THE INHIBITORY ACTIVITY OF DERIVATIVES OF SALICYLAMIDE AGAINST TWO SPECIES OF DERMATOPHYTES

by

ARCHIE YOUNG HAMILTON

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APPROVED:

[redacted]

Associate Professor of Bacteriology

In Charge of Major

[redacted]

Chairman of Department of Bacteriology

[redacted]

Chairman of School Graduate Committee

[redacted]

Dean of Graduate School

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INTRODUCTION

The study of a chemical compound that will inhibit the living processes of any pathogenic organism may serve a two-fold purpose. Investigation of the mechanism by which the compound inhibits the organism may shed new light on certain metabolic activities of the latter. Secondly, the possibility that the compound might have therapeutic value in the treatment of disease must also be considered.

The need for the study of new inhibitory compounds against the dermatophytes has become increasingly apparent. The more common and most widely used inhibitory agents against this group of fungi, propionic and undecylenic acids, undecylenates, salicylanilide and 5-chlorosalicylanilide, and salicylates, have not proven completely satisfactory in the treatment of dermatophytosis and have shed little light on the complex metabolic activities of these organisms.

In view of the fact that salicylates have long been prominent in the treatment of fungus infections and other diseases of the skin, it seemed worthwhile to examine the

inhibitory activity of a new group of salicylate derivatives against the dermatophytes.

HISTORICAL REVIEW

The dermatophytes (3, pp.590-598) are a closely related group of pathogenic fungi characterized by their ability to infect the keratinized areas of the body such as the skin, hair and nails. These organisms do not produce systemic infections and very rarely infect the subcutaneous tissues. The more common infections produced by these organisms are Athlete's foot (tinea pedis), infection of the nails (tinea unguium), infection of the glabrous skin of the body (tinea glabrosa) and ringworm of the scalp (tinea capitis). This group of fungi is composed of 3 genera, Microsporum, Trichophyton and Epidermophyton.

Salicylates (5, p.9) are organic chemical compounds derived from orthohydroxy benzoic acid. Most of the salicylates used for therapeutic purposes are formed by substitution on the carboxyl group, but the most widely used salicylate, acetylsalicylic acid, has the acetyl grouping substituted on the hydroxyl group.

The naturally occurring salicylates are widely distributed among various plants. These naturally occurring salicylates (5, pp.1-9) such as salicin and methyl salicylate were used as remedies in ancient times. Hippocrates used the juice of the willow tree in the treatment

of infections of the eyes and the leaves sodden in vinegar as a cataplasm for gout.

The modern history of salicylates began in 1874, when Kolbe and Lautmann (5, p.5) developed a procedure to produce synthetic salicylic acid. This was the groundwork which led to the utilization of salicylic acid and its many derivatives. The uses of these compounds in medicine have been numerous and the literature dealing with the therapeutic values of salicylates is voluminous. Extensive work has been done on the analgesic activity of the drugs, the treatment of rheumatic and skin diseases and the antipyretic properties of these compounds.

The use of salicylic acid in the treatment of dermatitis was established as early as 1888 (5, p.260) as reported by Heitzmann in "The value of salicylic acid in dermatology". McMurtry in 1913 (7, p.166) reported that salicylic acid possessed a wider range of usefulness than any therapeutic agent at the disposal of the dermatologist.

In the treatment of dermatitis, the action of salicylic acid is largely limited to its effect on the pathological tissue (7, p.168). In a 3 per cent or greater concentration, the acid is highly keratolytic as well as antiseptic. The action of this agent upon the horny layer of the epidermis is of immense value in cleaning

and preparing the area for local medication by the salicylic acid present or other therapeutic agents.

Binz (7, pp.168-169) reported that salicylic acid was an active poison for many types of protein and considered it a powerful parasiticide. In recent years the compound has been looked upon as a poor antiseptic agent and to some extent has been replaced by salicylate derivatives and other compounds.

Salicylanilide has become prominent as a commercial fungicide as well as a chemotherapeutic agent for dermatophytosis. Leonard and Pittman (6, pp.2338-2341) incorporated approximately 100 toxic agents into varnish and lacquer vehicles and tested the fungistatic properties of these compounds by natural exposure of the vehicles in a Panama jungle. Salicylanilide was found to possess good fungistatic properties, second only to ortho-hydroxy phenyl mercuric chloride.

Wade (12, p.266) reported that "Shirlan AG" (salicylanilide) was effective in the prevention of "brown rot" of apricots caused by the fungus Sclerotinia fructicola.

Topical application of 5-chlorosalicylanilide (8, p. 160) in a carbowax base produces a high per cent of cures in tinea capitis (ringworm of the scalp). A 5 per cent concentration of the compound in the ointment base was

more effective than 12 other compounds tested.

Antimicrobial agents may be general or specific in their action. (3, p.645; 10, pp.257-263). The general group of antimicrobial agents are so-called protoplasmic poisons and are toxic to a wide range of organisms. This group of compounds is composed mainly of acids, alkalies, salts, metallic and organometallic substances, inorganic ions, halogens, oxidizing agents, phenols, dyes, surface active agents, alcohols and other organic solvents. These compounds are non-specific in their action.

The specific antimicrobial agents are toxic to a limited number of organisms due to an interference with a physicochemical or metabolic mechanism which is peculiar to the susceptible organism.

"The manifestation of biological activity by a drug is a process which proceeds through various stages from the point of application of the drug to the final site of the chemical event which is the immediate cause of the desired effect" (10, p.59). These stages may involve the phenomenon of membrane passage, diffusion or transfer from the aqueous to the lipoid phase. In some instances it may be that only one step is significant. If this is a physicochemical step then a correlation can be observed between the biological activity and the physicochemical property concerned. These circumstances have given rise

to a number of empirical rules or hypotheses connecting physical properties and biological activity. In so far as it is known how the chemical structure affects the physical property, it is possible to correlate chemical constitution with biological activity.

The physical and chemical properties of a compound are determined by the number, kind and arrangement of the atoms within the molecule (13, p.4). It is not possible to make clear and precise distinctions between chemical and physical properties; however, the physical properties shown by a compound may determine the extent of absorption and concentration at the site of action and can have a significant relation to the observed biologic effect.

MATERIALS AND METHODS

Compounds Studied. The salicylamide derivatives used in this study were provided through the courtesy of Cutter Laboratories, Berkeley, California and were synthesized by Sayhun Laboratories, Santa Barbara, California. The majority of these are new compounds.

Salicylic acid, salicylamide and sodium undecylenate were used for comparative purposes, these being known anti-fungal agents.

The chemical data on these compounds provided by Sayhun Laboratories appear on pages 21 through 25.

Equipment. In addition to standard bacteriological laboratory equipment and sterile laboratory glassware, 100 ml screw cap containers, previously rinsed with distilled water were employed. These were sterilized by autoclaving and dried in an electric oven.

A 300 ml calibrated urinary irrigation cylinder was used as a dispensing burette. The accuracy of the cylinder was found to be within ± 2 per cent when 40 ml volumes were dispensed.

Medium. Sabouraud agar, used in the inhibition studies, and Sabouraud liquid medium, used in shake flask cultures, were prepared in 1 to 2 liter quantities. The

pH was adjusted at 5.9 to 6.0. The agar was then dispensed in 100 ml quantities into 250 Erlenmeyer flasks which were plugged with cotton. After autoclaving at 120°C for 20 minutes the pH was again determined. The desired pH after sterilization was 5.5 to 5.7. If the pH was not within this range the media was discarded.

Stock Solutions of the Compounds. The relative solubility of each compound in isopropyl alcohol, propylene glycol and acetone was roughly determined by adding an estimated 10 to 20 mg portion of each compound to 1 ml of each of the 3 solvents. In some cases heating in a boiling water bath and shaking vigorously by hand were required to facilitate solution. If a compound was soluble to this extent in all 3 solvents, isopropyl alcohol was the solvent of choice. Acetone was the second choice and propylene glycol the third choice solvent.

The concentrated stock solution of each compound was prepared by accurately weighing 160 mg in a 5 ml volumetric flask and diluting to volume with the selected solvent. In some cases, due to solubility limitations, 80 mg samples were used. These gave concentrated stock solutions of 32 and 16 mg per ml respectively. These solutions were stored in pyrex tubes and stoppered with tight fitting rubber stoppers to prevent evaporation.

Organisms. Stock cultures of Trichophyton mentagrophytes (NIH strain 598), obtained from the American Type Culture Collection, and Microsporum audouini, isolated and identified by the California Public Health Service Laboratory, were maintained on Sabouraud agar slants in a 28°C incubator. The cultures were transfered at frequent intervals, not greater than 30 days in any case. Ten to 14 day old cultures were used for inoculating purposes. At this age they showed abundant conidiospore formation.

Inhibition Studies Against Trichophyton mentagrophytes. The method used in testing the inhibitory activity of these slightly water soluble compounds was taken from the method described by Golden and Oster (9, p.283) for testing the fungistatic activity of alcohol soluble compounds.

Serial two-fold dilutions of each stock solution were prepared in sterile screw cap tubes using the proper solvent. Two-tenths ml of each dilution was then added to a sterile 100 ml screw cap container. Forty ml of melted Sabouraud agar was added to each container and the contents thoroughly mixed by lateral rotation. This resulted in a further 200 fold dilution of the compound. The medium in each container was then distributed equally between 2 sterile petri dishes. The medium was allowed to

harden and each plate was inoculated with spores from a 10 to 14 day old culture of Trichophyton mentagrophytes by means of a wire loop. The inoculum was then spread over the surface of the medium with a sterile glass rod. Duplicate control plates of Sabouraud agar and Sabouraud agar containing 0.2 ml of the solvent in 40 ml of medium were prepared and inoculated in the same manner.

The plates were then incubated at 28°C and observations made after 7 and 14 days of incubation. The point where growth ceased completely and the point where confluent growth ceased but a few colonies appeared were noted. The number of colonies were noted in the second case. The degree of confluent growth was also recorded.

The stock solutions and the serial two-fold dilutions of each compound were prepared so as to give the desired final concentrations in the medium in terms of micrograms per ml. In some cases, concentrations greater than 160 micrograms per ml were desired. The amount of stock solution added to the medium was then increased from 0.2 ml to 0.4 ml or 0.8 ml, the latter giving a final concentration of 640 micrograms per ml in the medium, the highest concentration of any compound tested. Whenever the solvent content in the medium was increased the proper control plates were prepared.

In the initial testing of each compound, a wide range of concentrations was generally employed to determine approximately the end point of activity of the compound. Once this point of activity was determined, a narrower range of concentrations was used in the final testing. In the initial testing, the range of concentrations used in the medium was dependent on the concentration of the stock solution. The highest concentration in the medium was usually 80 or 160 micrograms per ml and 3 or more two fold dilutions of this highest concentration were tested.

On the basis of the fungistatic tests, the compounds were arranged in order of decreasing activity in Table 5C.

In column 1 the compounds are arranged on a weight basis. In column 2 they are arranged on a molarity basis.

It is to be noted that no significant differences in the order of activity of the compounds is revealed when they are arranged on a molarity basis. The first 7 compounds on the latter basis are exactly the same as the 7 most active compounds on a weight basis.

Inhibition Studies Against Trichophyton Mentagrophytes in the Presence of Protein. The most active

compounds, those showing inhibitory activity against the organism at 20 micrograms per ml or less in Sabouraud medium after 14 days incubation[1], were retested in the presence of 10 per cent citrated human plasma as a source of protein. The procedure here was principally the same as that used in the inhibition studies in Sabouraud agar only. In place of 40 ml of melted Sabouraud agar, 36 ml was added to 0.2 ml of each serial two-fold dilution of the compound and cooled to about 50°C. Four ml of the plasma was then added to each container and mixed by lateral rotation. The mixture was then equally distributed between 2 sterile petri dishes and allowed to harden. The proper control plates using the plasma only and the plasma plus the solvent were prepared. The plates were inoculated, incubated and observations made as previously described under "Inhibition studies against Trichophyton mentagrophytes".

Inhibition Studies Against Microsporum Audouini. The most active compounds were tested against Microsporum audouini in the manner described for testing the activity

[1] Two exceptions, 5-iodo-3-phenylsalicylamide + N-(4-aminophenyl)-3-phenyl-salicylamide, HCl, showed significant inhibition after 7 days but did not inhibit confluent growth after 14 days incubation.

against Trichophyton mentagrophytes.

Concentration of the Compound Producing Turbidity or Precipitate in the Medium. Serial two-fold dilutions of each compound corresponding to the concentrations used in the inhibition studies against the organisms were prepared in 10 ml of melted Sabouraud agar and placed in a 50°C water bath. The optical density of each dilution was determined in a Beckman spectrophotometer at a wave length of 600 millimicrons. In this way the minimum concentration of each compound causing an increase in the turbidity of the medium was determined. It was assumed that any quantity of any compound in excess of this concentration was not in true solution, but was either in colloidal form or in the form of macroscopic aggregates.

Each dilution of the compound in the medium was poured in a petri dish and allowed to harden. The plates were observed immediately and after several days incubation by direct visual and microscopic examination for the presence of aggregates within the medium.

In addition, each dilution of the compound in the medium used in the inhibition studies against Trichophyton mentagrophytes was observed for microscopic and macroscopic aggregates immediately before the plates were

inoculated with the organism. The plates were again observed after 7 days of incubation. Control plates containing Sabouraud agar plus the solvent were used as a basis of comparison.

Fungicidal Activity of the Most Active Compounds. Attempts were made by various methods to determine the fungicidal activity of the most active compounds against Trichophyton mentagrophytes. The method described here was the only one yielding any satisfactory data.

The concentration of the compound showing the greatest degree of inhibitory activity or the minimum concentration giving complete inhibition in Sabouraud agar was prepared in sterile distilled water by the addition of the alcoholic stock solution.

The mycelial inoculum was prepared by inoculating a flask of liquid Sabouraud medium with spores from a 10 to 14 day old culture of the organism and incubating on a mechanical shaker at 28° to 32°C for 4 days. The mycelial suspension was then transferred to a graduated centrifuge tube and sedimented at high speed in the centrifuge and the supernatant removed. The mycelium was diluted with a sterile .016 M phosphate buffer at pH 7 to a concentration of 5 to 10 per cent by volume. The suspension was then added to a sterile waring blender container and blended at high speed for 3 minutes. The number of viable

mycelial fragments per ml was roughly determined by plating 1 ml volumes of a 1:100, 1:1,000 and 1:10,000 dilution in Sabouraud agar. Colony counts were made on each plate after 5 days incubation at 28°C.

One ml of the mycelial suspension was added to a 99 ml volume of the dilute water solution of each compound and the suspensions were well mixed. Sub-cultures were prepared after 8 days incubation at 28°C in one case and after 1, 3, 5 and 8 days in the second experiment by plating 0.1 ml of each dilution in 20 ml of Sabouraud agar. Colony counts were made after 5 days incubation at 28°C. Those plates showing no growth after 5 days incubation were held for 14 days before discarding. The amount of each compound carried over in plating each water dilution was not sufficient to produce inhibitory levels in any case.

Microscopic observation of wet mount preparations of the 4 day shake flask cultures showed no evidence of sporulation.

Mechanism of Inhibition by N-butyl-3-phenylsalicylamide. A preliminary study of the mechanism by which N-butyl-3-phenylsalicylamide inhibited the growth of Trichophyton mentagrophytes was undertaken.

A. Observations on Adsorption. It has been

reported[2] that this compound possessed fluorescent properties when observed under a long wave ultra violet light. This was confirmed in these studies. When the compound was present at a concentration of 20 micrograms per ml or more in an aqueous or alcoholic solution definite fluorescence was observed.

The affinity of the compound for the organism was demonstrated by the ability of the organism to adsorb small quantities of the compound. A concentration of 5 micrograms per ml of the compound was prepared in 10 ml of distilled water by addition of the alcoholic stock solution. A 10 ml water blank containing only the solvent was also prepared. Neither solution showed any evidence of fluorescence under the ultra violet light. A small clump of growth from a 14 day old culture of Trichophyton mentagrophytes was added to each solution and allowed to stand for 24 hours. Each clump of organisms was removed and observed under the ultra violet light. The organisms from the solution containing the compound showed intense fluorescence while the organisms from the solvent control blank showed no evidence of fluorescence.

A Sabouraud agar plate containing 10 micrograms per

2 E. B. McLean, Clinical Research Director, Cutter Laboratories. Personal communication.

ml of the compound and a control plate of the medium containing no compound were prepared and observed for fluorescence under the ultra violet light. Agar plugs were removed from a 14 day old culture of Trichophyton mentagrophytes with a cork borer. An agar plug was inverted on the surface of each plate, the organisms being in contact with the surface of the agar. After 1 hour the plugs were removed from the plates and observed under the ultra violet light.

Agar plugs from 5 day old cultures of Penicillium chrysogenum, Rhizopus tritici, Aspergillus niger, Mucor javanicus, Microsporum audouini and Trichophyton mentagrophytes were removed and treated in the manner described. Plugs cut from rolled cotton were treated in the same manner as agar plugs of the organisms. These were placed in a large petri dish and observed under the long wave ultra violet light.

The inhibitory activity of N-butyl-3-phenylsalicylamide was determined against each organism at a concentration of 80 and 160 micrograms per ml as described under "Inhibition studies against Trichophyton mentagrophytes".

B. Study of Effect on Respiration. The effect of N-butyl-3-phenylsalicylamide on the respiration of the mycelium of Trichophyton mentagrophytes was determined in

the Warburg respirometer. The procedure used here was taken in part from the method described by Bentley (1, p.365).

Mycelial cultures were prepared by inoculating Sabouraud liquid medium with spores from a 10 to 14 day old culture of the organism and incubating on a mechanical shaker at 28°C for 4 days. The mycelium was then washed 3 times with a .016 M phosphate buffer at pH 7 by centrifugation and resuspension of the organisms. The washings were done in sterile 50 ml graduated centrifuge tubes. After the final washing, the mycelium was diluted to a 10 per cent concentration by volume with the buffer and blended at high speed in a Waring blender for 3 minutes.

In experiments dealing with the effect of the compound on the respiration of the mycelium, 2.5 ml of the mycelial suspension was added to the Warburg flasks. Sterile 0.96 M glucose in 0.016 M phosphate buffer in a 0.5 ml quantity was added to each flask for exogenous respiration studies and 0.5 ml of the 0.016 M phosphate buffer added for endogenous respiration studies. Alcoholic solutions of the compound were prepared in concentrations of 0.06, 0.60, 3.0, 6.0, 12.0 and 30.0 mg per ml. Each solution was added in 0.05 ml quantities to the side arm of duplicate flasks. When added to the contents

of the flask, final concentrations of 1, 10, 50, 100, 200 and 500 micrograms per ml were obtained. Duplicate control flasks containing the same volume of alcohol but no compound were included in each experiment. The temperature of the water bath was maintained at 30°C and readings were made at 30 minute intervals for a period of 3 hours.

Aliquot samples of the mycelial suspension were placed in weighing vials and dried in an electric oven to a constant weight. The microliters of oxygen consumed per hour per mg of dry cells was calculated.

Chemical Data on the Compounds

Compounds	Molecular weight	Melting point °C	Solubility at 20°C	Nitrogen analysis % Calculated	Found
3-Phenyl-salicylamide	213.25	140-141	Less than 1% in water Soluble in alcohol	6.57	6.37
N-Methyl-3-phenyl-salicylamide	227.25	144-146	Less than 1% in water Soluble in alcohol	6.16	6.07
N-Ethyl-3-phenyl-salicylamide	241.25	133.5-134.5	Less than 1% in water Soluble in alcohol	5.45	5.43
N-Butyl-3-phenyl-salicylamide*	269.33		Less than 0.5% in water Soluble in alcohol		
N-Hexyl-3-phenyl-salicylamide	297.38	56-57	Less than 0.5% in water 5% in propylene glycol	4.74	4.63
N-Isopropyl-3-phenyl-salicylamide	254.30	118-119	Less than 1% in water Soluble in alcohol	5.51	5.29
N, N-Diethyl-3-phenyl-salicylamide*	269.33	90-91		5.20	5.12

Chemical Data on the Compounds (continued)

Compounds	Molecular weight	Melting point °C	Solubility at 20°C	Nitrogen analysis % Calculated	Found
N-(beta-Hydroxyethyl)-3-phenylsalicylamide	257.28	128-129	Less than 0.5% in water Soluble in ethanol	5.44	5.42
N-(beta-Hydroxypropyl)-3-phenylsalicylamide*	271.30		Soluble in alcohol		
N-(3-Methoxypropyl)-3-phenylsalicylamide	285.33	63-64	Less than 0.5% in water Soluble in alcohol and ether	4.91	5.00
2-Methoxy-3-phenyl-benzamide	227.25	177-178	Less than 0.5% in water Soluble in alcohol	6.16	6.14
2-Carboxymethoxy-3-phenylbenzamide	271.26	196-197	Less than 0.5% in water At least 5% in Na_2CO_3	5.16	4.98
2-(beta-Hydroxyethoxy)-3-phenylbenzamide	257.28	138-140	Less than 0.5% in water	5.44	5.30
3-Cyclohexyl-salicylamide	219.28	149-150	Less than 0.5% in water Soluble in chloroform	6.39	6.32

Chemical Data on the Compounds (continued)

Compounds	Molecular weight	Melting point °C	Solubility at 20°C	Nitrogen analysis % Calculated	Found
4-Phenyl-salicylamide	213.23	224-226	Less than 0.5% in water Less than 0.5% in propylene glycol	6.57	6.54
5-Phenyl-salicylamide	213.23	177-178	Less than 0.5% in water Approx. 0.5% in propylene glycol	6.57	6.56
N-Butyl-4-phenyl-salicylamide	269.33	129-130	Less than 0.5% in water Soluble in propylene glycol	5.20	5.12
N-Butyl-3-phenyl-5, x-diaminosalicylamide monohydrochloride	335.83	239-240	More than 10% in water	12.51	12.42
N-Butyl-3-phenyl-5, x-dinitrosalicylamide*	359.33	81-83	Less than 0.5% in water Soluble in acetone		
N-Butyl-3-phenyl-5, x-disulfonamide-salicylamide	427.48	227-228	More than 10% in water	9.83	9.62

Chemical Data on the Compounds (continued)

Compound	Molecular weight	Melting point °C	Solubility at 20°C	Nitrogen analysis % Calculated	Found
5-Chloro-3-phenyl-salicylamide	247.68	190-191	Less than 0.5% in water	5.65	5.59
5-Bromo-3-phenyl-salicylamide	293.10	202-203	Less than 0.5% in water	4.80	4.64
5-Iodo-3-phenyl-salicylamide	336.16	195-196	Less than 0.5% in water Soluble in methanol	4.18	3.99
5-Bromo-N-butyl-3-phenyl-salicylamide	348.24	76-77	Less than 0.5% in water More than 1% in propylene glycol	4.02	4.01
N-Phenyl-3-phenyl-salicylamide	289.32	113-115	Less than 0.5% in water Soluble in alcohol	4.84	4.92
N-(4-Carboxyphenyl)-3-phenylsalicylamide	333.33	> 235	Less than 0.5% in water Soluble in Na_2CO_3	4.20	4.03
N-(4-Nitrophenyl)-3-phenylsalicylamide	334.32	200-201	Less than 0.5% in water Insoluble in propylene glycol	8.38	8.16

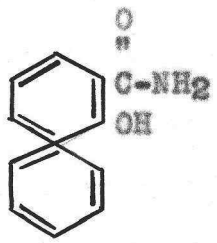
Chemical Data on the Compounds (continued)

Compound	Molecular weight	Melting point °C	Solubility at 20°C	Nitrogen analysis % Calculated	Found
N-(4-Hydroxyphenyl)-3-phenylsalicylamide	305.32	183-184	Insoluble in water Soluble in NaOH 0.5% in propylene glycol	4.59	4.65
N-(4-Aminophenyl)-3-phenylsalicylamide, HCl	340.81	239-241	Less than 0.5% in water Approx. 5% soluble in 50% propylene glycol	8.22	8.23
N-phenylethyl-3-phenyl-salicylamide*	317.36		Less than 0.5% in water Soluble in alcohol		
N-Cyclohexyl-3-phenyl-salicylamide	295.37	182-183	Less than 0.5% in water Insoluble in methanol	4.74	4.72

* Incomplete information on the compound.

EXPERIMENTAL RESULTS

Inhibition Studies Against Trichophyton Mentagrophytes. Thirty four compounds were selected for a study of their inhibitory activity against Trichophyton mentagrophytes. Thirty one of them were new salicylate derivatives obtained through the courtesy of Cutter Laboratories. Most of the compounds in this group were derivatives of 3-phenylsalicylamide, which has the following structure:



Three of the compounds, salicylic acid, salicylamide and sodium undecylenate, being known anti-fungal agents, were used for comparative purposes.

The fungistatic activity of all compounds was tested by incorporating various concentrations of each compound in Sabouraud agar and testing for ability to inhibit growth of the organism, as previously described.

Eighteen of the 34 compounds showed significant inhibitory activity against the organism after 14 days incubation. Two others showed significant activity after 7 days but did not inhibit confluent growth after 14 days incubation, these being 5-iodo-3-phenylsalicylamide and

5-phenyl-salicylamide. Minimum inhibitory concentrations ranged from 640 to 10 micrograms per ml. Twelve of the compounds were inhibitory at 20 micrograms per ml after 14 days incubation and 2 more showed significant inhibition at this level after 7 days with some evidence of activity after 14 days incubation. These results appear in Tables 1A through 5B.

A number of interesting observations on the relationship of structure to the activity of these compounds may be made. Tables 1A and 1B give the data obtained with the parent compound, 3-phenylsalicylamide, and derivatives in which straight carbon chains of varying length have been substituted on the amide nitrogen atom. As shown in these tables, the addition of a phenyl group in the 3 position of the benzene ring of salicylamide increased the activity of the compound very markedly. The addition of the phenyl group in the 4 or 5 position did not increase the activity to this extent as seen in Tables 3A and 3B. The 5-phenylsalicylamide showed some slight activity, but did not inhibit growth in the concentrations tested. The 4-phenyl derivative showed no inhibitory activity. Thus the 3 position for this substitution appears rather critical.

It is further shown in Tables 1A and 1B that with increasing length of the N substituted hydrocarbon side

chain, the activity increased, passing through a maximum in the region of 2 to 4 carbon atoms. Further increase in the length of the carbon chain to 6 atoms, resulted in a complete loss of activity as shown by N-hexyl-3-phenylsalicylamide.

Tables 2A and 2B contain derivatives of 3-phenylsalicylamide in which branched carbon chains or variously substituted chains are linked to the nitrogen atom. Here it may be seen that the addition of a hydroxyl group on the N substituted hydrocarbon side chain decreased the activity of the compound. N-ethyl-3-phenylsalicylamide was inhibitory at a much lower concentration than N-(beta-hydroxyethyl)-3-phenylsalicylamide. The peculiar behavior of the former compound at higher concentrations is discussed later.

The results in Tables 1 and 2 indicate that the compounds with straight hydrocarbon side chains on the N atom were somewhat more active than those with the branched or double side chains having the same number of carbon atoms. N-butyl-3-phenylsalicylamide showed a higher order of activity than N, N-diethyl-3-phenylsalicylamide. N-isopropyl-3-phenylsalicylamide showed a lower order of activity than N-ethyl-3-phenylsalicylamide.

Tables 3A and 3B contain derivatives of 3-phenylsalicylamide with simple carbon substitutions in the 2 position and a group of miscellaneous salicylamide derivatives with cyclic substituents on the benzene ring. Sodium undecylenate was included as a reference compound. In this group only 3-cyclohexylsalicylamide and the reference compound showed pronounced activity. Apparently the addition of a cyclohexyl group in place of a phenyl group in the 3 position of salicylamide increased the activity of the compound by about 2 fold.

Simple carbon chain substitutions on the 2-hydroxy group of 3-phenylsalicylamide destroyed the inhibitory activity as shown in tests with the first 3 compounds in Tables 3A and 3B.

In Tables 4A and 4B are the results of tests with derivatives of 3-phenylsalicylamide containing various substituents on the salicylamide benzene ring or on both rings. It may be seen that the addition of nitro, amino or sulfonamide groups on the benzene ring completely destroyed the activity of N-butyl-3-phenylsalicylamide. It is also shown in these tables that the addition of chlorine and bromine in the 5 position of the salicylamide benzene ring of 3-phenylsalicylamide increased the activity of the compound. The 5-chloro and 5-bromo derivatives of 3-phenylsalicylamide were more active than the parent

compound. 5-Iodo-3-phenylsalicylamide and 5-bromo-N-butyl-3-phenylsalicylamide were less active than the parent compounds. A possible explanation for this phenomenon will be presented in the discussion.

Tables 5A and 5B show the results of tests with derivatives of 3-phenylsalicylamide having cyclic carbon substituents on the amide nitrogen. The N-phenyl derivatives of 3-phenylsalicylamide varied somewhat in their inhibitory activity. N-phenyl-3-phenylsalicylamide and N-(4-hydroxyphenyl)-3-phenylsalicylamide were among the most active compounds tested. N-(4-aminophenyl)-3-phenylsalicylamide, HCl showed a significant degree of inhibition at a relatively low concentration while the N-(4-carboxyphenyl) and the N-(4-nitrophenyl) derivatives were inactive at the concentrations tested. The N-phenylethyl and N-cyclohexyl derivatives were also inactive.

Only a few of the more active compounds showed complete inhibition of growth at the concentrations tested. Many of the compounds showed a high order of activity at low concentrations and a decrease in activity as the concentration increased. Others gave partial inhibition at low levels of concentration with no apparent increase in activity with increasing concentrations. The relationship of activity to concentration is presented under "Concentration of the compound producing turbidity or

Table 1A

Inhibition Studies Against *Trichophyton Mentagrophytes*

Seven Day Observation

Compound	Concentration in micrograms per ml in the medium								
	640	320	160	80	40	20	10	5	2.5
Salicylic acid	- -	- -	4+ 4+	4+ 4+	4+ 4+				
	- -	- -	3+ 3+	4+ 4+	4+ 4+				
Salicylamide	- -	2+ 2+	4+ 4+	4+ 4+	4+ 4+				
	- -	1+ 1+	3+ 3+	3+ 3+	4+ 4+				
3-Phenylsalicylamide					- -	- -	4+ 4+	4+ 4+	
					- -	- -	4+ 4+	4+ 4+	
N-Methyl-3-phenyl-salicylamide			- 3c	- 1c	- 1c	- 1+	2+ 2+		
		5c 3c	- -	- 1c	2c 1c	- -	2+ 2+		
				- 1c	1c -	1c -	8c 3c	4+ 4+	
					- -	3c -	1+ 1+	3+ 3+	4+ 4+
N-Ethyl-3-phenyl-salicylamide			3+ 3+	2+ 2+	3+ 3+	2+ 2+			
		2+ 1+	3+ 3+	3+ 3+	3+ 3+	3+ 3+	4c 1c	1+ 1c	4+ 4+
			3+ 3+	3+ 10c	2+ 3+	2+ 1+	4c 6c	4+ 4+	
					3+ 3+	2+ 3+	1+ 3c	1+ 3c	3+ 3+
N-Butyl-3-phenyl-salicylamide		4c 1c	1c -	1c 2c	4c 1c	1c 1c	5c 5c	9c 20c	
					1c -	- 3c	4c 6c	10c 1+	2+ 2+
N-Hexyl-3-phenyl-salicylamide		4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+			
		3+ 3+	3+ 3+	4+ 4+					

Key:
- 1+ Growth covering 1/4 of plate
- 2+ Growth covering 1/2 of plate
- 3+ Growth covering 3/4 of plate
- 4+ Growth covering entire plate
- c Preceded by number represents number of colonies on plate
- \- No growth on plate

Control plates: Isopropyl alcohol, 1:200, 1:100, 1:50 4+

Propylene glycol, 1:200, 1:100, 1:50 4+

Sabouraud agar 4+

Data in table indicate results on duplicate plates.

Table 1B

Inhibition Studies Against Trichophyton Mentagrophytes

Fourteen Day Observation

Compound	Concentration in micrograms per ml in the medium								
	640	320	160	80	40	20	10	5	2.5
Salicylic acid	- -	7c 8c	4+ 4+	4+ 4+	4+ 4+				
	- -	1+ 1c	4+ 4+	4+ 4+	4+ 4+				
Salicylamide	- -	3+ 3+	4+ 4+	4+ 4+	4+ 4+				
	- -	3+ 4+	4+ 4+	4+ 4+	4+ 4+				
3-Phenylsalicylamide					- -	- -	4+ 4+	4+ 4+	
					- -	- -	4+ 4+	4+ 4+	
N-Methyl-3-phenyl-salicylamide			- 3c	- 3c	- 3c	- 1+	3+ 3+		
		5c 3c	- 1c	3c -	4c 3c	8c 1c	3+ 3+		
				- 2c	3c 3c	1c -	4+ 3+	4+ 4+	
					- -	1+ 1+	2+ 2+	4+ 4+	4+ 4+
N-Ethyl-3-phenyl-salicylamide			4+ 4+	3+ 3+	3+ 3+	3+ 3+			
		2+ 2+	3+ 3+	3+ 3+	4+ 4+	3+ 3+	1+ 8c	2+ 2+	4+ 4+
			4+ 4+	3+ 3+	4+ 4+	3+ 3+	6c 1+	4+ 4+	
					3+ 4+	3+ 3+	1+ 6c	4+ 4+	4+ 4+
N-Butyl-3-phenyl-salicylamide		4c 2c	1c -	2c 2c	6c 1+	1c 3c	5c 3c	9c 1+	
					1c -	- 4c	6c 6c	1+ 1+	4+ 4+
N-Hexyl-3-phenyl-salicylamide		4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+			
		4+ 4+	4+ 4+	4+ 4+					

Key: 1+ Growth covering 1/4 of plate
2+ Growth covering 1/2 of plate
3+ Growth covering 3/4 of plate
4+ Growth covering entire plate
c Preceded by number represents number of colonies on plate
- No growth on plate

Control plates: Isopropyl alcohol, 1:200, 1:100, 1:50 4+
Propylene glycol, 1:200, 1:100, 1:50 4+
Sabouraud agar 4+

Data in table indicate results on duplicate plates.

Table 2A

Inhibition Studies Against Trichophyton Mentagrophytes

Seven Day Observation

Compound	Concentration in micrograms per ml in the medium							
	320	160	80	40	20	10	5	2.5
N-Isopropyl-3-phenyl-salicylamide		5c 5c	1+ 1+	- -	1c 1c	2+ 2+		
		1+ 1+	1c -	1c -	- -	2+ 2+		
	1+ 2+	1c 1+	1+ 1+	2+ 2+	- -	1+ 1+	3+ 3+	4+ 4+
N,N-Diethyl-3-phenyl-salicylamide		- -	- -	3c 1c	14c 8c			
		- -	- 1c	6c 8c	5c 1+			
N-(beta-Hydroxyethyl)-3-phenylsalicylamide		- -	- -	3+ 3+	4+ 4+			
		- -	- -	4+ 4+	4+ 4+			
N-(beta-Hydroxypropyl)-3-phenylsalicylamide		- -	- -	- -	3+ 3+			
		- -	- -	- -	4+ 4+			
N-(3-Methoxypropyl)-3-phenylsalicylamide		- -	- -	- -	- -			
				- -	- -	7c 8c	1+ 1+	
				- -	1c 3c	3+ 3+	4+ 4+	

Key:
- 1+ Growth covering 1/4 of plate
- 2+ Growth covering 1/2 of plate
- 3+ Growth covering 3/4 of plate
- 4+ Growth covering entire plate
- c Preceded by number represents number of colonies on plate
- \- No growth on plate

Control plates: Isopropyl alcohol, 1:200, 1:100, 1:50 4+

Propylene glycol, 1:200, 1:100, 1:50 4+

Sabouraud agar 4+

Data in table indicate results on duplicate plates.

CA
CA

Table 2B

Inhibition Studies Against Trichophyton Mentagrophytes

Fourteen Day Observation

Compound	Concentration in micrograms per ml in the medium							
	320	160	80	40	20	10	5	2.5
N-Isopropyl-3-phenyl-salicylamide		3+ 3+	2+ 3+	1c -	1c 1c	4+ 4+		
		3+ 3+	2+ 2+	1c 1+	- -	3+ 3+		
	2+ 2+	2+ 2+	3+ 3+	4c 1+	- -	2+ 2+	4+ 4+	4+ 4+
N, N-Diethyl-3-phenyl-salicylamide		- -	- -	3c 1c	3+ 3+			
		- -	- 1c	6c 12c	4+ 4+			
N-(beta-Hydroxyethyl)-3-phenylsalicylamide		- -	- -	4+ 4+	4+ 4+			
		- -	- -	4+ 4+	4+ 4+			
N-(beta-Hydroxypropyl)-3-phenylsalicylamide		- -	- -	- -	4+ 4+			
		- -	- -	- -	4+ 4+			
N-(3-Methoxypropyl)-3-phenylsalicylamide		- -	- -	- -	1c -			
				- -	12c 1+	3+ 3+		
				- -	1c 4c	3+ 3+	4+ 4+	

Key:
- 1+ Growth covering 1/4 of plate
- 2+ Growth covering 1/2 of plate
- 3+ Growth covering 3/4 of plate
- 4+ Growth covering entire plate
- c Preceded by number represents number of colonies on plate
- - No growth on plate

Control Plates:
- Isopropyl alcohol, 1:200, 1:100, 1:50 4+
- Propylene glycol, 1:200, 1:100, 1:50 4+
- Sabouraud agar 4+

Data in table indicate results on duplicate plates.

Table 3A

Inhibition Studies Against *Trichophyton Mentagrophytes*

Seven Day Observation

Compound	Concentration in micrograms per ml in the medium							
	320	160	80	40	20	10	5	2.5
2-Methoxy-3-phenyl-benzamide	4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+	
2-Carboxymethoxy-3-phenylbenzamide	4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+		
2-(beta-Hydroxyethoxy)-3-phenylbenzamide	4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+		
3-Cyclohexyl-salicylamide		1+ 1+	3c 2c	- 3c	6c 1c - 2c	2c 1c 1c - 5c 2c	1c 5c 2+ 2+	4+ 4+
4-Phenyl-salicylamide	4+ 4+	4+ 4+	4+ 4+ 4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+	
5-Phenyl-salicylamide		8c -	1c -	4c -	3c 1c 1c 1c	4+ 4+ 3+ 3+	4+ 4+	4+ 4+
N-Butyl-4-phenyl-salicylamide	4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+	4+ 4+	4+ 4+		
Sodium undecylenate			- -	- -	- - - -	- 2c 2+ 1+	4c 3c 4+ 4+	

Key:
- 1+ Growth covering 1/4 of plate
- 2+ Growth covering 1/2 of plate
- 3+ Growth covering 3/4 of plate
- 4+ Growth covering entire plate
- c Preceded by number represents number of colonies on plate
- \- No growth on plate

Data in table indicate results on duplicate plates.

Control plates: Isopropyl alcohol, 1:200, 1:100, 1:50 4+

Propylene glycol, 1:200, 1:100, 1:50 4+

Sabouraud agar 4+

Table 3B

Inhibition Studies Against Trichophyton Mentagrophytes

Fourteen Day Observation

Compound	Concentration in micrograms per ml in the medium							
	320	160	80	40	20	10	5	2.5
2-Methoxy-3-phenyl-benzamide	4+ 4+	4+ 4+	4+ 4+ 4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+	
2-Carboxymethoxy-3-phenylbenzamide	4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+	4+ 4+	4+ 4+		
2-(beta-Hydroxyethoxy)-3-Phenylbenzamide	4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+	4+ 4+	4+ 4+		
3-Cyclohexyl-salicylamide		2+ 2+	6c 2c	3c -	5c 4c - 2c	3c 3c - - 3c 1+	4+ 4+ 3+ 3+	4+ 4+
4-Phenyl-salicylamide	4+ 4+	4+ 4+	4+ 4+ 4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+	
5-Phenyl-salicylamide		4+ 2+	4+ 3+	4+ 3+	3+ 3+ 2+ 2+	4+ 4+ 4+ 4+	4+ 4+	4+ 4+
N-Butyl-4-phenyl-salicylamide	4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+	4+ 4+	4+ 4+		
Sodium undecylenate			- -	- -	- - - -	3+ 3+ 4+ 4+	4+ 4+	

Key: 1+ Growth covering 1/4 of plate
2+ Growth covering 1/2 of plate
3+ Growth covering 3/4 of plate
4+ Growth covering entire plate
c Preceded by number represents number of colonies on plate
- No growth on plate

Control plates: Isopropyl alcohol, 1:200, 1:100, 1:50, 4+
Propylene glycol, 1:200, 1:100, 1:50 4+
Sabouraud agar 4+

Data in table indicate results on duplicate plates.

Table 4A

Inhibition Studies Against Trichophyton Mentagrophytes

Seven Day Observation

Compound	Concentration in micrograms per ml in the medium							
	320	160	80	40	20	10	5	2.5
N-Butyl-3-phenyl-5, x-diaminosalicylamide monohydrochloride	4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+			
N-Butyl-3-phenyl-5, x-dinotrosalicylamide	4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+			
N-Butyl-3-phenyl-5, x-disulfonamide-salicylamide	4+ 4+ 3+ 3+	4+ 4+ 3+ 3+	4+ 4+	4+ 4+	4+ 4+			
5-Bromo-3-phenyl-salicylamide		1+ 2c	5c 8c	2+ 3+	7c 8c - 1c	- - - 1c	2+ 2+	4+ 4+
5-Chloro-3-phenyl-salicylamide		6c 1c 1c 1c	3c 1c 1c 1c	2c 1c 1c 3c	5c - 2c 1c - -	- - - 1c - -	1+ 2+	4+ 4+
5-Iodo-3-phenyl-salicylamide			9c 8c	5c 1+	12c 9c 2+ 3+	7c 1c 1c -	2c 1c 3c 1c	3+ 3+
5-Bromo-N-butyl-3-phenyl salicylamide					3+ 3+	3+ 3+ 3+ 3+	3+ 3+ 3+ 3+	3+ 3+ 3+ 3+

Key: 1+ Growth covering 1/4 of plate
2+ Growth covering 1/2 of plate
3+ Growth covering 3/4 of plate
4+ Growth covering entire plate
c Preceded by number represents number of colonies on plate
- No growth on plate

Control plates: Isopropyl alcohol, 1:200, 1:100, 1:50 4+
Propylene glycol, 1:200, 1:100, 1:50 4+
Sabouraud agar 4+

Data in table indicate results on duplicate plates.

Table 4B

Inhibition Studies Against Trichophyton Mentagrophytes

Fourteen Day Observation

Compound	Concentration in micrograms per ml in the medium							
	320	160	80	40	20	10	5	2.5
N-Butyl-3-phenyl-5, x-diaminosalicylamide monohydrochloride	4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+			
N-Butyl-3-phenyl-5, x-dinitrosalicylamide	4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+			
N-Butyl-3-phenyl-5, x-disulfonamide-salicylamide	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+	4+ 4+	4+ 4+			
5-Bromo-3-phenyl-salicylamide		2+ 1+	1+ 1+	2+ 3c	1c - 1c 7c	1c - - -	4+ 4+	4+ 4+
5-Chloro-3-phenyl-salicylamide		7c 1c 1c 2c	1+ 3c 1c 1c	2c 1c 1+ 4c	1+ - 1+ 1+ - -	- - - 1c - -	4+ 4+	4+ 4+
5-Iodo-3-phenyl-salicylamide			2+ 1+	2+ 3+	2+ 3+ 3+ 3+	2+ 2+ 2+ 1+	2+ 2+ 2+ 2+	4+ 4+
5-Bromo-N-butyl-3-phenyl-salicylamide					4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+	4+ 4+ 4+ 4+

Key: 1+ Growth covering 1/4 of plate
2+ Growth covering 1/2 of plate
3+ Growth covering 3/4 of plate
4+ Growth covering entire plate
c Preceded by number represents number of colonies on plate
- No growth on plate

Control plates: Isopropyl alcohol, 1:200, 1:100, 1:50 4+
Propylene glycol, 1:200, 1:100, 1:50 4+
Sabouraud agar 4+

Data in table indicate results on duplicate plates.

Table 5A

Inhibition Studies Against *Trichophyton Mentagrophytes*

Seven Day Observation

Compound	Concentration in micrograms per ml in the medium							
	320	160	80	40	20	10	5	2.5
N-Phenyl-3-phenyl-salicylamide		7c 1c	- 5c	1c 1c	2c 6c	2c 1c		
						1c 1c	2c 5c	
					1c 2c	1c 3c	1c 1c	1+ 2+
N-(4-Carboxyphenyl)-3-phenylsalicylamide	1+ 4c	1+ 1+	2+ 2+	2+ 3+	2+ 2+			
	2+ 2+	2+ 2+						
N-(4-Nitrophenyl)-3-phenylsalicylamide	4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+			
	3+ 3+	3+ 3+						
N-(4-Hydroxyphenyl)-3-phenylsalicylamide		- -	- -	- -	- -	- -		
			- -	1c -	2c -	- -	8c 12c	
					- -	- 2c	- -	4+ 4+
N-(4-Aminophenyl)-3-phenylsalicylamide, HCl	1c -	- 1c	4c 2c	4c 6c	3c 1c			
	1c 1c	- -	1c 2c	1c 1c				
N-(Phenylethyl)-3-phenylsalicylamide		4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+		
	4+ 4+	4+ 4+	4+ 4+					
N-Cyclohexyl-3-phenyl-salicylamide			4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+	
	4+ 4+	4+ 4+	4+ 4+					

Key: 1+ Growth covering 1/4 of plate
2+ Growth covering 1/2 of plate
3+ Growth covering 3/4 of plate
4+ Growth covering entire plate
c Preceded by number represents number of colonies on plate
- No growth on plate

Control plates: Isopropyl alcohol, 1:200, 1:100, 1:50 4+
Propylene glycol, 1:200, 1:100, 1:50 4+
Sabouraud agar 4+

Data in table indicate results on duplicate plates.

Table 5B

Inhibition Studies Against *Trichophyton Mentagrophytes*

Fourteen Day Observation

Compound	Concentration in micrograms per ml in the medium							
	320	160	80	40	20	10	5	2.5
N-Phenyl-3-phenyl-salicylamide		12c 1c	- 5c	2c 1c	2c 6c	2c 1c		
						3c 1c	2+ 1+	
					1c 2c	1c 4c	1+ 1+	3+ 3+
N-(4-Carboxyphenyl)-3-phenylsalicylamide	4+ 3+	4+ 4+	4+ 4+	4+ 4+	4+ 4+			
	3+ 2+	3+ 2+						
N-(4-Nitrophenyl)-3-phenylsalicylamide	4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+			
	4+ 4+	4+ 4+						
N-(4-Hydroxyphenyl)-3-phenylsalicylamide		- -	- -	1c 2c	4c 4c	5c 1c		
			- -	3c 1c	2c -	- 1c	2+ 8c	
					- 1c	- 2c	1c 1+	4+ 4+
N-(4-Aminophenyl)-3-phenylsalicylamide, HCl	3c 1c	- 1c	4c 3c	1+ 1+	3+ 2+			
	1c 1c	- -	2c 2c	1+ 1+	1+ 1+	4+ 3+	4+ 4+	
N-(Phenylethyl)-3-phenylsalicylamide		4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+		
	4+ 4+	4+ 4+	4+ 4+					
N-cyclohexyl-3-phenyl-salicylamide			4+ 4+	4+ 4+	4+ 4+	4+ 4+	4+ 4+	
	4+ 4+	4+ 4+	4+ 4+					

Key: 1+ Growth covering 1/4 of plate
2+ Growth covering 1/2 of plate
3+ Growth covering 3/4 of plate
4+ Growth covering entire plate
c Preceded by number represents number of colonies on plate
\- No growth on plate

Control plates: Isopropyl alcohol, 1:200, 1:100, 1:50 4+
Propylene glycol, 1:200, 1:100, 1:50 4+
Sabouraud agar 4+

Data in table indicate results on duplicate plates.

Table 5C
Decreasing Order of Inhibitory Activity
Against Trichophyton Mentagrophytes

Minimum inhibition conc. in μg per ml	Weight basis	Molarity basis
10	5-Chloro-3-phenylsalicylamide	N-(4-Hydroxyphenyl)-salicylamide
10	5-Bromo-3-phenylsalicylamide	5-Bromo-3-phenylsalicylamide
10	N-(4-Hydroxyphenyl)-3-phenylsalicylamide	N-Phenyl-3-phenylsalicylamide
10	N-Butyl-3-phenylsalicylamide	N-Butyl-3-phenylsalicylamide
10	N-Phenyl-3-phenylsalicylamide	N-Ethyl-3-phenylsalicylamide
10	5-Cyclohexyl-salicylamide	5-Chloro-3-phenylsalicylamide
10	N-Ethyl-3-phenylsalicylamide	3-Cyclohexyl-salicylamide
20	3-Phenylsalicylamide	N-(3-Methoxypropyl)-3-phenylsalicylamide
20	Sodium undecylenate	N-Isopropyl-3-phenylsalicylamide
20	N-Isopropyl-3-phenylsalicylamide	Sodium undecylenate
20	N-(3-Methoxypropyl)-3-phenylsalicylamide	3-Phenylsalicylamide

(continued on the following page)

Table 5C, continued

Minimum inhibition conc. in μg per ml	Weight basis	Molarity basis
20-40	N-Methyl-3-phenylsalicylamide	N-Methyl-3-phenylsalicylamide
40	N-(beta-Hydroxypropyl)-3-phenylsalicylamide	N, N-Diethyl-3-phenylsalicylamide
40	N, N-Diethyl-3-phenylsalicylamide	N-(beta-Hydroxypropyl)-3-phenylsalicylamide
80	N-(4-Aminophenyl)-3-phenylsalicylamide, HCl	N-(4-Aminophenyl)-3-phenylsalicylamide, HCl
80	N-(beta-Hydroxyethyl)-3-phenylsalicylamide	N-(beta-Hydroxyethyl)-3-phenylsalicylamide
320	Salicylic acid	Salicylic acid
640	Salicylamide	Salicylamide

Table 6A
Inhibition Studies Against *Trichophyton Mentagrophytes*
in the Presence of 10% Human Plasma
Seven Day Observation

Compound	Concentration in micrograms per ml in the medium				
	320	160	80	40	20
3-Phenylsalicylamide		- -	- -	3c 1+	4+ 4+
			- -	2+ 1+	
N-Methyl-3-phenylsalicylamide		- -	1+ 1+	3+ 3+	4+ 4+
		- -	2+ 2+		
N-Ethyl-3-phenylsalicylamide		3+ 3+	3+ 3+	3+ 3+	4+ 4+
	4+ 4+	4+ 4+			
N-Butyl-3-phenylsalicylamide		- -	3c 1c	1+ 1+	1+ 2+
		- 1c	1+ 1+	2+ 2+	
N-Isopropyl-3-phenylsalicylamide		- -	1+ 1+	3+ 3+	3+ 3+
		- -	3+ 3+		
N-(3-Methoxypropyl)-3-phenylsalicylamide		- -	- -	1+ 1+	3+ 3+
			- -	3+ 3+	
5-Chloro-3-phenylsalicylamide		- 1c	- -	3+ 3+	4+ 4+
	1+ 1+	3c -	- -	3+ 3+	
5-Bromo-3-phenylsalicylamide		1+ 1+	1+ 1+	3+ 3+	4+ 4+
	1+ 1+	2+ 2+			
5-Iodo-3-phenylsalicylamide		3+ 3+	3+ 3+	4+ 4+	4+ 4+
	3+ 3+	3+ 3+			
3-Cyclohexyl-salicylamide		1c 2c	3c 2c	7c 2+	3+ 3+
	1c 1c	- 1c	3c 1c	2+ 2+	

(continued on the following page)

Table 6A, continued

Compound	Concentration in micrograms per ml in the medium									
	320		160		80		40		20	
N-Phenyl-3-phenylsalicylamide			2+	2+	3+	3+	4+	4+	4+	4+
	2+	2+	1+	1+						
N-(4-Hydroxyphenyl)-3-phenylsalicylamide			-	-	-	-	3+	3+	4+	4+
			-	-	2c	-	4+	4+		
N-(4-Aminophenyl)-3-phenylsalicylamide, HCl	-	-	-	1c	3+	2+	4+	4+	4+	4+
	-	-	1c	1c	1+	1+				
Sodium undecylenate			-	-	1+	1c	3+	2+	4+	4+
			-	-	2+	2+				

Key:
- 1+ Growth covering 1/4 of plate
- 2+ Growth covering 1/2 of plate
- 3+ Growth covering 3/4 of plate
- 4+ Growth covering entire plate
- c Preceded by a number represents number of colonies on plate
- - No growth on plate

Controls:

Isopropyl alcohol, 1:200, 1:100, 1:50	4+
Propylene glycol, 1:200, 1:100, 1:50	4+
Sabouraud agar	4+

Table 6B
Inhibition Studies Against Trichophyton Mentagrophytes
in the Presence of 10% Human Plasma

Fourteen Day Observation

Compound	Concentration in micrograms per ml in the medium				
	320	160	80	40	20
3-phenylsalicylamide		- -	- -	4+ 4+	4+ 4+
			- -	4+ 4+	
N-Methyl-3-phenylsalicylamide		- -	2+ 2+	4+ 4+	4+ 4+
		- 2c	4+ 4+		
N-Ethyl-3-phenylsalicylamide		4+ 4+	4+ 4+	4+ 4+	4+ 4+
	4+ 4+	4+ 4+			
N-Butyl-3-phenylsalicylamide		- -	1+ 1+	2+ 2+	3+ 2+
		- 1c	1+ 2+	3+ 3+	
N-Isopropyl-3-phenylsalicylamide		- -	2+ 2+	4+ 4+	4+ 4+
		2c -	3+ 3+		
N-(3-Methoxypropyl)-3-phenylsalicylamide		- -	- -	3+ 3+	4+ 4+
			- -	3+ 3+	
5-Chloro-3-phenylsalicylamide		1+ 1+	- -	4+ 4+	4+ 4+
	2+ 2+	1+ 8c	- 1c	4+ 4+	
5-Bromo-3-phenylsalicylamide		3+ 3+	3+ 3+	4+ 4+	4+ 4+
	3+ 3+	3+ 3+			
5-Iodo-3-phenylsalicylamide		3+ 3+	3+ 3+	4+ 4+	4+ 4+
	4+ 4+	4+ 4+			
3-Cyclohexylsalicylamide		4c 5c	3c 2c	3+ 3+	3+ 3+
	3c 1c	- 4c	1c 6c	3+ 4+	

(continued on the following page)

Table 6B, continued

Compound	Concentration in micrograms per ml in the medium				
	320	160	80	40	20
N-Phenyl-3-phenylsalicylamide		2+ 2+	3+ 3+	4+ 4+	4+ 4+
	3+ 3+	2+ 2+			
N-(4-Hydroxyphenyl)-3-phenylsalicylamide		- -	1c -	3+ 3+	4+ 4+
		- -	3c 2c	4+ 4+	
N-(4-Aminophenyl)-3-phenylsalicylamide, HCl	- -	2c 2c	3+ 3+	4+ 4+	4+ 4+
	- -	1c 2c	3+ 3+		
Sodium undecylenate		- -	3+ 2+	3+ 3+	4+ 4+
		- -	4+ 4+		

Key:

1+ Growth covering 1/4 of plate
2+ Growth covering 1/2 of plate
3+ Growth covering 3/4 of plate
4+ Growth covering entire plate
c Preceded by a number represents number of colonies on plate
- No growth on plate

Controls:

Isopropyl alcohol, 1:200, 1:100, 1:50	4+
Propylene glycol, 1:200, 1:100, 1:50	4+
Sabouraud agar	4+

precipitate in the medium".

Inhibition Studies Against Trichophyton Mentagrophytes in the Presence of Protein. On the basis of the inhibition studies against Trichophyton mentagrophytes in Sabouraud agar, 14 of the compounds were selected for further study of their inhibitory activity against the organism in the presence of 10 per cent citrated human plasma as a source of protein.

The presence of the protein in the medium reduced the activity of all compounds tested. Three of the compounds, N-ethyl-3-phenylsalicylamide, N-phenyl-3-phenylsalicylamide and 5-iodo-3-phenylsalicylamide, showed virtually a complete loss of inhibitory activity. The activity of the other compounds was decreased by 4 to 16 fold. The order of activity of many of the compounds of this group was changed by the addition of the protein as shown in Tables 6A and 6B. Those which were the most active under these conditions were 3-phenylsalicylamide, N-(3-methoxypropyl)-3-phenylsalicylamide, 5-chloro-3-phenylsalicylamide, N-(4-hydroxyphenyl)-3-phenylsalicylamide and the reference compound, sodium undecylenate.

Inhibition Studies Against Microsporum Audouini. The 14 compounds selected for study of the inhibitory

activity against Trichophyton mentagrophytes in the presence of protein were also tested against Microsporum audouini in Sabouraud agar.

The results in Tables 7A and 7B show Microsporum audouini to be somewhat more sensitive to the compounds than Trichophyton mentagrophytes at the same concentrations. Four of the compound, N-(4-hydroxyphenyl)-3-phenylsalicylamide, N-(3-methoxypropyl)-3-phenylsalicylamide, 5-chloro-3-phenylsalicylamide and 3-cyclohexylsalicylamide inhibited Microsporum audouini at concentrations about half as great as those required for Trichophyton mentagrophytes. The other compounds were inhibitory at the same concentrations, although the inhibition was more complete against Microsporum audouini.

Concentration of the Compound Producing Turbidity or Precipitate in the Medium. In view of the fact that the water solubility of a compound has a marked influence on biological activity (13, p.5), it was deemed necessary to determine the approximate solubility of the compounds in the medium. By turbidity measurements and direct visual and microscopic observation of the dilutions of each compound in the medium as previously described, the approximate concentration where turbidity or visible aggregates appeared was observed.

Table 7A

Inhibition Studies Against Microsporum Audouini

Seven Day Observation

Compound	Concentration in micrograms per ml in the medium					
	80	40	20	10	5	2.5
3-Phenylsalicylamide		- -	- -	1+ 1+	2+ 2+	
			- -	3+ 3+		
N-Methyl-3-phenylsalicylamide		- -	- -	1+ 1+	1+ 2+	
			- -	5c 2c		
N-Ethyl-3-phenylsalicylamide		1+ 1+	2+ 1+	- -	2+ 2+	
		1+ 1+	1+ 1+	- -	1+ 1+	
N-Butyl-3-phenylsalicylamide		- -	1c -	- -	1c 2c	
			- -	1c -	3c 5c	
N-Isopropyl-3-phenylsalicylamide		- -	- -	1+ 1+	2+ 2+	
			- -	1+ 1c		
N-(3-Methoxypropyl)-3-phenylsalicylamide		- -	- -	1+ 1+	1+ 3c	
			- -	2+ 1+		
5-Chloro-3-phenylsalicylamide		- -	- -	- -	- 1c	
				- -	- -	2+ 2+
5-Bromo-3-phenylsalicylamide		1+ 1+	- -	- -	1+ 1+	
	1+ 1+	2+ 1+	- -	- -	1c 3c	
5-Iodo-3-phenylsalicylamide		1c -	- -	- -	1+ 1+	
	3c 1c	2c 3c	1c -	- 1c		
3-Cyclohexyl-salicylamide		- -	- -	- -	2c -	
				- -	1c -	1+ 1+

(continued on the following page)

Table 7A, continued

Compound	Concentration in micrograms per ml in the medium					
	80	40	20	10	5	2.5
N-Phenyl-3-phenylsalicylamide		- -	1c -	2c 3c	3c 2c	
	1c 1c	- 2c	2c -	1c -	1c 1c	
N-(4-Hydroxyphenyl)-3-phenylsalicylamide		- -	- -	- -	- -	
				- -	1c -	1+ 1+
N-(4-Aminophenyl)-3-phenylsalicylamide, HCl	- -	- 1c	1+ 1+	2+ 2+	2+ 2+	
	- -	1c 3c				
Sodium undecylenate		- -	- -	- -	2+ 2+	
			- -	5c 2c		

Key: 1+ Growth covering 1/4 of plate
2+ Growth covering 1/2 of plate
3+ Growth covering 3/4 of plate
4+ Growth covering entire plate
c Preceded by a number represents number of colonies on plate
- No growth on plate

Controls: Isopropyl alcohol, 1:200 3+
Propylene glycol, 1:200 3+
Sabouraud agar 3+

Data in table indicate results on duplicate plates.

Table 7B
Inhibition Studies Against *Microsporum Audouini*
Fourteen Day Observation

Compound	Concentration in micrograms per ml in the medium					
	80	40	20	10	5	2.5
3-Phenylsalicylamide		- -	- -	4+ 4+	4+ 4+	
			- -	4+ 4+		
N-Methyl-3-phenylsalicylamide		- -	- -	3+ 2+	4+ 4+	
			- -	2+ 3+		
N-Ethyl-3-phenylsalicylamide		1+ 1+	1+ 1+	- -	4+ 4+	
		3+ 3+	3+ 3+	- -	3+ 3+	
N-Butyl-3-phenylsalicylamide		1c -	3c -	1c -	2+ 3+	
			1c -	1c -	2+ 2+	
N-Isopropyl-3-phenylsalicylamide		- -	- -	2+ 1+	3+ 3+	
			- -	2+ 2+		
N-(3-Methoxypropyl)-3-phenylsalicylamide		- -	- -	2+ 2+	4+ 4+	
			- -	3+ 2+		
5-Chloro-3-phenylsalicylamide		- -	- -	- -	- 1c	
				- -	8c 3c	4+ 4+
5-Bromo-3-phenylsalicylamide		3+ 3+	2+ 1+	1c -	4+ 4+	
	1+ 2+	2+ 2+	5c 1+	3c -	4+ 4+	
5-Iodo-3-phenylsalicylamide		2+ 2+	2+ 2+	1+ 1+	4+ 4+	
	2+ 2+	1+ 2+	2+ 2+	1+ 2+		
3-Cyclohexyl-salicylamide		- -	- -	- -	2c -	
				- -	1c -	4+ 4+

(continued on the following page)

Table 7B, continued

Compound	Concentration in micrograms per ml in the medium					
	80	40	20	10	5	2.5
N-Phenyl-3-phenylsalicylamide		2c 1c	3c 2c	6c 8c	3+ 3+	
	1c 1c	- 3c	2c 1c	3c -	4+ 3+	
N-(4-Hydroxyphenyl)-		- -	- -	- -	- 1c	
3-phenylsalicylamide				- -	5c 1	4+ 4+
N-(4-Aminophenyl)-	- -	1+ 1+	3+ 4+	4+ 4+	4+ 4+	
3-phenylsalicylamide, HCl	- -	1c 1+				
Sodium undecylenate		- -	- -	4+ 4+	4+ 4+	
			- -	4+ 4+	4+ 4+	

Key: 1+ Growth covering 1/4 of plate
2+ Growth covering 1/2 of plate
3+ Growth covering 3/4 of plate
4+ Growth covering entire plate
c Preceded by a number represents number of colonies on plate
- No growth on plate

Controls: Isopropyl alcohol, 1:200 4+
Propylene glycol, 1:200 4+
Sabouraud agar 4+

Data in table indicate results in duplicate plates.

The results appear in Table 8, where the compounds were grouped according to their apparent solubilities in the medium, Group 1 being the least soluble, Group 6 the most soluble. A number of observations on the relationship of structure to apparent solubility may be noted. The data show that the addition of dinitro, disulfonamide and diamino groups to the molecule greatly increased the solubility of the parent compound. This is seen by comparing compounds in Groups 2, 5 and 6. It is further shown that the addition of a hydroxyl group on the N substituted side chain increased the apparent solubility as is evident in comparing the sixth compound in Group 2 with the fifth compound in Group 4.

The addition of halogens to the salicylamide benzene ring decreased the apparent solubility of the parent compound. This may be seen by comparing the first compound in Group 1 and the third and fourth compounds in Group 2 with the first compound in Group 3.

Substitution of a non-polar group on the amide nitrogen atom decreased the apparent solubility, as may be seen by comparing compound one in Group 2 with the first compound in Group 3.

The substitution of a phenyl group in the 3 position of salicylamide markedly reduced the solubility of the parent compound, as is evident when the first compound in

Group 3 is compared with the fourth compound in Group 5.

The 3-cyclohexyl derivative of salicylamide is apparently less soluble than the 3-phenyl salicylamide as evident when the fifth compound in Group 2 is compared with the first compound in Group 3.

The relationship of apparent solubility to inhibitory activity may be readily observed from Table 8. The compounds in Group 1, being the most insoluble, were relatively inactive as fungistatic agents, with none producing significant inhibition after 14 days incubation. Group 2 contains the bulk of the most active compounds except for N-(4-hydroxyphenyl)-3-phenylsalicylamide which appears in Group 4. The apparent solubility in Group 2 was approximately twice that of Group 1.

All compounds in Group 3 were active with one exception, N-butyl-4-phenylsalicylamide. As stated previously, the phenyl substitution in the 3 position is quite critical to the activity of these compounds. In Group 3 the apparent solubility is approximately twice that of Group 2. The compounds were generally not as active as those in the previous group.

In Group 4, all compounds showed significant inhibitory activity. However, with one exception they were less active than the active compounds in Groups 1, 2 and 3. The apparent solubility here is about twice that of

Group 3.

In Groups 5 and 6, only one compound, excluding the reference compounds, showed a significant degree of inhibition. In these groups the apparent solubility was greatest.

In general, the results seem to indicate a definite correlation between the inhibitory activity and the apparent solubility of the compounds. The majority of the most active compounds possessed very limited apparent solubility in the medium (Group 2). As the apparent solubility gradually increased the activity of the compounds tended to decrease until it finally was no longer detectable. Exceptions may be noted but this appears to be the general trend.

The compounds were further classified according to their behavior as fungistatic agents. The second classification is designated by the letter immediately following the compound in the table.

Those compounds designated with the letter "A" gave partial inhibition at low levels of concentration with no apparent increase in activity with increasing concentration. Three of the compounds in this group showed a uniform turbidity with no visible aggregates in the medium when the concentration of the compound was increased beyond the point of apparent solubility. The other compound

showed evidence of small microscopic aggregates at this level. It appears from the results that the maximum solubility level and the minimum level of the compound required for inhibition are very near the same. As the concentration was increased, the inhibitory activity did not increase because the amount of compound in true solution was not increased. Presumably the uniform turbidity noted with these compounds at higher concentrations was due to the formation of a colloidal solution. The compound in this group showing microscopic aggregates at concentrations above the apparent solubility, N-methyl-3-phenylsalicylamide, behaved differently than the other class "A" compounds in that the precipitation of the compound was influenced by mechanical disturbance of the medium, such as streaking of the plates during inoculation. If the surface of the medium was streaked harshly with a rough instrument, numerous microscopic crystals formed in the line of streaking. If streaked with a glass rod, the amount of crystal formation was much less.

Those compounds designated by the letter "B" showed a high order of activity at low concentrations and a decrease or complete loss of inhibitory activity with increasing concentrations. All compounds in this group showed definite evidence of aggregation in the medium beyond the point of apparent solubility of the compound.

The results suggest that the compounds were precipitating out at the higher concentrations, thus reducing the amount of compound in true solution in the medium. This appears to account for the loss of activity at the higher concentrations.

The compounds designated by the letter "C" are those which produced complete inhibition of growth at the lower concentrations with no apparent loss of activity at higher concentrations. The results indicate that the minimum concentration of the compound required for complete inhibition was below the point of apparent maximum solubility so that increasing the concentrations gave increased fungistatic activity up to the point of complete inhibition.

Thus it appears that the fungistatic behavior of Class A, B and C compounds can be explained satisfactorily on the basis of their apparent solubility in the medium and whether they formed precipitate or uniform turbidity when the concentration was progressively increased.

Fungicidal Activity of the Most Active Compounds. The 14 compounds previously described, were selected for testing their fungicidal activity against Trichophyton mentagrophytes.

The test method for the evaluation of alcohol soluble

Table 8

Concentration of the Compound Producing Turbidity or Precipitate in the Medium

Compound	Maximum concentration in μg^* per ml showing no turbidity or precipitate in Sabouraud agar	Minimum concentration in μg^* per ml showing turbidity or precipitate in Sabouraud agar	Observation
(Group 1)			
5-Iodo-3-phenylsalicylamide	5	10	Precipitate
5-Bromo-N-butyl-3-phenylsalicylamide	5	10	Precipitate
4-Phenylsalicylamide	5	10	Precipitate
N-Cyclohexyl-3-phenylsalicylamide	2.5	5	Precipitate
N-Phenylethyl-3-phenylsalicylamide	5	10	Precipitate
N-Hexyl-3-phenylsalicylamide	5	10	Precipitate
2-(beta-Hydroxyethoxy)-3-phenylbenzamide	5	10	Precipitate
(Group 2)			
N-Butyl-3-phenylsalicylamide (A)	10	20	Turbidity
N-Phenyl-3-phenylsalicylamide (A)	10	20	Turbidity
5-Chloro-3-phenylsalicylamide (B)	10	20	Precipitate
5-Bromo-3-phenylsalicylamide (B)	10	20	Precipitate

(continued on following page)

Table 8, continued

Compound	Maximum concentration in μg* per ml showing no turbidity or precipitate in Sabouraud agar	Minimum concentration in μg* per ml showing turbidity or precipitate in Sabouraud agar	Observation
3-Cyclohexyl-salicylamide (B)	10	20	Precipitate
N-Ethyl-3-phenyl-salicylamide (B)	10	20	Precipitate
5-Phenyl-salicylamide	10	20	Precipitate
2-Carboxymethoxy-3-phenylbenzamide	10	20	Precipitate
(Group 3)			
3-Phenyl-salicylamide (C)	20	40	Turbidity
N-Isopropyl-3-phenyl-salicylamide (B)	20	40	Precipitate
N-Methyl-3-phenyl-salicylamide (A)	20	40	Precipitate
N-Butyl-4-phenyl-salicylamide	20	40	Precipitate
(Group 4)			
N-(4-Hydroxy-phenyl)-3-phenyl-salicylamide (C)	40	80	Turbidity
N-(3-Methoxy-propyl)-3-phenyl-salicylamide (C)	40	80	Precipitate
N-(4-Aminophenyl)-3-phenylsalicylamide, HCl (A)	40	80	Turbidity

(continued on following page)

Table 8, continued

Compound	Maximum concentration in μg* per ml showing no turbidity or precipitate in Sabouraud agar	Minimum concentration in μg* per ml showing turbidity or precipitate in Sabouraud agar	Observation
N-(beta-Hydroxyethyl)-3-phenylsalicylamide (C)	40	80	Turbidity
N, N-Diethyl-3-phenylsalicylamide (C)	40	80	Turbidity
(Group 5)			
Salicylic acid	80	160	Turbidity
2-Methoxy-3-phenylbenzamide	80	160	Turbidity
N-Butyl-3-phenyl-5, x-disulfonamidesalicylamide	160	320	Precipitate
Salicylamide	320	640	Turbidity
N-Butyl-3-phenyl-5, x-dinitrosalicylamide	320	640	Precipitate
(Group 6)			
Sodium undecylenate	>80	>80	
N-(beta-Hydroxypropyl)-3-phenylsalicylamide	>160	>160	
N-Butyl-3-phenyl-5, x-diaminosalicylamide monohydrochloride	>640	>640	

* μg: micrograms

fungicides described by Golden and Oster (4, pp.359-362) seemed applicable to a study of the fungicidal activity of these new salicylates. This method employs 1 cm discs cut from a petri dish culture of Trichophyton mentagrophytes on Sabouraud agar with a sterile cork borer. The discs are transferred with aseptic precautions to 10 ml of the test fungicide in 95 per cent ethyl alcohol. After exposure for 1 minute to the fungicide, the discs are transferred to 10 ml of sterile broth and shaken for 3 minutes to remove any water-soluble or miscible material. They are then washed for 5 minutes in a 30 per cent acetone-water solution, thereby removing the fungicide adhering to the mycelium. Following the acetone washing step, the discs are once again immersed in sterile broth to remove all traces of acetone, and spread, culture side down, on the surface of Sabouraud agar slants. The slants are incubated at 28°C for 3 weeks and observed for growth.

In an attempt to demonstrate fungicidal activity by this method, solutions of N-butyl-3-phenylsalicylamide containing 32 mg per ml in isopropyl alcohol were employed. Exposure of the discs cut from 14 day old cultures of Trichophyton mentagrophytes to the test solutions for 3 minutes did not produce killing of the organism. Longer periods of exposure resulted in killing of the organism by the solvent alone, as shown by the

controls. The fungicidal activity of the compounds was next tested in propylene glycol solution at 32 mg per ml. Six minute exposure periods were used but the organisms were still not completely killed.

The compound adsorbed by the organism on exposure to the solution could not be removed by the washing method previously described or by numerous washings in isopropyl alcohol, acetone and ether as evidenced by intense fluorescence of the discs under the ultra violet light. Even though significant amounts of the compound remained on the organism, the latter was still viable when inoculated on Sabouraud agar plates.

In another attempt to demonstrate fungicidal activity, solutions of N-butyl-3-phenylsalicylamide and N-phenyl-3-phenylsalicylamide containing 32 mg per ml in peanut oil were prepared. Agar discs from a 14 day old culture of Trichophyton mentagrophytes were exposed to the solutions for 24, 48 and 72 hour periods. The discs were then washed in a 30 per cent acetone-water solution for 5 minutes and rinsed 3 times in sterile distilled water. They were then streaked, culture side down, on the surface of Sabouraud agar plates. After 5 days incubation at 28°C, growth was observed on the plate indicating incomplete killing of the organism. Controls using oil with no compound were treated in the same

manner. Here again, a significant amount of the compound remained on the discs which had been exposed to oil solutions of the compound as evidenced by fluorescence under the ultra violet light.

In view of the unsatisfactory results obtained from the above methods, it was decided to test the fungicidal activity of the compounds in aqueous solution at the concentration found to give the greatest inhibition of growth in Sabouraud agar. This method would permit prolonged exposure periods not possible when organic solvent solutions of the compound were employed. It was considered that such a method would indicate the relative fungicidal activity of the compounds at these concentrations, however weak such activity might be. The procedure was carried out as described in "Materials and methods".

The results in Table 9A show that 5 of the compounds were fungicidal at the concentration tested after 8 days incubation at 28°C against the relatively dilute inoculum. Three others, N-butyl-3-phenylsalicylamide, N-phenyl-3-phenylsalicylamide and N-(4-hydroxyphenyl)-3-phenylsalicylamide, showed a 50 per cent or greater reduction in the number of viable mycelial fragments. Sodium undecylenate was the only compound which did not inhibit growth of the mycelium in the solution. It should be explained

here that the mycelium of Trichophyton mentagrophytes will show significant growth when incubated in distilled water with no added nutrients. Presumably small amounts of nutrient material are carried over with the inoculum.

Additional data obtained by the same method, but employing a heavier inoculum are shown in Table 9B. The decrease in number of viable mycelial fragments follows the same general pattern as shown in Table 9A. Only one compound, N-(4-aminophenyl)-3-phenylsalicylamide, HCl, gave complete sterilization of the heavier inoculum, while 8 others showed a 50 per cent or greater reduction in the number of viable mycelial fragments. N-isopropyl-3-phenylsalicylamide was somewhat more active here than the previous results indicated as shown in Table 9A.

In the experiment shown in Table 9B, the concentration of sodium undecylenate was increased 4 fold over that in the previous experiment and again did not inhibit growth of the mycelial fragments. The other compound not inhibiting growth of the mycelium was N-ethyl-3-phenylsalicylamide.

The pH of the water solution of each compound was determined and found to be between pH 6.9 and 7.3 with one exception, that being N-(4-aminophenyl)-3-phenylsalicylamide, HCl which gave a pH of 4.41.

In a separate experiment, an 80 microgram per ml

solution of N-(4-aminophenyl)-3-phenylsalicylamide, HCl, was adjusted to neutrality with dilute sodium hydroxide and the fungicidal activity of the compound retested as described. No activity of the compound was then apparent as it did not prevent growth of the mycelial inoculum after 7 days incubation at 28°C. Apparently the effect observed in the earlier experiments was due to the relatively low pH.

It seems evident, then, that this group of salicylamide derivatives, although quite active as fungistatic agents when tested against the two dermatophytes, showed relatively weak fungicidal activity for these organisms at the concentrations employed, which were limited by the slight water solubility of the compounds.

Mechanism of Growth Inhibition by N-butyl-3-phenylsalicylamide.

A. Observations on Adsorption. In an attempt to observe the mycelium and spores of Trichophyton mentagrophytes exposed to a solution of N-butyl-3-phenylsalicylamide, by means of ultra violet microscopy, it was noted that the organism would adsorb the compound from very dilute aqueous solutions. When the organism was treated with a concentrated aqueous solution, which was presumably colloidal, the solution itself was fluorescent and

Table 9A
Fungicidal Activity of the Most Active Compounds
Eight Days Incubation

Compound	Concentration µg per ml	Viable mycelial fragments per ml
3-Phenylsalicylamide	20	None
N-Methyl-3-phenylsalicylamide	40	40,000
N-Ethyl-3-phenylsalicylamide	10	36,000
N-Butyl-3-phenylsalicylamide	20	26,000
N-Isopropyl-3-phenylsalicylamide	20	48,000
N-(3-Methoxypropyl)-3-phenylsalicylamide	40	None
5-Chloro-3-phenylsalicylamide	10	None
5-Bromo-3-phenylsalicylamide	10	33,000
5-Iodo-3-phenylsalicylamide	10	48,000
N-Phenyl-3-phenylsalicylamide	10	9,000
3-Cyclohexylsalicylamide	20	None
N-(4-Hydroxyphenyl)-3-phenylsalicylamide	80	18,000
N-(4-Aminophenyl)-3-phenylsalicylamide, HCl	80	None
Sodium undecylenate	20	Too numerous to count

Control: 50,000 viable mycelial fragments per ml at beginning of experiment.

Table 9B

Fungicidal Activity of the Most Active Compounds

Compound	Concentration μg per ml	Viable mycelium after incubation periods: 1 day	3 days	5 days	8 days
3-Phenylsalicylamide	20	200,000	100,000	54,000	60,000
N-Methyl-3-phenylsalicylamide	40	110,000	110,000	124,000	120,000
N-Ethyl-3-phenylsalicylamide	10	175,000	180,000	176,000	Too numerous to count
N-Butyl-3-phenylsalicylamide	20	71,000	89,000	39,000	28,000
N-Isopropyl-3-phenylsalicylamide	20	190,000	194,000	84,000	40,000
N-(3-Methoxypropyl)-3-phenylsalicylamide	40	64,000	10,000	3,000	6,000
5-Chloro-3-phenylsalicylamide	10	175,000	97,000	96,000	47,000
5-Bromo-3-phenylsalicylamide	10	125,000	125,000	138,000	104,000
5-Iodo-3-phenylsalicylamide	10	125,000	125,000	138,000	104,000
N-Phenyl-3-phenylsalicylamide	10	28,000	38,000	16,000	3,000
3-Cyclohexyl-salicylamide	20	78,000	78,000	14,000	8,000
N-(4-Hydroxyphenyl)-3-phenylsalicylamide	80	116,000	110,000	96,000	26,000
N-(4-Aminophenyl)-3-phenylsalicylamide, HCl	80	38,000	3,000	1,000	None
Sodium undecylenate	80	180,000	190,000	Too numerous to count	

Control: 184,000 viable mycelium per ml at beginning of experiment.

interfered with observation of the mycelium and spores under the microscope. If the concentration of the compound was reduced to 10 micrograms per ml or less, the solution was not fluorescent. The organism when treated with this dilute aqueous solution, after standing for some time, showed definite fluorescence as compared to control cultures not exposed to the compound.

In view of this observation, the adsorption of the compound from a dilute aqueous solution was retested as described in "Materials and methods". Here again, the organism was observed to adsorb the compound from the dilute solution which did not show fluorescence. The organism, when observed under the ultra violet light after 24 hours, showed intense fluorescence.

In an attempt to determine the minimum concentration at which the compound showed fluorescence in Sabouraud agar by examination of plates of the medium under the ultra violet light, it was observed that the spores inoculated on a plate of medium became fluorescent after several days although no growth occurred. The medium itself was not fluorescent. In view of this observation, it was decided to see if this adsorption phenomenon was specific for organisms which were sensitive to the compound.

This led to the experiment described in the

"Materials and methods" on the relationship of adsorption of the compound to inhibitory activity. Several fungi were tested for their ability to adsorb the compound by inverting culture discs of the organisms on the surface of agar plates containing 10 micrograms per ml of N-butyl-3-phenylsalicylamide. After an hour the discs were examined under the ultra violet light for fluorescence.

Three of the organisms readily adsorbed the compound from the medium as evidenced by increased fluorescence, these being Trichophyton mentagrophytes, Microsporum audouini and Rhizopus tritici. These organisms were shown to be inhibited by the compound, although the inhibition was only partial in the case of Rhizopus tritici, which showed some growth in the presence of 160 micrograms per ml in Sabouraud agar.

Penicillium chrysogenum, Aspergillus niger and Mucor javanicus showed only faint fluorescence and were not inhibited by the compound at concentrations of 160 micrograms per ml in Sabouraud agar.

These results suggest that the activity of the compound may be closely related to this adsorption affinity existing between the compound and the organism.

B. Study of Effect on Respiration. As part of a preliminary study on the mechanism of action of N-butyl-3-phenylsalicylamide, the effect of this compound on the

respiration of Trichophyton mentagrophytes was investigated. Experiments were performed in a conventional Warburg respirometer. The effect of the compound was determined at concentrations of 1, 10, 50, 100, 200, and 500 micrograms per ml as previously described.

As shown in Table 10, the compound, in a wide range of concentrations, did not inhibit the respiration of the mycelium either in the presence of glucose or in the absence of the substrate. On the contrary the compound apparently had a stimulatory effect on both endogenous and exogenous respiration.

In view of the data in Table 10, and in view of the fact that N-butyl-3-phenylsalicylamide inhibits growth of this organism at 10 micrograms per ml, one might conclude that the mechanism of this inhibition is not dependent upon a general interference with the respiration of the organism, either in the presence or absence of glucose. The effect of the compound on the respiration of the organism in the presence of substrates other than glucose was not investigated.

The results further suggest that the inhibition of the organism by the compound might be a specific or antimetabolite type reaction, in contrast to a non-specific or general protein denaturant. Generally, inhibitors of

the latter type decrease the respiration in the absence or presence of all substrates.

The differences in the respiration of the mycelium between different experiments is due to some unknown factor or factors. An effort was made to keep the materials and methods constant in each experiment, although some uncontrolled factor was apparently involved, probably differences in the mycelial culture. The relatively slight effect of glucose on the respiratory rate of this organism is in agreement with the work of Bentley (8, p. 368). In experiment 3 the observations on respiration in the presence of glucose were made several hours before the endogenous measurements. The same mycelial culture was used, but in the latter case the suspension had been held in the phosphate buffer for this period of time. This may account for the differences in respiration in the presence and absence of glucose.

Table 10

The Effect of N-Butyl-3-phenylsalicylamide on the Respiration of Trichophyton Mentagrophytes

Experiment number	Substrate	Control	Alcohol control	Concentration of the compound micrograms per ml 1	10	50	100	200	500
1	None	4.33							
	Glucose	5.38							
2	None		6.12	7.41	7.58				
	Glucose		7.80	7.96	8.40				
3	None		9.20	10.00	9.87	9.88	8.85		
	Glucose		6.25	8.02	8.15	7.72	5.92		
4	None		3.25				5.80	5.16	5.44
	Glucose		3.44				5.74	5.83	5.46
5	None		3.08		3.53	3.30		3.71	
	Glucose		3.12		3.76	3.60		4.55	
6	Glucose		3.07		4.02	5.43	5.89	4.97	5.95

control: Endogenous and exogenous studies in the absence of alcohol or compound.
The data in the table are in microliters of oxygen per hour per mg dry weight of cells.

DISCUSSION

One of the major obstacles to a complete study of the fungistatic and fungicidal activity of these new salicylates is the low water solubility of the most active compounds. Yet, in view of the correlation between activity and solubility of the compounds in the medium, the property of slight solubility is an important factor in conferring marked fungistatic activity at low levels of concentration. This phenomenon has been observed with anti-bacterial agents, whereby low water solubility and activity of the compound are in close correlation (13, p.5).

This property of insolubility may well explain the lack of activity of many of the compounds of this group which on the basis of their structure should be extremely active fungistatic agents. It was stated earlier that the addition of chlorine and bromine in the 5 position of 3-phenylsalicylamide increased the order of activity of the compound. The addition of iodine in the 5 position decreased the order of activity of the compound. Substitution of bromine in the 5 position of N-butyl-3-phenylsalicylamide virtually destroyed the activity of the compound as previously stated. In each case, the solubilities of the halogenated derivatives in the medium

were decreased. In view of this, and in view of the fact that all compounds studied seemed to be as active at the point of maximum apparent solubility as they were at higher concentrations, it must be assumed that the solubility of the compounds was decreased beyond the minimum level required for fungistatic activity.

The low solubility of these compounds in aqueous solutions prevented a thorough study of their fungicidal activity. The use of organic solvents prevented the use of sufficient exposure times and the concentrations obtained in aqueous solutions were much lower than those usually employed in fungicidal tests.

The phenomenon of adsorption of the compound by the organism seems to be closely related to the activity of the compound. As stated previously, the sensitive organisms appear to adsorb the compound more readily than the resistant fungi. The fact that cotton exhibited similar properties to the sensitive organisms in that it possessed the ability to adsorb the compound may have some bearing on the mechanism of action. The cell wall of fungi is composed of chitin (5, p.3) which is a complex polysaccharide and contains nitrogen in the form of glucosamine. Cotton is a pure polysaccharide having numerous linked glucose units. It may well be that the chitin of the dermatophytes and the other sensitive

fungi, as well as cotton, possess functional groups which have an affinity for the compound. This does not fully explain the mechanism of inhibition of Trichophyton mentagrophytes by N-butyl-3-phenylsalicylamide.

The possibility that some of these compounds may be of value in the treatment of dermatophytosis must not be overlooked. In view of the decreased activity of the compounds in the presence of 10 per cent citrated human plasma as a source of protein, it is unlikely that these compounds will be of value in the treatment of dermaphytosis by systemic administration. Nevertheless, this does not exclude the possibility of these agents being effective when used as topical applicants.

In view of the results obtained in these studies, it appears that further investigation along certain lines would be extremely fruitful. These are as follows:

1. Extensive investigation of the mechanism of action of N-butyl-3-phenylsalicylamide or other compounds of this group, in hope that the studies may further elucidate the biochemical reactions of the organism, which in turn might provide further knowledge on the treatment of dermatophytosis by these compounds or by other closely related derivatives.

2. Toxicity studies of the compounds in experimental animals.

3. Investigation of the value of these compounds in the treatment of feline ringworm, as topical applicants and systemic therapeutic agents.

4. The synthesis and study of new salicylate derivatives patterned after these compounds.

SUMMARY

In summary, it was shown that a new group of salicylates, most of which were derivatives of 3-phenylsalicylamide, were extremely active as fungistatic agents against Trichophyton mentagrophytes and Microsporum audouini. When compared with a salt of undecylenic acid, which is recognized to be among the most active and commonly used inhibitory agents against this group of fungi, 9 of the 31 compounds exhibited fungistatic properties at lower concentrations when compared on a weight and molar basis.

The fungistatic activity of the most active compounds were tested in the presence of 10 per cent citrated human plasma as a source of protein. The presence of the protein reduced the activity of all compounds, the majority of them showing a 4 to 16 fold decrease in activity. Three of them showed virtually a complete loss of activity under these conditions.

The fungicidal activity of the most active compounds was determined. Eight of these were shown to possess fungicidal properties exceeding that of sodium undecylenate when compared on a weight and molarity basis.

In a preliminary study of the mechanism of inhibition of growth by N-butyl-3-phenylsalicylamide, it was shown that Trichophyton mentagrophytes would adsorb the

compound from dilute aqueous solutions or from the surface of an agar plate. There appeared to be some correlation between this adsorption phenomenon and sensitivity of an organism to the compound.

The compound, in a wide range of concentrations, was not inhibitory to the respiration of the organism when determined in a conventional Warburg respirometer. A slight but definite stimulation of the exogenous and endogenous respiration was observed at all concentrations tested. Apparently the mechanism of inhibition by the compound does not depend on a general interference with respiration.

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