THE POTENTIALITIES OF CERTAIN CHEMICAL COMPOUNDS AS FOOD PRESERVATIVES

by

SHIU CHI LAU

A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1947

APPROVED

Professor and Head of Department of Food Technology
In Charge of Major

Chairman of School Graduate Committee

Associate Dean, Graduate Division

ACKNOWLEDGMENT

Grateful acknowledgment is made of the helpful suggestions and guidance, as Major Professor, of Professor E. H. Wiegand and of the financial support of the project by the Refrigeration Research Foundation.

I am indebted to Dr. J. S. Butts, Head, Department of Agricultural Chemistry, and to Dr. J. R. Haag, for their provision of test animals and counsel; to Dr. M. P. Chapman for his help in the autopsy studies; to Mr. E. C. Bubl for his help in setting up the acute toxicity trials; to Dr. W. B. Bollen for kindly furnishing some test organisms; and to Professor O. J. Worthington and other members of the staff of the Department of Food Technology for their helpful advice and criticism.

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THE POTENTIALITIES OF CERTAIN CHEMICAL COMPOUNDS AS FOOD PRESERVATIVES

INTRODUCTION

The purpose of this investigation was to study various compounds obtainable from commercial sources with respect to their usefulness in increasing the storage life of perishable food products, especially with respect to the storage of such products in refrigerated warehouses. In the food industries fruits and vegetables are harvested in season; a major portion of the harvest is sold for fresh consumption while the other portion is processed for future use by canning, freezing, and drying. These three processes of canning, freezing, and drying are inherently complete processes and the foods thus prepared are more or less permanently preserved. However, in between these two classes of foods, the fresh and the processed, there is the intermediary class which is intended for remanufacture or later processing. This class of food needs to be stored or temporarily preserved in some way. The means now employed are either to freeze them and keep them in freezer storage (29° F and lower) or else preserve them (fruits) with sulfur dioxide. The first method is now faced with major difficulties, however, because during the past eight years freezer storage has become

increasingly congested (14). This freezer storage congestion has become so acute that food packers are faced with the problem of not being able to store the pack planned for later months unless some appreciable out-movement takes place. When it comes to displacing less needed items in congested freezer storage the logical first sacrifice would be those of least economic value or those with the weakest consumer demand. The class of foods intended for remanufacture falls under this category. For this class of foods, if a satisfactory agent was found that could preserve them from microbiological deterioration at cool storage temperatures (29-36° F), then they could be channeled into cool storage warehouses and thus relieve the critical congestion of freezer storage space. It was with this thought in mind that the project was undertaken.

Another aspect of the case is that there is a real demand for a good chemical food preservative. Just recently the National Wine Association petitioned for a hearing to provide a tolerance for monochloroacetic acid in wine (1). When manufacturers petition for permission to use a preservative in such a product as wine, which is a comparatively stable food product, the demand for a preservative must be quite pressing.

Food manufacturers have been using sodium

benzoate in their products, the only food preservative allowed in foods under the provisions of the Federal Food, Drug and Cosmetic Act. However, this compound is not satisfactory from many points of view, the chief objection being its weak preservative effect in neutral or slightly acid food materials (26).

This project was undertaken to see if a compound can be found that will effectively and efficiently prolong the storage life of perishable food materials in cooler storage, and, also, one that will take the place of sodium benzoate.

In looking for a food preservative one must be able to recognize the attributes that make the preservative good or desirable. Gershenfeld and Perlstein (11) list these attributes as follows:

1. It must be effective against the types of microorganisms causing decomposition or spoilage.

2. It must be soluble in the concentration used.

3. It must not be toxic in the concentration in which it is employed.

4. It must be compatible, must not alter the character of the substance to which it is added, must not impart any odor, color, taste, etc. to the product, and it must be practically neutral so that it will not alter the pH of the product.

5. It should be available or potentially available and not prohibitive in cost.

6. The developed inhibiting effect must be lasting and therefore it may not be possible to depend on volatile substances, the effects of which disappear after evaporation.

With these points in mind the project was undertaken to see if, among the number of new chemical compounds now available, there is one that possesses qualities which nearly approach these ideals. Once that compound has been found and its structure known then systematic modifications can be made of its structure in an attempt to increase its activity, lower its toxicity, or facilitate ease of application.

EXPERIMENTAL

The studies in this paper include: (1) A determination of the concentration of a selected group of chemical compounds in broth media and in agar media which will destroy and inhibit the growth of representative food spoilage microorganisms during the incubation period; (2) An evaluation of the preservative action of the most promising compounds on natural food substances at (i) incubation temperature and at (ii) cool storage temperatures; (3) Toxicity trials on the most promising compound; (4) Synthesis and attempts at synthesis of improved homologues or derivatives of the most promising compound; (5) Tests on the synthesized compounds for (i) their potency against food spoilage microorganisms, (ii) their toxicity for animals, (iii) methods of analysis, (iv) incompatibilities and potentiating complement.

1. Test chemical compounds

The list of chemicals shown in Table 1 were tested for their antifungal properties. These chemicals were obtained from commercial sources and were chosen because they had appeared in the literature as compounds that may be of promise for the purposes of this study.

In the selection of these compounds a bibliography of over a hundred titles has been consulted. These have included articles appearing in scientific journals as well as those in trade journals. The criteria for singling out any compound for testing were descriptions or data appearing in the literature which point favorably to their activity and/or nontoxicity. Historical or legendary connections of the compounds with the food industries were also taken into consideration.

It must be pointed out that the list of test chemicals was not static, but rather extremely expansible, because current literature was being continually consulted and, as soon as what appeared to be a promising compound was brought to notice, steps were immediately made to include that compound in the test list. In this connection it should be stated here that the test list does not claim to include all the compounds that had appeared in the literature as promising food preservatives. Undoubtedly some compounds had been missed in the literature. Others were not included either because they were unavailable from commercial or experimental sources or because it was not possible during the time of the experiment to contact sources of supply.

Table 1

List of compounds tested for antifungal potency

Trade Name	Chemical Identity
Phygon	2,3-dichloro-1,4-naphthoquinone
Synkamin	4-amino-2-methyl-1-napthol hydrochloride
Synkavite	2-methyl-1,4-naphthohydroquinone diphosphoric acid ester tetrasodium salt
Roccal	Alkyl dimethyl benzyl ammonium chloride
SP-25	Alkyl dimethyl benzyl ammonium chloride
Phemerol	p-tert-octylphenoxyethoxy-ethyl-dimentyl benzyl ammonium chloride
Hyamine 1622	Di-isobutyl phenoxy ethoxy ethyl dimethyl benzyl ammonium chloride
Hyamine 10-X	Di-isobutyl cresoxy ethoxy ethyl dimethyl benzyl ammonium chloride
Emulsept	N(acyl calamino formyl methyl)-pyridinium chloride
Dowacide G	Sodium pentachlorophenol
Dowecide A	Sodium orthophenoxylphenol
G-4	2,2'-dihydroxy-5,5'-dichloro-diphenyl methane
G-11	2,2'-dihydroxy-3,5,6-3',5',6'-hexachloro-diphenyl methane
Aerosol OT	Di-octyl sodium sulfo succinate
Alkanol B	Sodium alkyl naphthalene sulfate
PABA	Para amino benzoic acid
Mycoban	Sodium propionate
Mycoban	Calcium propionate
Kowel No. 309	Sodium sulfite-sodium benzoate mixture
Mone	2-methyl-1,4-naphthoquinone
[illegible]	Xyloquinone
[illegible]	Hexylresorcinol
[illegible]	Monochloroacetic acid
[illegible]	Acetic acid
[illegible]	Sodium benzoate
[illegible]	2,3,5-triiodobenzoic acid
[illegible]	3,5-diiodo-2-hydroxybenzoic acid
[illegible]	3,5-diiodo-4-hydroxybenzoic acid
[illegible]	Propylene chloride
[illegible]	Propylene chloride
[illegible]	Propylene glycol
[illegible]	Lauryl chloride
[illegible]	Cinnamic acid
[illegible]	Tannic acid
[illegible]	Lauryl sodium sulfate
[illegible]	Furfural
[illegible]	Trioxane
[illegible]	Sodium sulfite

2. Test and index organisms

The scope of the project dictated that the chemicals to be tested, once obtained, should undergo a quantitative screening process whereby those of little promise as food preservatives would be eliminated from further tests, thus leaving time for more intensive studies of the promising compounds. For this preliminary screening process the chemicals were tested quantitatively for their inhibiting and lethal activity against a food spoilage microorganism.

The plan as at first conceived was to use an organism of known identity and one which may commonly be found in food spoilage. Two molds, Aspergillus repens and Aspergillus ruba, were tested in this connection. However, these two organisms proved unsatisfactory in that they were not fast growers, producing but scanty growth in various nutrient media under an incubation temperature of 30 degrees C. Because other known organisms which might be suitable for the purpose were not immediately available various molds were isolated from foods and their growth characteristics studied. A green mold isolated from gooseberry jam was found to be a vigorous and rapid grower, producing a profusion of bright green spores and a thick mycelial

mat in two days. This mold was observed to be able to grow under a wide variety of conditions and was chosen as the index organism in the preliminary trials. It was later identified to belong to the genus Penicillium.

Other index organisms used in this study were obtained from the American type culture collection. They were: Aspergillus niger, Aspergillus Sydowi, Penicillium expansum, Saccharomyces cerevisiae, Saccharomyces pastorianus, and Zygosaccharomyces marxianus (Hansen). These microorganisms were selected through reference to the literature and were cited in these for their association with food spoilage and also for their representative value as more or less typical members of food spoilage groups (4,5,6,15,18,19).

These index microorganisms of known identity were used in the detailed testing of the more promising compounds.

3. Preliminary evaluation of the fungistatic and fungicidal activity of chosen chemical compounds

The fungistatic and fungicidal activities of the chosen compounds were examined in liquid nutrient media by measuring one milliliter of a solution of the compound directly into the bottom of sterile tubes. To this was added 9 ml. of broth which had just been inoculated with mold spores. Sabouraud's dextrose broth was the

medium used and the spores were obtained from four- to six-day-old agar cultures of the test organism.

The concentrations of the chemicals used were 10, 7.5, 5.0, 2.5, 2.0, 1.5, and 1.0 mg. per ml. respectively. Since 9 ml. of broth was added to each one ml. of test solution per tube the final concentrations of chemical in the tubes were one-tenth of the above figures.

In making up these solutions of test chemicals water was used as the solvent whenever possible. In the case of certain acids or phenols which were not soluble in water the water soluble sodium salts were prepared. When this could not be done ethyl alcohol was used as the solvent. In those cases where ethyl alcohol was used dilutions were so chosen that not more than 0.3 ml. of alcohol was added to any one tube.

When the test organism failed to grow in the tube of the highest dilution then retests were run using lower concentrations. These concentrations were 0.75, 0.50, 0.40, 0.30, 0.20, 0.15, 0.10, 0.05, and 0.02 mg. per milliliter.

The incubation time was three days at 30 degrees C. after which the tubes were examined for growth. From those tubes showing no growth, subcultures were made into tubes containing sterile broth. The concentration of the chemical which inhibited growth in the original tube

was taken to be the inhibiting concentration and the concentration of the chemical showing no growth in both original and subcultured tubes were taken as the lethal concentration.

Table 2 summarizes the data obtained in determining the effectiveness with which the chosen chemicals inhibited growth and destroyed the test organism.

In addition to testing the chemicals on just one microorganism in liquid media the chemicals were further tested on three other microorganisms using the agar-streak method of Waksman and Reilly (27). In this test each compound was dissolved in water or alcohol. When alcohol was used as the solvent the dilutions were chosen in such a manner that no more than 0.3 ml. of alcohol was added to any plate containing 10 ml. of agar.

Each of a series of 7 sterile 10-cm. Petri dishes was marked off into three sectors, and to each of six dishes was added a portion of the solution to be tested, usually 1.0, 0.75, 0.6, 0.5, 0.4, 0.3, and 0.2 ml. respectively. None of the sample was added to the seventh, or control, dish.

Sabouraud's dextrose agar was melted, cooled to 45 deg. C., added in ten milliliter quantities to each dish, and mixed thoroughly with the sample by rocking the dish.

After the agar had solidified the test organisms were streaked on the plate, each within a marked sector. Three discrete streaks of each organism were made by means of an inoculating wire. The wire was sterilized in a flame and dipped into a uniform suspension of the test organism, and the three streaks made on the agar without recharging the inoculating wire. The wire was flamed and recharged between plates.

The plates were incubated at a temperature of 28 deg. C. for 36 to 40 hours, and the results recorded.

The end point was taken as the highest dilution at which growth was completely inhibited. The result was expressed in "activity units," the amount of material which, added to 1 ml. of the test medium, just inhibited the growth of the test organism.

Unitage is calculated by dividing the volume of agar plus added compound in the plate by the weight of the compound added to that plate which showed the end point. Dividing these "activity units" into a million will give the effective inhibiting concentration of the chemicals in parts per million.

Table 2

Inhibiting and lethal concentrations of test chemicals against Penicillium sp. in Sabouraud's dextrose broth (concentrations in ppm)

Compound	Inhibiting concentration	Lethal concentration
Phygon	2	5
Synkamin	200	200
Synkavite	200	200
2-methyl-1,4-naphthoquinone	20	100
Xyloquinone	50	100
Roccal	10	20
SP-25	15	20
Phemerol	10	25
Hyamine 1622	25	25
Hyamine 10-X	25	25
Emulsept	50	100
Dowacide G	40	60
Dowacide A	100	300
Hexylresorcinol	100	200
G-4	200	500
G-11	300	500
Monochloacetic acid	750	1,000
Acetic acid	#1,000	#1,000
Sodium benzoate	#1,000	#1,000
2,3,5-triiodobenzoic acid	250	250
3,5-diiodo-2-hydroxybenzoic acid	250	250
3,5-diiodo-4-hydroxybenzoic acid	250	250
PABA	#1,000	#1,000
Mycoban (sodium)	#1,000	#1,000
Mycoban (calcium)	#1,000	#1,000
Propylene chloride	#1,000	#1,000
Propylene oxide	#1,000	#1,000
Propylene glycol	#1,000	#1,000
Lauryl chloride	#1,000	#1,000
Cinnamic acid	#1,000	#1,000
Tannic acid	#1,000	#1,000
Lauryl sodium sulfate	250	500
Aerosol OT	100	200
Alkanol B	#1,000	#1,000
Furfural	400	600
Trioxane	#1,000	#1,000
Sodium sulfite	500	500
Eswell No. 309	#1,000	#1,000

#Ineffictive at highest concentration of test

4. Feeding tests

As soon as Phygon, 2,3-dichloro-1,4-naphthoquinone, was obtained and preliminary tests had shown it to be promising material for further study, arrangements were made to run tests on it for toxicity. The toxicity trials consisted in feeding the compound to six-week-old rats by incorporating it in their solid ration at the rate of 1.0% and 0.2% by weight respectively.

Results on these tests showed that those rats that were put on a ration containing 1.0% 2,3-dichloro-1,4-naphthoquinone rapidly showed toxic symptoms. One test animal died after being on the diet for two days; two others died after twelve days. The lone survivor was chloroformed after fourteen days because it too showed toxic symptoms.

The other group of animals receiving 0.2% 2,3-dichloro-1,4-naphthoquinone in their feed also showed toxic symptoms. These animals took less feed than was normal. When they showed but 25 to 50 percent of what should be normal gains in weight at the end of 25 days on this experimental diet the animals were sacrificed.

Table 3

Fungistatic activity of test chemicals in Sabouraud's dextrose agar

COMPOUND	Saccharomyces pastorianus	Saccharomyces cerevisiae	Aspergillus niger
	units/g.	units/g.	units/g.
Phygon	201,000	201,000	201,000
2-methyl-1,4-naphthoquinone	34,333	26,000	26,000
Kyloquinone	21,000	21,000	11,000
Synkamin	26,000	21,000	15,286
Synkavite	26,000	21,000	15,286
Roccal	34,333	34,333	6,000
SP-25	34,333	34,333	6,000
Phemerol	6,000	101,000	5,100
Hyamine 1622	6,000	101,000	5,100
Hyamine 10-X	6,000	101,000	5,100
Emulsept	#5,100	5,100	#5,100
Dowacide G	26,000	17,666	17,666
Dowacide A	17,666	14,333	14,333
Hexylresorcinol	17,666	17,666	21,000
G-4	26,000	6,000	1,100
G-11	5,100	1,100	1,100
Monochloroacetic acid	14,333	14,333	#1,100
Acetic acid	2,100	2,100	1,100
Sodium benzoate	#1,100	#1,100	#1,100
2,3,5-triiodobenzoic acid	#1,100	#1,100	#1,100
3,5-diiodo-2-hydroxy benzoic acid	#1,100	#1,100	#1,100
3,5-diiodo-4-hydroxy benzoic acid	#1,100	#1,100	#1,100
PABA	#1,100	#1,100	#1,100
Mycoban (sodium)	#1,100	#1,100	#1,100
Mycoban (calcium)	#1,100	#1,100	#1,100
Propylene chloride	#1,100	#1,100	#1,100
Propylene oxide	#1,100	#1,100	#1,100
Propylene glycol	#1,100	#1,100	#1,100
Lauryl chloride	#1,100	#1,100	#1,100
Cinnamic acid	#1,100	#1,100	#1,100
Tannic acid	#1,100	#1,100	#1,100
Lauryl sodium sulfate	4,010	14,333	2,100
Aerosol OT	5,100	25,100	10,100
Alkanol B	1,433	1,433	1,100
Furfural	2,100	2,100	2,100
Trioxane	#1,100	#1,100	#1,100
Sodium sulfite	#1,100	#1,100	#1,100

Activity lower than indicated figure

5. Antifungal evaluation of the more promising compounds

As might be expected in a study of this nature many of the chemicals tested showed very little promise and were discarded in the preliminary trials. Certain members of the assembled compounds, however, showed some promise and these were set aside for further testing. As may be seen from Table 2 and Table 3 the most potent compounds against the test microorganisms naturally fell into three groups: the quaternary ammonium compounds, the naphthoquinones, and the phenols.

Although the naphthoquinones were very potent they were only obtained for tests after a study of the other compounds had proceeded to quite an advanced stage. For this reason the naphthoquinones only appear irregularly in Tables 4, 5, and 6, which summarize data obtained on the activities of various compounds against food spoilage microorganisms.

Table 4 shows the effective inhibiting and lethal concentrations of various compounds on seven representative food spoilage yeasts and molds in Sabouraud's dextrose broth. Table 5 shows the effective preservative concentrations of various compounds on different food materials. Table 6 summarizes the influence of storage temperatures on the preservative power of the chemicals.

The data assembled in Table 6 were collected because it was conceived that some chemicals might lose their preservative activity at low temperatures.

Table 4

Inhibiting and Lethal Concentration of Test Chemicals in Sabouraud's Dextrose Broth

(concentrations in parts per million)

COMPOUND	Aspergillus SYDOWI		Aspergillus FLAVUS		Penicillium species		Penicillium expansum		Saccharomyces cerevisiae		Saccharomyces pastorianus		Saccharomyces marxianus	
Activity --	In.	L.	In.	L.	In.	L.	In.	L.	In.	L.	In.	L.	In.	L.
Roccal	30	30	20	20	20	40	10	10	15	15	15	15	30	40
Phemerol	30	30	20	20	20	30	10	10	15	15	15	15	30	40
Hyamine 10-X	30	30	20	20	20	40	10	10	15	15	15	15	30	40
Hyamine 1622	30	30	20	20	30	30	10	10	15	20	20	30	30	40
Hexylresorcinol#	150	150	150	200	70	70	60	80	150	200	100	100	150	200
Dowacide G	100	150	80	100	80	80	80	80	--	--	--	--	150	200
Aerosol OT	Ineffective at maximum concentrations of tests													

Note: In. denotes inhibitory concentration L. denotes lethal concentration

#Sodium salt

Table 5

Effective preservative concentration of test chemicals on foods
(concentrations in ppm)

COMPOUND	Raspberries pH 3.3	Blackberries 3.0	Cherries 3.9	Apricots 3.7	Prunes 4.2	Pears 4.0	Apples 3.3	Beets 5.5
Roccal	100	100	>200	>100	>200	150	50	100
Phemerol	100	100	>200	>100	>200	150	50	150
Hyamine 10-X	75	100	125	>100	200	100	25	200
Hyamine 1622	100	75	125	>100	200	150	50	150
Hexylresorcinol#	150	100	300	100	--	--	50	--
Dowacide G	--	--	300	--	--	--	--	--
Aerosol OT	--	--	300	--	--	--	--	--
Phygon	5	5	50	--	--	--	--	40

Sodium salt

Table 6

Inhibiting Concentrations of Test Chemicals on various Foods at 28° and 3° C.

(concentrations in parts per million)

COMPOUND	Beets		Blackberries		Cherries		Peaches		Sabouraud's dextrose broth	
	28°	3°	28°	3°	28°	3°	28°	3°	28°	3°
Hyamine 1622	150	50	---	150	200	100	---	---	20	15
Emulsept	200	150	--	150	--	150	---	---	60	40.
Phemerol	150	50	200	100	200	150	---	---	25	15
Hyamine 10-X	200	120	150	150	150	125	---	---	30	20
G-4	---	---	---	--	---	200	---	100	60	40
G-11	---	---	---	---	---	250	---	150	100	75
2-methyl-1,4-naphthoquinone	---	---	40	15	60	40	---	---	25	15
Phygon	40	30	20	10	40	30	---	---	5	5

6. Synthesis

Phygon, 2,3-dichloro-1,4-naphthoquinone, a water insoluble substance, has been found to be highly potent against the common food spoilage microorganisms. It has also been found to be toxic. Since the chemical structure of this compound is known the possibility exists that through modification of its structure an improved compound may be obtained. Accordingly attempts were made to synthesize a water soluble derivative. At the same time it was hoped that modification of its structure would lower its toxicity.

In planning experiments to synthesize a water soluble derivative out of an insoluble organic compound one of the first reactions to be thought of was sulfonation. However, a review of the literature discloses the fact that 2,3-dichloro-1,4-naphthoquinone cannot be sulfonated and still retain its identity (3) since even mild sulfonating agents will displace the chlorine atoms in both positions two and three of the naphthoquinone ring.

It is probably because both the chlorine atoms are labile that the soluble sodium salt cannot be successfully prepared. Dissolving 2,3-dichloro-1,4-naphthoquinone in sodium hydroxide solution produced a

colored solution that turned red on standing. This solution possessed but very slight antifungal activity.

Notwithstanding these limitations to the production of a soluble derivative the following reactions were attempted. Before these reactions were attempted the literature was searched. However, only records on reduction of 2,3-dichloro-1,4-naphthoquinone to 2,3-dichloro-1,4-naphthohydroquinone could be found.

A. Reduction

For this reaction the methods of Claus (8) and Graebe (12) were used. Claus's method using dilute stannous chloride was not successful, Graebe's method of reduction with hydriodic acid and red phosphorus was successful. The method follows:

An excess of hydriodic acid in water is added to 2,3-dichloro-1,4-naphthoquinone. Red phosphorus is added and the reaction mixture kept at 50 to 55 degrees Centigrade for 60 to 90 minutes, with stirring. The mixture is then filtered, washed with water, then the naphthoquinone dissolved with alcohol and separated from the phosphorus residue.

On evaporation of the alcohol, or through precipitation with water, 2,3-dichloro-1,4-naphthohydroquinone is obtained. The crude product is then

recrystallized from alcoholic hydriodic acid yielding colorless prisms. These prisms acquire a reddish color on long exposure to air, M.P. 135-140° C. with decomposition (12).

This compound is moderately soluble in hot alcohol, 100 ml. of alcohol at 50 degrees Centigrade dissolving 10 grams. This solubility is much greater than the parent 2,3-dichloro-1,4-naphthoquinone which only dissolves to the extent of 0.256 gram in 100 ml. hot alcohol.

In acetic acid also this prepared 2,3-dichloro-1,4-naphthoquinone is much more soluble than the parent substance, 8.34 grams dissolving in 100 ml. of acetic acid at 50 degrees Centigrade. The solubility of 2,3-dichloro-1,4-naphthoquinone in acetic acid at this temperature is 1 gram in 100 ml.

This prepared 2,3-dichloro-1,4-naphthohydroquinone is insoluble in cold water.

B. Nitration

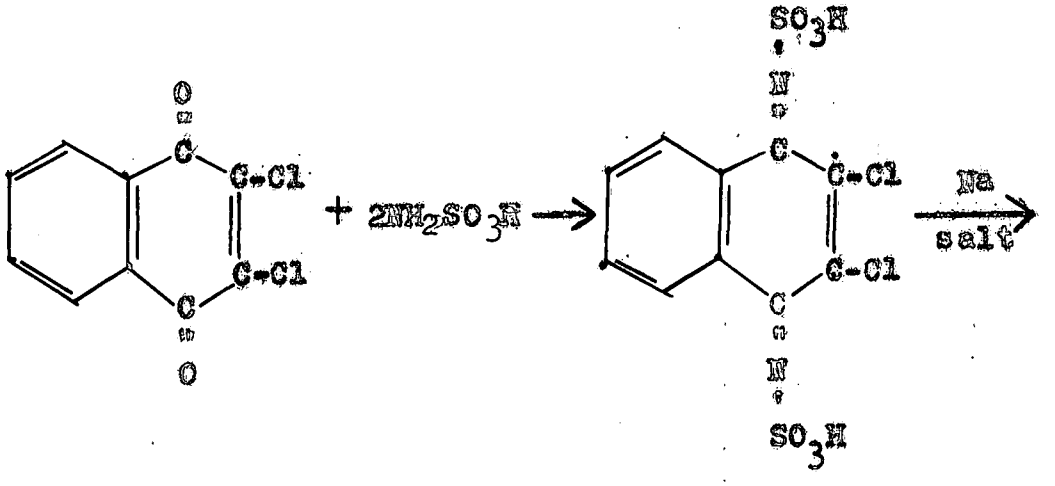
The method of Hartman and Smith (13) for the nitration of beta-acetylaminonaphthalene was followed. The original method called for the addition of 500 ml. glacial acetic acid to 1.62 moles of the compound to be nitrated. The nitrating medium was 200 grams of

concentrated nitric acid added dropwise over a period of 45 minutes

In carrying out this procedure with 2,3-dichloro-1,4-anphthoquinone no evidence of reaction was observed. The starting material was recovered and identified by mixed melting point experiment.

C. Reaction for dioxime formation

Geiger (10) and Potter (24) both found that the nitrogen analogues of the quinones and hydroquinones retain physiological activity. With this in mind it was thought that the following reaction might be profitably attempted.



In the study two molecular proportions of sulfamic acid were added to 2,3-dichloro-1,4-naphthoquinone. This mixture was treated in various ways.

When this mixture was heated in a water bath at fifty degrees Centigrade for eight hours no apparent reaction took place. The melting point of the resulting product was unchanged.

When 2,3-dichloro-1,4-naphthoquinone was first dissolved in hot alcohol and an aqueous solution of sulfamic acid added and this mixture refluxed in a water bath for eight hours a dark-colored solution with some purple-colored needles was obtained. Evaporation off of the alcohol brought down more purple needles. When a Beilstein test for halogen atoms was made on the recrystallized needles a negative test for halogen was obtained. This shows that the reaction led to decomposition.

Mild heat treatment of a mixture of 2,3-dichloro-1,4-naphthoquinone and sulfamic acid in which acetic acid was added as a solvent did not bring about any reaction. Unaltered 2,3-dichloro-1,4-naphthoquinone and sulfamic acid may be recovered from the mixture.

When this mixture was refluxed over a water bath or subjected to more severe heat treatment decomposition of 2,3-dichloro-1,4-naphthoquinone resulted. This was

evidenced by the compound turning dark and by the fact that a negative Beilstein test was obtained.

D. Production of 2-methyl-1,4-naphthohydroquinone-3-sodium sulfonate

In addition to attempts at synthesis of a water soluble derivative of 2,3-dichloro-1,4-naphthoquinone a water soluble derivative of 2-methyl-1,4-naphthoquinone was prepared. The prepared product was 2-methyl-1,4-naphthohydroquinone-3-sodium sulfonate (20). The method followed was that described by Baker, Davis, McElroy and Carlson (2).

Experiments with this prepared compound showed that, mole for mole, it is as potent as 2-methyl-1,4-naphthoquinone against the test organisms in Sabouraud's dextrose media. This compound proved more stable than the commercially available water soluble derivatives of 2-methyl-1,4-naphthoquinone: 4-amino-2-methyl-1-naphthol hydrochloride and 2-methyl-1,4-naphthoquinone diphosphoric acid ester tetrasodium salt. However, when this prepared compound was tested for its activity in the presence of natural food substances it showed but very little activity. This incompatibility with natural food substances rules it out as a potential food preservative.

7. 2,3-dichloro-1,4-naphthohydroquinone

A. Antifungal activity

Tests with this compound against the index food spoilage microorganisms in synthetic media and in natural food substances showed that it has high activity. This activity is summarized in Table 7.

Table 7

Activity of 2,3-dichloro-1,4-naphthohydroquinone

Substrate	Organism	Effective inhibiting concentration in ppm. 28° C.	3° C.
Sabouraud's dextrose broth	Saccharomyces cerevisiae	20	5
Sabouraud's dextrose broth	Aspergillus niger	20	15
Potato dextrose broth	Saccharomyces cerevisiae	10	5
Potato dextrose broth	Aspergillus niger	10	5
Apple juice	Aspergillus niger	5	2
Red raspberry	Native flora	50	40
Prunes in light syrup	Native flora and P. expansum	80	20
Prune juice	Penicillium expansum	20	5
Strawberry juice	Saccharomyces pastorianus	5	2
Grape wine of 7% alcohol content	Saccharomyces cerevisiae	2	2
Grape wine of 7% alcohol content	Saccharomyces ellipsoideus	10	2
Apricot puree	Aspergillus niger	35	10
Bean soup	Aspergillus niger	5	5
Apples in light syrup	Native flora and A. niger	50	20

B. Toxicity trials

(1) Feeding tests

Oral administration of this compound to rats at the rate of one-tenth of one percent of their entire solid ration was well tolerated. Three six-week-old rats were fed on this ration beginning January 2, 1947. After thirteen weeks on this diet the two males weighed 342 and 361 grams respectively. Comparison of their rate of growth with standard growth curves showed that growth had been essentially normal. However, at the end of the feeding trial the fur of the animals had put on a light orange tinge. This orange color in the fur of the males changed back to normal after they were put back on stock ration for 18 days.

On April 24, 19 days after the animals were put back on stock ration, the males were autopsied. No gross pathology was evident nor did sections of the liver, kidney or stomach show any microscopic pathology.

The female, which was a small animal to begin with, also exhibited no toxic symptoms while on this diet. On March 3, sixty days after she was on the diet, she gave birth to a normal litter of nine.

The fur of the female also acquired a slight orange color after she had been on the experimental diet. This color was more persistent on the female and even after 19 days back on stock ration her fur still maintained that orange tinge.

During the entire period of the trial the three test animals ate a total of 5012 grams of their ration. That is, 5.012 grams of 2,3-dichloro-1,4-naphthohydroquinone were taken by the three animals.

(ii) Stomach tube tests

Acute toxicities were also determined in rats by stomach tube administration of the compound suspended in water and alcohol. Ten rats each weighing around 200 grams were used for this experiment. They received 10, 20, 60, 80, and 100 mg. of the compound respectively. Two test animals were used for each level.

Absorption after administrations was rapid since toxic symptoms appeared within 5-10 minutes. The animals receiving the highest dosage (100 mg. per animal) were affected the most rapidly; one animal died two and a half hours after administration and the second the night after administration.

The mean lethal dose was 20 mg. per rat or

approximately 100 mg./kg. since one of the rats receiving 20 mg. died. The rats receiving 10 mg. of the compound appeared normal. Subsequent autopsy of these animals revealed no gross pathology.

Autopsy of the animals killed by the compound showed that the main symptom was hemorrhage. Death was always due to glomerulonephritis, the principal lesions being hemorrhages. In the acute cases there was very evident hemorrhage in the stomach pyloris; evidences of hemorrhage were found in the liver and intestine, though there was no evidence of hemorrhage in the caecum.

C. Assay method for 2,3-dichloro-1,4-naphthohydroquinone

An important consideration in the study of a food preservative or potential food preservative is a method for analysis of the material. From the standpoint of regulatory organizations an assay method is essential. This is easily understandable since without a method of assay there can be no effective control.

For a compound such as 2,3-dichloro-1,4-naphthohydroquinone where minute quantities are required an assay method must be found that is very sensitive. The colorimetric methods are among the most sensitive of

analytical methods since photometers have been developed to such a point that high-precision instruments are available to even modest laboratories. Because of this the literature was searched for a colorimetric method that might be adapted for use on 2,3-dichloro-1,4-naphthohydroquinone. An article appearing in the 1941 issue of "Science" by A. Novelli described a colorimetric assay method for 2-methyl-1,4-naphthoquinone (21). This method was tried on 2,3-dichloro-1,4-naphthoquinone and its reduced form 2,3-dichloro-1,4-naphthohydroquinone. It was found that with the naphthoquinone the index green coloration was not obtained; with the naphthohydroquinone the method was as sensitive as for 2-methyl-1,4-naphthoquinone.

The method as adopted was as follows:

1. 1 ml. of a water suspension of 2,3-dichloro-1,4-naphthohydroquinone was measured into a test tube.

2. Three drops of 0.5% 2,4-dinitrophenylhydrazine in dilute hydrochloric acid (5N) was added.

3. The mixture was heated in a boiling water bath for thirty seconds, then cooled.

4. Three drops of concentrated ammonium hydroxide was added to the cooled mixture. On addition of the ammonium hydroxide the color of the solution changed from yellow to orange.

5. 1 ml. of amyl alcohol was added and the mixture shaken.

In the presence of 2,3-dichloro-1,4-naphthohydroquinone a green color is obtained in the alcohol layer. The intensity of the green color varies quantitatively with the amount of 2,3-dichloro-1,4-naphthohydroquinone present.

2-methyl-1,4-naphthoquinone and xyloquinone also gave this color reaction. However, the three compounds may be differentiated because each gave different color reactions when the ammonium hydroxide was added and prior to the addition of amyl alcohol. 2,3-dichloro-1,4-naphthohydroquinone gave an orange-red color; xyloquinone gave a violet color, and 2-methyl-1,4-naphthoquinone gave a green color.

The sensitivity of this method for 2,3-dichloro-1,4-naphthohydroquinone was 0.005 mg. That is, five parts per million of 2,3-dichloro-1,4-naphthohydroquinone can be detected by this method.

A specific method to differentiate 2,3-dichloro-1,4-naphthohydroquinone from 2-methyl-1,4-naphthoquinone and xyloquinone is to add glacial acetic acid to the ammonium hydroxide-amyl alcohol mixture. The green alcohol layer will turn red-orange if the color were due to 2,3-dichloro-1,4-naphthohydroquinone. If the

green color were due to the other two compounds, a yellow color will be obtained.

Since 2,3-dichloro-1,4-naphthohydroquinone is insoluble in water and very soluble in ether, extracting it from foods is comparatively simple. Ten grams of the food material are taken and extracted with 100 ml. ether. The ether layer is separated and evaporated in a water bath. Ten ml. water is used to wash the residue left after evaporation of the ether, and one ml. of this wash water assayed for 2,3-dichloro-1,4-naphthohydroquinone by the colorimetric method just described.

D. Incompatibilities and potentiating complements

In the study of compounds whose values lie in their activities against microorganisms it is desirable to know their weaknesses as well as their strength. For this reason it is desirable to determine the compounds or substances commonly found in foods that will antagonize or reduce the activity of 2,3-dichloro-1,4-naphthohydroquinone.

Posner (22,23) found that many ketones have the ability to react with sulfhydryl compounds; Klarmann and Wright (16) found that organic matter impaired germicidal performance of disinfectants to more or less extent; and Colwell and McCall (9) found that reducing agents, such

as sodium acid sulfite and sodium pyrosulfate, suppress antibacterial and antifungal properties of 2-methyl-1,4-naphthoquinone. Woolley (28) found that 2-methyl-1,4-naphthoquinone acts to negate the antibacteriostatic and antifungistatic action of 2,3-dichloro-1,4-naphthoquinone.

Following the precedent of these investigators tests were designed to find out to what extent 2,3-dichloro-1,4-naphthohydroquinone is antagonized in its fungistatic activity by organic matter by sulfhydryl compounds and by reducing substances. Table 8 summarizes the results obtained.

In testing the effect of organic matter on fungistatic activity of the test compound two sources of organic matter were used; the organic matter as found in natural strawberry juice and the organic matter as represented by protein preparations. In the first case the ingredients for Sabouraud's dextrose agar were weighed out in the standard amounts and proportions. However, instead of using water as solvent, strawberry juice with a solid content of 8.2% and a pH of 4.2 was used. In the second case a mixture of dry protein was added to Sabouraud's dextrose agar in an amount equal to 5% by weight of the prepared media. The protein mixture consisted of equal parts of cystine, casein, yeast extract,

and tryptone.

In testing the effect of sulfhydryl and reducing compounds on the fungistatic activity of 2,3-dichloro-1,4-naphthohydroquinone, 2-molecular concentrations of sodium thioglycollate, cystine hydrochloride, sodium thiosulfate and sodium acid sulfite respectively were added to 50 milligrams of 2,3-dichloro-1,4-naphthohydroquinone and the whole diluted to 50 milliliters. Appropriate amounts of these binary mixtures were then measured into sterile petri dishes and their antifungal potency determined by the agar-streak method (27).

As may be seen from Table 8 thioglycollate and protein are most antagonistic to 2,3-dichloro-1,4-naphthohydroquinone. However, this antagonism varies with different organisms.

Strawberry juice enhanced the fungistatic value of 2,3-dichloro-1,4-naphthohydroquinone probably because of the lowering of the pH. It has previously been found that 2,3-dichloro-1,4-naphthohydroquinone is more active at lower pH's.

Cystine hydrochloride, sodium thiosulfate and sodium acid sulfite did not reduce the activity of the compound to a very great extent. These findings bear out those of Colwell and McCall (9) who observed that naphthoquinones with an attached 3-position are more stable as

Table 8

Effect of 2,3-dichloro-1,4-naphthohydroquinone singly and in the presence of organic matter, sulfhydryl and reducing compounds on the growth of Saccharomyces cerevisiae, S. pastorianus, and Aspergillus niger.

TEST MIXTURE	Concentration of 2,3-dichloro-1,4-naphthohydroquinone required to inhibit growth of the organisms: S. pastorianus	S. cerevisiae	A. niger
	ppm	ppm	ppm
2,3-dichloro-1,4-naphthohydroquinone alone	10	10	15
In presence of 5% mixed protein	60	30	15
In presence of strawberry juice	5	5	5
In presence of sodium thioglycollate	Ineffective at 100 ppm/		
In presence of cysteine hydrochloride	30	10	15
In presence of sodium thiosulfate	20	20	30
In presence of sodium acid sulfite	15	10	20

antimicrobial agents than those naphthoquinones whose three-position is free.

While on the subject of incompatibilities and antagonisms it is well to note also that there are substances which potentiate the activity of 2,3-dichloro-1,4-naphthohydroquinone.

As has been stated in an earlier page the activity of 2,3-dichloro-1,4-naphthohydroquinone is increased at lower pH's. It has been observed that acetic acid increases its activity, and to an extent much in excess to that which might be attributable to a lowering of the pH. Table 9 will show how acetic acid potentiates the fungistatic activity of 2,3-dichloro-1,4-naphthohydroquinone against Aspergillus niger in Sabouraud's dextrose broth.

Table 9

Potentiating activity of acetic acid for 2,3-dichloro-1,4-naphthohydroquinone against Aspergillus niger in Sabouraud's dextrose broth

Concentration of acetic acid added to Sabouraud's dextrose broth	pH	Inhibiting concentration of 2,3-dichloro-1,4-naphthohydroquinone
ppm		ppm
0	6.80	15
0	4.62#	15
0	4.48#	15
180	4.65	10
250	4.55	5
350	4.55	5
500	4.48	2

#pH adjusted with HCl

DISCUSSION

This paper attempts to present in a concise manner the various experiments undertaken and performed toward a study of potential preservatives that might be of use in the food industries.

The very nature of the project necessitated a diversified approach that has delved into the realms of bacteriology, organic chemistry, and toxicology. On reading this paper a critic may say that it is nothing but a hodgepodge of minor experiments none of which has been outstanding and none of which has yielded any result of importance. Indeed he may go on to say that these experiments have been of a superficial nature, that none of them has been pushed to completion. From the bacteriological point of view he might ask why were not more test organisms used and why that group of very important food spoilage microorganisms, the bacteria, was not included in the study?

From the organic chemistry point of view the critic may ask why were there not more attempts made at synthesis, why was no study made of the physiological disposition of the promising chemical when fed to animals? In the work on synthesis why were the compositions of the products not determined?

From the toxicology point of view why were not more animals used? Why was there no experiment set up to determine the minimum lethal dose or chronic toxicity by mouth of the chemicals?

The answer to all these questions is that time and facilities have been limited. A project of this sort may properly be undertaken by a number of collaborating laboratories, each specializing in its own field of bacteriology, organic chemistry, pharmacology, and toxicology.

When this project was first undertaken it was realized that it would entail techniques in bacteriology, organic chemistry, and toxicology. Nevertheless it was decided to undertake an exploratory study and to find out what could be accomplished in the time available.

Answering the questions raised by the hypothetical critic, it may be stated that more test organisms were not used because the six yeasts and molds chosen as index organisms have been cited in the literature as hardy and were therefore regarded to represent their groups. Bacteria were not included because the first aim of this study was to deal with foods to be held at cool storage temperatures, especially foods for remanufacture. Under these conditions, and among this particular class of foods, molds and yeasts are the predominant spoilage

organisms.

Looking over what has been accomplished in this study it can be said that it has uncovered a compound that may have great possibilities in the food field. This compound should also be of interest to the pharmacologist and to the toxicologist.

2,3-dichloro-1,4-naphthoquinone, the parent compound from which the reduced naphthohydroquinone was derived, has been found by Woolley (28) and in this experiment to be very toxic. This compound antagonized the bacteriostatic and fungistatic activities of synthetic vitamin K, 2-methyl-1,4-naphthoquinone (28). However, animals poisoned by this compound did not show signs of hemorrhage macroscopically as did the animals fed an excess of the reduced form. The question then raised is whether the naphthohydroquinone structure is hemorrhagic whereas the naphthoquinone structure poisons by blocking other enzyme systems? If by merely reducing the naphthoquinone to the naphthohydroquinone form the mode of action of a compound in the animal body is entirely changed without impairing its antimicrobial activity, the possibility exists that by some other changes a poisonous compound may be made nonpoisonous. In this respect the preparation of the imine or the oxime forms would prove of great interest.

The quinones and naphthoquinones offer great challenge to the investigator of food preservatives. There are many quinones and naphthoquinones among the naturally occurring antibiotics. Among these are fumagatin, spinulosin, citrinin, actinomycin, and puberulic acid. In this connection it is interesting to note that in May 1946, Raab (25) reported on the antibacterial action of phenanthrene-related substances, and a few months later investigators in Italy followed with a report that phenanthrene-quinone was so superior in its antibacterial effect that further studies with related compounds were planned (17).

SUMMARY

1. The inhibitory action of 38 chemical compounds on the growth of representative food spoilage yeasts and molds was determined quantitatively.

2. 2,3-dichloro-1,4-naphthoquinone, a commercial fungicidal spray, has been found to have powerful inhibitory and lethal activity against the test organisms both in synthetic media and in natural food substances.

3. 2,3-dichloro-1,4-naphthoquinone was found to have two properties which do not adapt the compound for use as a preservative in foods. When fed to test animals (rats) it proved highly toxic; it is insoluble in water and thus presents an application problem.

4. Systematic modification of the structure of 2,3-dichloro-1,4-naphthoquinone produced the reduced form: 2,3-dichloro-1,4-naphthohydroquinone. This compound proved superior to the parent compound in the following respects: (1) Oral administration of this compound to rats at the rate of one-tenth of one percent of the entire solid ration was tolerated without toxic symptoms. The males showed normal gains during the entire thirteen-week period of the test. The female, while on the experimental diet, produced a litter of nine in due course of time, and the offspring were normal. (2) This compound was much more soluble than the parent naphthoquinone;

thirty-nine times more soluble in alcohol, and eight times more soluble in acetic acid. (3) The new compound was as powerful an anti-fungal agent as the parent naphthoquinone.

5. 2,3-dichloro-1,4-naphthohydroquinone was also found to be insoluble in water.

6. In order to produce a more water-soluble derivative of this compound the following reactions were attempted:

a. Nitration

b. Production of the dioxime form

7. The reactions could not be made to take place.

8. Synthetic vitamin K, 2-methyl-1,4-naphthoquinone, was found to possess high antifungal properties.

9. A water-soluble derivative, 2-methyl-1,4-napthohydroquinone-3-sodium sulfonate, was prepared. This prepared compound was also highly active against the test organisms in synthetic media. However, this activity was found to be markedly reduced in the presence of natural food substances.

10. Studies on a method of assay for 2,3-dichloro-1,4-naphthohydroquinone showed that 0.005 mg. of this compound may be detected by a comparatively simple color reaction. This method can be adapted to a quantitative test and is carried out as follows: A water suspension

of the naphthohydroquinone is mixed with a solution of 2,4-dinitro-phenyl-hydrazine in dilute hydrochloric acid and is gently heated. A green color is then produced with ammonia and amyl alcohol. The color produced is stable. Means are described for differentiating the various naphthoquinones and quinones which give this color reaction.

11. It was pointed out that a project of this sort may properly be undertaken by a number of collaborating laboratories, each specializing in its own field of bacteriology, organic chemistry, or toxicology.

12. The interesting aspects of the physiological and preservative action of the chemical homologues are mentioned.

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