<th>Concentration of Diiodo-PAS</th>
<th>4 days</th>
<th>8 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.00%</td>
<td>10 mm.</td>
<td>0</td>
</tr>
<tr>
<td>1.00%</td>
<td>8 mm.</td>
<td>0</td>
</tr>
<tr>
<td>0.50%</td>
<td>5 mm.</td>
<td>0</td>
</tr>
<tr>
<td>0.25%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.10%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.05%</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Attempts to Incorporate
Sodium Diiodo-p-Aminosalicylate
into the Media

Attempts to incorporate the sodium salt of the diiodo acid into melted Sabouraud's media resulted in a suspension of the precipitated salt which could not be used due to questionable homogeneity.
Study of Irritation Resulting from the Intracutaneous Injection of Diiodo-p-Aminosalicylic Acid

One criteria of a good antimycotic is that it is relatively non-irritating. The use of laboratory tests to evaluate the degree of irritation produced by a compound can eliminate the need to extend unsuitable products to clinical testing.

Irritation studies connected with drug irritation, physiological response, and characterization of analgesics have been made (24, 25, 26) based upon the observation that intravenously injected colloidal dyes promptly stain injured skin areas as a result of increased capillary permeability at the site of injury (27). The depth of the colored area produced increases with the severity of the irritation, providing a basis for estimating the degree of irritation produced.

The rating of the intensity of color and the degree of irritation corresponding to it is a matter of relativity and is difficult to express. Although methods have been suggested for the assigning of numerical ratings (28), the final evaluation requires an adjectival rating. Table II shows the adjective correlating to the numerical evaluation of the extent of irritation, as outlined by Hoppe et al (29), that was employed in this work.
Table II. Estimation of Irritation by the Intensity of Staining by Trypan Blue at the Site of Injection of an Irritant Solution in Rabbits

<table>
<thead>
<tr>
<th>Appearance of Injected Area</th>
<th>Maximum Average Irritation Score</th>
<th>Adjective Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>No color</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>Faint, but discernable color</td>
<td>1-3</td>
<td>Mild</td>
</tr>
<tr>
<td>Distinct blue color throughout</td>
<td>4-7</td>
<td>Moderate</td>
</tr>
<tr>
<td>Deep blue color throughout</td>
<td>8</td>
<td>Marked</td>
</tr>
<tr>
<td>Ischemic central area surrounded by blue halo</td>
<td>16</td>
<td>Marked</td>
</tr>
</tbody>
</table>

Procedure

Eight albino rabbits, sex at random, weighing approximately 4 Kg. were used. The animals were fastened securely in a supine position and the hair carefully clipped from the abdominal area with a fine-bladed clipper, four to six hours before the test was to be made. At the time of the test the animals were again secured in the supine position and the bare abdominal areas were marked into 8 or 9 squares of approximately 20 sq. cm. each, by means of a soft wax pencil, and numbered for identification. A volume of 0.3 cc. of each test solution was injected intracutaneously into random areas, carefully avoiding areas that were irritated during the clipping. Chloroform was applied topically by laying a saturated pledget of cotton on the area for fifteen seconds.

The solutions of sodium diiodo-p-aminosalicylate (pH 6.9) used were prepared in both normal saline and distilled water. Since the 2% concentration crystallized from normal saline as the solution cooled, it was discontinued after two trials. The 1% concentration of the
iodinated compound, which was soluble in warm normal saline, was injected at a temperature of approximately 40°C.

As a comparative standard for rating irritation, solutions of quinine hydrochloride of 1%, 0.5% and 0.25% concentration in normal saline were injected simultaneously into the marked areas. Hoppe et al (29) gave these solutions of quinine ratings of "marked", "moderate" and "mild" irritation in the order of decreasing concentration.

For control purposes normal saline solution and distilled water were injected simultaneously.

Ten minutes after the intracutaneous injections were completed, 1 cc./kg. of 1% trypan blue in normal saline was injected into the marginal ear vein of the rabbit. The intensity of the color produced in each area was observed at one-half, one, three, and twenty-four hour intervals. The results of the tests are shown in Table III.

It was found that some of the irritated areas began to be stained within one minute after injection of the trypan blue. Maximum coloration was reached within three hours after injection. Sclerosis of tissue at the site of injection was noted in several instances at the end of twenty-four hours. In three cases in which 2% diiodo-p-amino-salicylic acid solutions were used, sclerosis occurred after twenty-four hours, at the sites of injections which had been scored as "moderate" or "marked" at the end of three hours. The areas marked "2" and "4" in Figure 1 illustrate the ischemia that usually preceded sclerosis.

It is apparent from the results shown in Table III that the effect of distilled water is irritating and that it exhibits an additive irritating effect to that of the chemical agent in solution.
From this work it was concluded that sodium diido-p-aminosalicylate was only mildly irritating in concentrations of 1% or less. An accurate evaluation of the 2% concentrations could not be made due to the solubility factor involved.
Table III. Compiled Results of the Irritation Resulting From the Intracutaneous Injection of Diiodo-\(p\)-Aminosalicylic Acid and Other Chemical Agents on the Rabbit, Evaluated by the Trypan Blue Staining Method

<table>
<thead>
<tr>
<th>Solution Injected</th>
<th>Number of Trials</th>
<th>Numerical Rating</th>
<th>Adjective Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diiodo-PAS 2% in Saline</td>
<td>2</td>
<td>16</td>
<td>marked(^a)</td>
</tr>
<tr>
<td>Diiodo-PAS 2% in Water</td>
<td>4</td>
<td>16</td>
<td>marked(^a)</td>
</tr>
<tr>
<td>Diiodo-PAS 1% in Saline</td>
<td>4</td>
<td>1-3</td>
<td>mild</td>
</tr>
<tr>
<td>Diiodo-PAS 1% in Water</td>
<td>2</td>
<td>8</td>
<td>marked</td>
</tr>
<tr>
<td>Diiodo-PAS 0.5% in Saline</td>
<td>4</td>
<td>1-3</td>
<td>mild</td>
</tr>
<tr>
<td>Diiodo-PAS 0.5% in Water</td>
<td>2</td>
<td>8</td>
<td>marked</td>
</tr>
<tr>
<td>Diiodo-PAS 0.25% in Saline</td>
<td>4</td>
<td>1-3</td>
<td>mild</td>
</tr>
<tr>
<td>Diiodo-PAS 0.25% in Water</td>
<td>2</td>
<td>8</td>
<td>marked</td>
</tr>
<tr>
<td>Diiodo-PAS 0.1% in Saline</td>
<td>4</td>
<td>1-3</td>
<td>mild</td>
</tr>
<tr>
<td>Diiodo-PAS 0.1% in Water</td>
<td>2</td>
<td>4</td>
<td>moderate</td>
</tr>
<tr>
<td>Quinine-HCl 1% in Saline</td>
<td>7</td>
<td>8</td>
<td>marked</td>
</tr>
<tr>
<td>Quinine-HCl 1% in Water</td>
<td>1</td>
<td>16</td>
<td>marked(^a)</td>
</tr>
<tr>
<td>Quinine-HCl 0.5% in Saline</td>
<td>7</td>
<td>4</td>
<td>moderate</td>
</tr>
<tr>
<td>Quinine-HCl 0.5% in Water</td>
<td>1</td>
<td>16</td>
<td>marked(^a)</td>
</tr>
<tr>
<td>Quinine-HCl 0.25% in Saline</td>
<td>7</td>
<td>1-3</td>
<td>mild</td>
</tr>
<tr>
<td>Quinine-HCl 0.25% in Water</td>
<td>1</td>
<td>16</td>
<td>marked(^a)</td>
</tr>
<tr>
<td>Saline Control</td>
<td>8</td>
<td>0</td>
<td>none</td>
</tr>
<tr>
<td>Water Control</td>
<td>8</td>
<td>2-4</td>
<td>mild-moderate</td>
</tr>
<tr>
<td>Chloroform</td>
<td>8</td>
<td>8</td>
<td>marked</td>
</tr>
</tbody>
</table>

\(^a\)Sclerosis occurred at site of injection
Figure 1. The Erypan Blue Irritation Test Demonstrating the Local Action of Diiodo-p-Aminosalicylic Acid on Rabbit Skin as Compared to the Local Effect of Known Irritants

Designation of the numbered areas in Figure 1 is as follows: 1. Normal Saline, 2. Diiodo-PAS 2% in water, 3. Distilled Water, 4. Diiodo-PAS 2% in Saline, 5. Quinine-HCl 1% in Saline, 6. Quinine-HCl 0.5% in Saline, 7. Chloroform, 8. Quinine-HCl 0.25% in Saline.
Acute Toxicity of Diiodo-\(p\)-Aminosalicylate on the White Rat

Although the likelihood of absorption of antifungal compounds in toxic quantities from relatively limited areas of skin is slight, the Council on Pharmacy and Chemistry of the American Medical Association recommends the determination of the toxic values of antimycotic substances.

For the purpose of this work it was decided to determine the median lethal dose (ID\(_{50}\)) of sodium diiodo-\(p\)-aminosalicylate for the white rat.

Procedure

White Wistar rats with weights ranging from 125 to 325 Gm. were used. The animals were fasted for twelve hours prior to the test, divided into nine groups of six animals with approximately an equal distribution of weight and sex.

The animals received 2\% sodium diiodo-\(p\)-aminosalicylate solution (pH 6.9) by intraperitoneal injection in doses ranging from 0.270 Gm./Kg. to 0.400 Gm./Kg. Dosages were calculated to the nearest 0.1 Gm. of body weight. The animals were observed for twenty-four hours and the mortalities recorded. The results are summarized in Table IV. The median lethal dose was calculated by the graphic method of DeBeer (30). The ID\(_{50}\) obtained was 0.342 Gm./Kg. Limits of error: 95.6\%-104.8\%
Table IV. The Acute Toxicity of Sodium Diodo-p-aminosalicylate When Injected Intraperitoneally Into White Rats

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Dosage Gm./Kg.</th>
<th>Number of Rats Injected</th>
<th>Percent Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.270</td>
<td>6</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>0.300</td>
<td>6</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
<td>0.320</td>
<td>6</td>
<td>16.67</td>
</tr>
<tr>
<td>4</td>
<td>0.330</td>
<td>6</td>
<td>50.00</td>
</tr>
<tr>
<td>5</td>
<td>0.350</td>
<td>6</td>
<td>50.00</td>
</tr>
<tr>
<td>6</td>
<td>0.360</td>
<td>6</td>
<td>66.67</td>
</tr>
<tr>
<td>7</td>
<td>0.370</td>
<td>6</td>
<td>83.33</td>
</tr>
<tr>
<td>8</td>
<td>0.380</td>
<td>6</td>
<td>100.00</td>
</tr>
<tr>
<td>9</td>
<td>0.400</td>
<td>6</td>
<td>100.00</td>
</tr>
</tbody>
</table>

It was observed that the rats exhibited similar characteristic symptoms before death. Within fifteen minutes after injection of the compound, lacrimation, salivation, and hyperneic breathing began. Death was preceded by convulsions of short duration resembling those produced by anoxia. Immediately following death, the muscles of the neck, back and extremities of the animal were in a state of pronounced rigidity. The heart at death was in systolic standstill with the auricles, vena cava, and coronary vessels engorged with blood. Death was apparently due to stagnant type anoxia, as a result of cardiac failure.
II. DISCUSSION

In this work it was found that the use of the glass reference electrode in the potentiometric determination of the iodine content of the diiodo-p-aminosalicylic acid was faster and simpler than the use of the mercurous sulfate half cell. The potential changes were fast and showed very little fluctuation.

Drain, Goodacre, and Seymour's investigation of the toxicity of diiodo-p-aminosalicylic acid to the tubercle bacilli showed that it was active in low concentrations (18). A greater effect upon fungi might be expected, but Klarman et al. (5,6) showed that there was no consistent correlation between the action of chemical agents on bacteria and fungi.

During the preparation and attempts to show the antifungal characteristics of this compound, solubility and stability properties were noted that would make it of doubtful value as an antmycotic. The alcoholic solutions of the diiodo-acid were limited to two per cent concentrations and precipitation occurred if water was introduced to the solutions. The solubility of the sodium salt was approximately two per cent in water with warming at approximately pH 7, but the iodinated compound was precipitated by the presence of other salts in the solution.

The diiodo-p-aminosalicylic acid as a result of instability was not found to meet the practical standards for an antmycotic. Solutions of the iodinated compound became darker and developed a flocculent brown precipitate at room temperatures. This was thought to be a result of decarboxylation, a common problem in the manufacture of PAS pharmaceuticals (31).
The irritation resulting from the intracutaneous injection of sodium diiodo-p-aminosalicylate solutions of 1% or less concentrations in saline was found to be no greater than that resulting from the injection of distilled water alone.

The acute toxicity of sodium diiodo-p-aminosalicylate for the white rat was found to differ considerably from that reported for p-aminosalicylic acid. McClosky, Smith and Frias (32) have reported that intravenous injections of the latter in doses as high as 2 Gm./Kg. were well tolerated by the white rat. In the present study it was found that intraperitoneal injections of the former in doses as low as 0.4 Gm./Kg. were invariably fatal to the white rat. Post-mortem examination of these animals revealed that the compound exerted a direct toxic action on the heart. This study indicates that iodination of the parent compound increases the acute toxicity.
III. SUMMARY

1. Diiodo-p-aminosalicylic acid was prepared by three methods and analyzed for iodine content.

2. The compound was tested for fungicidal characteristics in alcoholic and aqueous solutions.

3. Sodium diiodo-p-aminosalicylate was tested for its fungistatic characteristics, both in the presence and absence of serum protein.

4. The irritation resulting from the injection of sodium diiodo-p-aminosaliclylate solution intracutaneously in the rabbit was determined.

5. The median lethal dose of diiodo-p-aminosalicylate for the white rat was determined.
IV. CONCLUSIONS

Diiodo-p-aminosalicylic acid was prepared by three methods, and the yields reported. The use of hydrogen peroxide and iodine crystals was the simplest, most practical and best yielding method of preparation used. The addition of pyridine, in excess, to the reaction solvent increased the yield of the iodinated compound when iodine monochloride was used.

The compound did not show the qualities of a suitable antimycotic. Only slight inhibition of fungal growth under the most lenient conditions was demonstrated.

The irritation resulting from the intracutaneous injection of sodium diiodo-p-aminosalicylate solutions into the rabbit was found to be mild, at concentrations of one per cent or less, in normal saline solution.

The median lethal dose of sodium-p-aminosalicylate for the white rat was found to be 0.342 Gm./Kg.
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