Report on Methyl paraben & Propyl paraben 8/31/72

MONOGRAPH
ON
METHYL PARABEN
AND
PROPYL PARABEN

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METHYL PARABEN

and

PROPYL PARABEN

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METHYL PARABEN
and
PROPYL PARABEN

Summary

In a study by Matthews et al. in which dogs were given, orally, 1.0 g methyl or propyl paraben/kg body weight/day for one year, between 96% and 100% of the daily administered dose was accounted for in the urine, chiefly in the form of conjugated end products. They observed that the parabens are readily and completely absorbed from the gastrointestinal tract (162).

Schuebel and Manger reported that the first step of paraben detoxication is saponification, resulting in formation of PHBA. They suggest that most of this acid is decomposed in the body, the benzene ring being broken, while the rest is eliminated through the kidneys in the form of salts or as free acid. They found no evidence of accumulation of the parabens or their metabolites in the animal body (262).

Jones et al. found no accumulation of methyl or propyl paraben or their metabolites in dogs which had been given 1.0 g/kg/day for one year. They were able to account for 96% of the daily administered dose in the urine in the form of free or conjugated PHBA. In vitro studies using esterases found in the liver and kidneys implicated those organs as the sites of paraben detoxication. By giving a fasted man 70 mg of methyl paraben/kg body weight, they determined that the paraben is handled similarly in human metabolism (136).

In a study by Sabalitschka and Neufeld-Crzellitzer in which they gave a human subject 2 g of propyl paraben daily for 5 days, they found that 17.4% of the administered dose was excreted in the form of PHBA. They computed that about 55% of the paraben was excreted paired with sulfuric acid. It was suggested that breaking of the benzene ring takes place to some extent during detoxication (249).

Nathan and Sears reported that methyl paraben, when applied directly to the nerve, blocks nerve conduction (180).

Bubnoff et al. found that i.v. and orally administered methyl paraben exhibits an anticonvulsive effect in rats with cocaine-induced cramps (39).

There have been relatively few feeding studies on the toxic effects/levels of the paraben esters, either methyl or propyl; although there is quite a bit of data available on the acute toxicity of these compounds. This body of scientific literature is unique in two senses: first, it is primarily German and Italian; second, the literature entries are cross-referenced and all too frequently quoted without citation so that despite the large number of entries in the literature, there is really very little information available.

Short-term feeding studies have been undertaken with methyl paraben on rats, rabbits, cats, dogs, and man; similar studies involving propyl paraben have been conducted on rabbits, dogs, and man. Long-term studies involving both of these esters have been conducted with rats as test subjects.

Bijlsma reported in 1928 on growth depression in rats fed methyl paraben at 200 and 500 mg/kg/day for an unspecified period; however, he was unable to repeat these results (24). Schuebel and Manger reported that rabbits withstood 500 mg of methyl paraben/kg/day for 6 days; but, when this level was raised by 1000 mg/kg per day, the rabbits were affected by the 9th day at a level of about 3000 mg/kg/day (262). In contrast to this, they mentioned that a cat fed methyl paraben at 500 mg/kg/day for 6 days became ill immediately upon challenge and remained so for the duration of the dosage (262). Bijlsma found that a dog fed methyl paraben at 105 mg/kg/day for 4 days was unaffected (24), and Schuebel and Manger found that this dosage could be increased to 500 mg/kg/day for 7 days with no effect on dogs (262); however, when this edosage was increased to 2000-3000 mg/kg/day, the dogs were dramatically affected (262). Matthews, et al., in the definitive short-term study on methyl paraben showed that dogs fed 500 and 1000 mg/kg/day were unaffected after 1 year on the regimen (162). Sabalitschka, et al., report that a man was unaffected after taking 2000 mg of methyl paraben daily for 1 month (249); Bracessi confirmed these observations (35).

Schuebel and Manger gave rabbits 500 mg of propyl paraben/kg/day for 6 days, and these animals showed no ill effects (262). However, at a level of 3000 mg/kg/day, there was evidence of toxicity (262). They also reported that dogs showed ill effects only at levels of 400 mg of propyl paraben/kg/day and higher (262). Matthews, et al., reported that propyl paraben fed at dosages of 500 and 1000 mg/kg/day for 1 year to dogs resulted in no untoward effects (162). Sabalitschka, et al., report that a man was unaffected after taking 2000 mg of propyl paraben daily for 1 month (249).

Long-term feeding of both methyl and propyl paraben to rats by Matthews, et al., revealed that, at dosage of 1000 mg/kg/day for 96 weeks, neither methyl nor propyl paraben showed toxicity for rats (162). At a level of 4000 mg/kg/day for 96 weeks, both esters definitely depressed the growth rate of rats; although the depression was more noticeable in the male rats (162). Sokol reported that propyl paraben stimulated growth in rats when fed at a dosage of 150 mg/kg/day for 18 months, but at a dosage of 1500 mg/kg/day for the same period, a growth depression without specific pathology resulted (272).

In a report submitted by the Heyden Chemical Company, it was found that a mixture of paraben esters (60% propyl - 40% ethyl) fed to rats over a period of 18 months at levels of 14.3 and 143 mg/kg/day resulted in a slight growth increase. At a level of 1430 mg/kg/day for 18 months, this same mixture depressed growth from month 4 to month 8; however, over the entire period of the study, growth of control and test rats was comparable at this level (8).

Boyland and Homburger both report that methyl paraben does not exhibit carcinogenic activity (34, 121). Matthews, et al., failed to show that guinea pigs became sensitized to either methyl or propyl paraben, but they did show that methyl paraben solutions in propylene glycol exerted an irritant action at a concentration above 5%; propyl paraben showed the same effect at concentrations above 12% (162).

There have been several reports in the literature on paraben dermatitis. The consensus seems to be that these paraben esters can cause a dermatitis in susceptible individuals which may be diagnosed with great difficulty as a result of the cross-reactivity of the parabens. Reportedly, this situation is more common in Europe where the parabens are permitted in higher concentrations (260, 255, 83).

B. Food Grade
See C

C. Food Chemicals Codex

Assay	Not less than 99% $C_8H_8O_3$
Melting Range	125° and 128°
Limits of Impurities	
Acidity	Passes Test
Arsenic (as As)	Not more than 3 ppm
Heavy metals (as Pb)	Not more than 10 ppm
Residue on ignition	Not more than 0.05%

VI. Description

A. General Characteristics

Small, colorless crystals or a white, crystalline powder. It is odorless or has a faint characteristic odor and has a slight burning taste.

B. Physical Properties

Melting point	125° - 128°
Boiling point	270° - 280°

Solubility

Solvent	g/100 g solvent
Water, 25°C	0.25
Water, 15°C	0.16
Water, 80°C	3.2
Ethanol, 25°C	52.0
Ethanol, 10%, 25°C	0.5
Ethanol, 50%, 25°C	18.0
Propylene glycol, 25°C	22.0
Propylene glycol 10%, 25°C	0.3
Propylene glycol 50%, 25°C	2.7
Glycerin, 25°C	1.7
Peanut Oil, 25°C	0.5
Ether	14.2

Methyl paraben is also soluble in acetone, slightly soluble in fixed oils, benzene, and carbon tetrachloride.

C. Stability

Methyl paraben should be stored in tight containers. Since esters of p-hydroxybenzoic acid are hygroscopic, their containers should be stored in a dry place.

VII. Analytical Methods

Genest and Chapman developed a method for extracting preservatives from various foods. Liquid-liquid extraction is performed with chloroform, evaporated and dissolved in ethanol. The solution is then analyzed by paper chromatography. Methods are described for extraction from catsup,

METHYL PARABEN

Chemical Information

I. Nomenclature

A. Common Name
Methyl paraben

B. Chemical Names
1. Methyl p-hydroxybenzoate
2. p-Hydroxybenzoic acid methyl ester

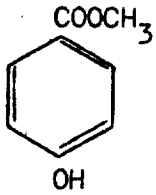
C. Trade Names
1. Methyl parasept
2. Nipagin M
3. Tegosept M

D. Chemical Abstracts Services Registry Number
000099763

II. Empirical Formula

$C_8H_8O_3$ $(CH_3OOCC_6H_4OH)$

III. Structural Formula



IV. Molecular Weight

152.14

V. Specifications

A. Chemical (USP)

Appearance	Small crystals, or white powder
Odor	Faint, Characteristic
Assay (dry basis)	99% Minimum
Melting Range	125°C - 128°C
Acidity	Slightly acid to litmus
Loss on drying (5 hrs. over silica gel)	0.5% Maximum
Residue on ignition	0.05% Maximum
Chloride	350 ppm Maximum
Sulfate	200 ppm maximum

jellies, jams, fruit drinks, margarine, cheese, and preserved fish (101).

Hoyem developed a method using paper chromatography and ultraviolet spectrophotometry. The spotted filter paper is placed in a chromatography tank containing n-butanol, ammonia and water (5:2:3) for 20 hours. The paper is then exposed to an ultraviolet lamp for development of the spots. This procedure can be quantitated by eluting the spots with either 0.01 N HCl or 0.01 N NaOH and measuring at Lambda-max on a UV spectrophotometer (124).

Thin-layer chromatography (TLC) has been extensively used to separate and identify the parabens. Rangone and Ambrosio used silica plates and developed them in borate buffer, adding a given volume of organic solvent as required. The following buffer-solvent combinations have proved most suitable for separation of the various preservatives; buffer-methyl alcohol (90:10), buffer-ethylcellosolve (90:10), buffer-ethyl acetate (saturated solution at 25°), buffer-ethyl ether (saturated solution at 15°), buffer-tetrahydrofuran (95:5), buffer-dioxane (90:10). The spots were then analyzed using a 250 millimicron UV source. The spots were removed with methanol using a vacuum technique and were than quantitated using UV spectrophotometry. The coefficient of variation was 3% for the straight-line determination (211). Another TLC system has been reported with polyamide-silica gels used as the stationary phase and solvents such as n-hexane-benzene-glacial acetic acid (1:1:1), water-28% ammonia solution (20:5). This method can be used to identify the parabens, sorbic acid, and dehydroacetic acid (49).

Gas chromatography has been used to detect methyl p-hydroxybenzoate and separate it completely from a mixture of sorbic acid, benzoic acid, salicylic acid, dehydroacetic acid, menadione and ethyl, butyl, and propyl p-hydroxybenzoate without any pretreatment. The column was packed with 30% DC 550 silicone on celite 545, the column temperature was 190° and the carrier gas was hydrogen (127).

Parabens present in urine and body tissues may be determined by a method using 4-amino-antipyrine. A buffered solution of the sample was centrifuged under conditions appropriate to the type of sample, 4-amino-antipyrine and potassium ferrocyanide. The color formed was measured at 490 millimicrons (136). This method was simplified by Inamine, Obatake and Matsuda (128).

VIII. Occurrence

Methyl paraben is produced by esterifying p-hydroxybenzoic acid with methanol, using an acid catalyst such as sulfuric acid and an excess of methanol. The materials are heated in a glass lined reactor under reflux. The acid is then neutralized with caustic soda, and the product crystallized by cooling, centrifuged, washed, dried under vacuum, milled and blended, all in corrosion resistant equipment to avoid metallic contamination.

PROPYL PARABEN

Chemical Section

I. Nomenclature

A. Common Name
Propyl paraben

B. Chemical Names
1. Propyl p-hydroxybenzoate
2. p-Hydroxybenzoic acid propyl ester

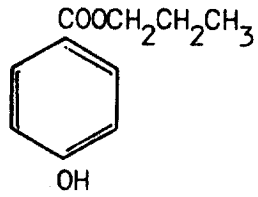
C. Trade Names
1. Nipasol
2. Chemocide PK
3. Propyl Chemosept
4. Solbrol P
5. Propyl Parasept

D. Chemical Abstracts Service Registry No.
000094133

II. Empirical Formula

$C_{10}H_{12}O_3$

III. Structural Formula



IV. Molecuair Weight

180.20

V. Specifications

A. Chemical USP

Appearance	small crystals, or white powder
Odor	none
Assay	99% minimum
Melting range	95-98 deg. C
Acidity	acid to litmus
Loss on drying (5 hrs. over silica gel)	0.5% maximum
Residue on ignition	0.05%
Chloride	350 ppm maximum
Sulfate	200 ppm maximum

B. Food

See A above

C. Food Chemicals Codex

Assay	Not less than 99% $C_{10}H_{12}O_3$
Melting range	Between 95° - 98°
Limits of Impurities	
Acidity	Passes test
Arsenic	Not more than 5 ppm (0.0003%)
Heavy metals (as Pb)	Not more than 10 ppm (0.001%)
Residue on ignition	Not more than 0.05%

VI. Prescription

A. General Characteristics

Propyl paraben appears as small, colorless crystals or a white powder.

B. Physical Properties

Melting point 96-97°

Solubility Data

Solvent	Solubility (g/100 g solvent)
Water, 25°C	0.04
Water, 15°C	0.023
Water, 80°C	0.45
Water, 100°C	2.5
Ethanol, 25°C	95.0
Ethanol, 10%, 25°C	0.1
Ethanol, 50%, 25°C	18.0
Propylene Glycol, 25°C	26.0
Propylene Glycol, 10%, 25°C	0.06
Propylene Glycol, 50%, 25°C	0.9
Glycerin, 26°C	0.4
Peanut Oil, 25°C	1.4
Ether	33.3

C. Stability

Should be stored in tight containers.

VII. Analytical Methods

See Methylparaben

VIII. Occurrence

Propyl paraben is produced by esterifying p-hydroxybenzoic acid with n-propanol, using an acid catalyst such as sulfuric acid and an excess of propanol. The materials are heated in a glass lined reactor under reflux. The acid is then neutralized with caustic soda, and the product crystallized by cooling, centrifuged, washed, dried under vacuum, milled and blended, all in corrosion resistant equipment to avoid metallic contamination.

METHYL PARABEN

Biological Data

I. Acute Toxicity

Substance	Animal	Rt. of Admin.	Dosage (mg/kg)	Determination	Ref.
Methyl Paraben	Mouse	S.C.	333	LD_{50}	24
Methyl Paraben	Mouse	S.C.	125	LD_{50}	121
Methyl Paraben	Mouse	S.C.	1200	LD_{50}	2
Methyl Paraben	Rat	I.P.	960	LD_{50}	272
Methyl Paraben	Rat	P.O.	8000	LD_{50}	272
Methyl Paraben	Rabbit	P.O.	3000	LD_{100}	261
Methyl Paraben	Dog	P.O.	3000	LD_{100}	261

II. Short-Term Studies

Rats

Litters of young rats were divided into equal control and test groups. The test groups were fed 250 mg/kg or 500 mg/kg/day of methyl paraben for an unreported period of time. Growth of these rats was retarded, and the animals died during a cold spell when laboratory heating system failed. Despite repeated attempts, Bijlsma could not repeat these results (24).

Rabbits

Rabbits were fed methyl paraben at a dosage initially of 500 mg/kg/day for 6 days. On the 7th day through the 10th day, the level was raised 1000 mg/day. Rabbits were definitely affected by the 9th day and exhibited somnolence and ataxic motions within several hours of dosage with methyl paraben. Schuebel and Manger reported that the 3000 mg/kg level was toxic for these rabbits (262).

Cat

A white cat weighing 2.7 kg was fed methyl paraben at a dosage of 500 mg/kg/day for 6 days. The animal exhibited nausea, vomiting, and a general malaise within 15 minutes of dosage and remained in this condition throughout the duration of the 6 day dosage period (262).

Dogs

A dog fed 105 mg of methyl paraben/kg/day for 4 consecutive days showed no pathologic effects (24). Dogs fed methyl paraben at a dosage of 500 mg/kg/day for 7 days exhibited no toxic effects, but when this dosage was increased to 2000 or 3000 mg/kg/ they were affected (vomiting, tumors, etc.) (262).

Shuebel and Manger as well as most of the other workers reporting on the parabens tend to make statements that give the impression that conclusions on toxicity of these compounds are based on large numbers of observations. However, in the literature, data presented pertain to very few test subjects, frequently only a single animal. An additional problem in interpretation of some, if not most, of this literature is that results and procedures are not specified to the degree desirable to evaluate. This is particularly true of histological findings.

A group of 7 mongrel pups (5 test, 2 control) were housed individually and fed a dry diet ad lib supplemented with meat scraps. Six days weekly for 1 year, 3 of the dogs were given methyl paraben at a dosage of 1000 mg/kg/day and 2 were given 500 mg/kg/day in the form of a gelatin capsule. One month from the end of the experiment, blood samples were taken and determined for accumulation of methyl paraben (162).

No toxic symptoms were observed in any of the animals. All blood samples were normal. One of the females receiving 500 mg/kg/day was mated toward the end of the experiment and delivered a litter of healthy pups. All dogs were killed and autopsied. No abnormalities were found on macroscopic and microscopic examinations of the organs (162).

Man

Sabalitschka, et al., reported that a volunteer ingesting methyl paraben at a dosage of 2000 mg/day for 1 month was unaffected (249). Bracessi reported that he took 2000 mg of methyl paraben/day and concluded, after increasing this dosage over an indefinite period, that methyl paraben was essentially harmless to man (35).

III. Long-Term Studies

Rats

Two groups of 24 (12 male, 12 female) weanling Wistar rats were caged in groups of 4 and, upon maturity, separated so that there were only 2 animals/cage. Food and water were provided ad lib. One group was fed a stock diet, the other group a diet containing 2% methyl paraben. Food intake and weight were checked every 2 weeks (162).

After 40 weeks, no effects on growth were noted. This experiment was terminated after 96 weeks, and another experiment identical in set-up to the first (except for the inclusion of an 8% methyl paraben level) was initiated. This experiment also lasted 96 weeks (162).

Rats receiving 8% methyl paraben in the diet showed a slower rate of weight gain than did control animals. This difference was marked during the early part of the experiment and tended to dissappear later; the effect was more marked in males than in females. Animals receiving 2% methyl paraben were indistinguishable from controls. It would appear, according to Matthews, et al., that the toxic threshold for methyl paraben is at least 3000 mg/kg/day (162).

IV. Special Studies

Carcinogenic

A test group of 20 weanling female mice was administered 0.1 ml of a 1% methyl paraben solution in Carbowax 1000 (polyethylene glycol) twice weekly into the vagina by means of a syringe/blunt needle apparatus. A control group of 20 weanling mice were administered 0.1 ml of Carbowax 1000 similarly. The regimen was continued for 18 months, at which time all mice were sacrificed (34).

Eleven control animals survived 12 months, and 7 survived the full 18 months. Carcinomas were initiated in 5 mice. Of the methyl paraben-treated mice, 16 survived 12 months, and 8 survived the full 18 months. There were no carcinomas in any of these animals (34).

Other substances of known carcinogenic activity were also tested (DMBA). Although the test showed that carcinomas resulted from compounds not generally regarded as having carcinogenic activity, the significance is that methyl paraben did not initiate any carcinomas (34).

A carcinogenic study reported by Homburger which involved several techniques for ascertaining carcinogenicity, i.e., subcutaneous injection/secondary host transfer, intravenous injection/observation of lung adenomas, and co-carcinogenesis, did not show that methyl paraben had carcinogenic activity (121).

Sensitization

Ten male guinea pigs fed a stock diet supplemented with greens had hair removed (clipped) from back and flanks. A solution of 0.1% methyl paraben in physiological saline was prepared and injected 3 times weekly in the back and upper flanks. A total of 10 injections was given; the first injection was 0.5 ml; subsequent injections were 0.1 ml. Two weeks after the 10th injection a retest injection was made using 0.05 ml of a freshly prepared solution. Twenty-four hours later, the animals were observed for a reaction. There was no sensitization evident (162).

Methyl paraben was dissolved in propylene glycol in increasing concentrations and applied to the skin of 50 human subjects. Methyl paraben, when applied from 4-8 hours every other day for 10 applications, produced no irritation at the 5% level; however, higher concentrations than 5% did produce irritation (162).

Studies on the contact of aqueous solutions of methyl paraben with the conjunctiva showed that an aqueous solution less than 0.2% methyl paraben produced no effect in the rabbit. A 0.2% solution produced a slight, transitory conjunctival hyperemia (270).

In man, aqueous solutions of 0.1-0.3% produced a moderate hyperemia accompanied by slight lachrymation and a sensation of slight burning. These symptoms disappeared within approximately 1 minute. This regimen was repeated several times a day in more than 100 patients without resulting in any inconveniences to test subjects (270).

Schorr reports that, since 1966, there have been 8 cases of paraben allergy reported from the US in the scientific literature. The importance of parabens as a cause of long-standing, progressive, and severe iatrogenic contact dermatitis rests not in incidence of disease production but rather in the insidious pattern and ease with which paraben allergy can be mis-diagnosed. Patients suffering from paraben allergy show cross-sensitivity to the 4 common paraben esters (methyl, ethyl, propyl, and butyl) when given a patch test (260).

Schamberg reports that an additional factor creating difficulty in recognizing paraben sensitivity is that the patch test may be negative to paraben-containing steroid creams as a result of the anti-inflammatory effect of corticosteroid. In Europe, the incidence of paraben sensitivity is much higher, possibly as a result of greater allowable concentration under their laws governing preservatives (255).

Biological Data

I. Acute Toxicity

Substance	Animal	Rt. of Admin.	Dosage (mg/kg)	Determination	Ref.
Propyl Paraben	Mouse	S.C.	1650	LD_{50}	2
Propyl Paraben	Rat	P.O.	8000	LD_{50}	272
Propyl Paraben	Rat	I.P.	640	LD_{50}	272
Propyl Paraben	Rabbit	P.O.	6000	LD_{100}	261
Propyl Paraben	Dog	P.O.	6000	LD_{100}	261

II. Short-Term Studies

Rabbits

Rabbits given propyl paraben per os at a dosage of 500 mg/kg/day for 6 days showed no ill effects. The first toxic effects appeared distinctly at a level of 3000 mg/kg/day (262).

Dogs

A dog administered propyl paraben daily in the diet over a 7 day period at increasing dosages from 1000-4000 mg/kg/day showed effects attributable to propyl paraben toxicity only at the 4000 mg/kg/ level (262).

A group of 6 mongrel pups (4 test, 2 control) were housed individually and fed a dry diet ad lib supplemented with meat scraps. Six days weekly for 1 year, 3 of the dogs were given 1000 mg of propyl paraben/ kg/day, and one was given 500 mg/kg/day in the form of a gelatin capsule. One month before the end of the experiment, blood samples were taken from the dogs at the 1000 mg/kg/day dosage and determined for accumulation of propyl paraben (162).

No toxic symptoms of any kind were observed. Blood samples revealed no trace of propyl paraben accumulation (162).

Man

Sabalitschka, et al., report that a volunteer ingesting propyl paraben at a dosage of 2000 mg of propyl paraben/man/day for 1 month resulted in no visible toxic effects (249).

III. Long-Term Studies

Rats

Two groups of 24 (12 male, 12 female) weanling Wistar rats were caged in groups of 4, and, upon maturity, separated so that there were only 2 animals to a cage. Food and water were provided ad lib. One group was

fed a stock diet, the other group a diet containing 2% propyl paraben. Food intake and weight was checked every 2 weeks (162).

After 40 weeks, no effects on growth were noted. The experiment was terminated after 96 weeks. Another experiment, identical to the first, was set up in which the dosage levels of paraben were 2 and 8%, and this experiment was also run for 96 weeks (162).

At the conclusion of each experiment, all animals were sacrificed, and kidney, liver, heart, lung, spleen, and pancreas tissues were removed for pathologic study. Rats dying during the course of the experiment were autopsied (162).

Animals receiving 8% propyl paraben in the diet showed a slower rate of weight gain than did controls. This difference was marked during the early phase of the experiment and tended to disappear later; it was also more noticeable in males than in females. Animals receiving the 2% dosage were indistinguishable from controls. Matthews opined that the first signs of toxicity would not be evident until an intake of at least 3000 mg/kg/day had been reached (162).

Propyl paraben fed to rats over an 18 month period at 150 mg/kg/day resulted in no ill effects and possibly some growth stimulation. At 1500 mg/kg/day growth depression resulted, but no irregular or pathologic changes could be found (272).

Weanling albino rats were divided into 4 test groups and were treated as follows: (8)

Group I Forty rats were fed a mixture of 60 parts propyl paraben and 40 parts ethyl paraben at a level of 14/mg/kg/day for an 18 month study.

Group II Twenty rats were fed this mixture at a level of 140 mg/kg/day for 18 months.

Group III Twenty rats were fed this mixture at a level of 1400 mg/kg/day for 18 months.

Control Twenty rats were fed a stock diet for 18 months.

At the end of 2 months, 10 group I animals were sacrificed for gross pathology, and 3 were selected for histologic study. At the end of 4 months, 10 additional rats were sacrificed, and 3 were examined histologically (lungs, liver, spleen, stomach, small intestine, kidney, and adrenals) (8).

Group 2, 3, and control animals were continued throughout the 18 month period. At the end of the experiment, all of the animals were sacrificed and 3 animals from each group were examined histologically (8).

All animals were weighed weekly, and gross pathologic and histologic examination results were recorded. The animals were fed the ethyl-propyl paraben mixture daily with the dosage level adjusted to their previous weekly weight (8).

The mixture resulted in an increase in growth rate at the 14 mg/kg/day and the 140 mg/kg/day level over the 18 month period. At the highest level, 1400 mg/kg/day, the mixture resulted in a growth depression by the 4th month. By the 8th month, growth rate of the rats at the highest dosage level was comparable with that of the control rats. This may have been indicative of a tolerance factor, or it may have been the result of age/metabolic change in the test animals (8).

Histological (pathological) findings revealed that, even at a level of about 1430 mg/kg/day for 18 months, there were no significant differences among test rats and control rats (8).

IV. Special Studies

Sensitization

Ten male guinea pigs fed a stock diet supplemented with greens had hair clipped from their backs and flanks. A solution of 0.1% propyl paraben (Na salt) in physiological saline was prepared and injected 3 times weekly into the back and/or upper flanks. A total of 10 random injections was given; the 1st injection was 0.05 ml, the remaining were 0.1 ml. Two weeks after the 10th injection, a retest injection was made using 0.05 ml of a freshly-prepared solution. Twenty-four hours later, the guinea pigs were observed for a reaction. No sensitization reaction to propyl paraben was observed (162).

Irritation Effect

Propyl paraben was dissolved in propylene glycol in increasing concentrations and applied to the skin of 50 human subjects. Propyl paraben, when applied from 4-8 hours every other day for 10 applications, produced no irritation at the 12% level; however, concentrations higher than 12% resulted in some irritation (162).

PARABENS

Biochemical Aspects

I. Breakdown

No information available from sources obtained.

II. Absorption-Distribution

Orally administered methyl and propyl paraben are readily and completely absorbed from the gastrointestinal tract. Matthews found that the use of the sodium salts appears to enhance absorption, since the fatal dose for the sodium salts is significantly lower than when the free ester is used (162).

III. Metabolism and Excretion

In vitro studies using esterases found in the liver and kidneys implicate those organs as the sites of paraben detoxication (136).

Oral administration of 100 mg of methyl or propyl paraben to rats (av. wt. 200 g) resulted in rapid detoxication, with metabolites appearing in the urine within one half hour after ingestion. Metabolites identified were p-hydroxybenzoic acid, p-hydroxyhippuric acid, an ester and ether glycuronides, and an ethereal sulphate. Methyl paraben was not detected in the urine. The concentration of phenolic acid in the blood remained extremely low throughout the experiment (71).

The metabolism of methyl and propyl paraben was investigated in rabbits by oral administration of a single dose of 0.4 or 0.8 g/kg. Thirty-nine percent of the administered methyl paraben was excreted as free p-hydroxybenzoic acid (PHBA), while the rest appeared as glycine (15%), glucuronic acid (ester- 7%, ether- 15%), and sulfuric acid (10%) conjugates of PHBA. Urinary excretion of the metabolites was rapid, 86% in 24 hours. With propyl paraben, 30% of the dose was excreted as free PHBA, while the glycine, glucuronic acid (ester and ether), and sulfuric acid conjugates of PHBA were found at levels of 24%, 7%, 13%, and 7% respectively. Seventy percent of the 0.4 g/kg dose of propyl paraben was excreted in 9 hours, 85% in 24 hours, and 88% in 48 hours. The rate of excretion was slightly lower with the larger dose (295, 296, 297).

In a study in which dogs were given, orally, 1.0 g methyl or propyl paraben/kg body wt/day for one year, between 96% and 100% of the daily administered dose was accounted for in the urine, chiefly in the form of conjugated end products (162).

Schuebel and Manger found that oral administration of methyl and propyl paraben to cats, rabbits, and dogs led to urinary excretion of 15-40% and 4-21%, respectively, of the dose in the form of free PHBA. No methyl or propyl paraben or free PHBA was excreted in the feces, indicating that the parabens were totally absorbed. It was proposed that the first step of paraben detoxication is saponification. This results in formation of PHBA, which is eliminated as such and in the form of salts through the kidneys. Part of this acid is combined with sulfuric acid or glycine. The authors suggest that most of this acid is decomposed in the body, the benzene ring being broken. The alcohol formed by the saponification may be of significance toxically if the rate of saponification is high enough; however, it is unlikely that enough paraben would be obtained from the diet to provide a significant quantity of alcohol. The authors found no evidence of accumulation of the parabens or their metabolites in the animal body (262).

Jones et al. found that dogs given a single oral dose of 1.0 g methyl paraben/kg/body weight excreted 66% of the administered dose in the urine within 24 hours (89% in 48 hrs), 21% as free PHBA and 33% as glucuronic acid conjugates. With a 1.0 g/kg dose of propyl paraben, 53% of the dose was excreted in the urine within 24 hours (58% in 48 hrs), 12% as free PHBA and 12% as glucuronic acid conjugates. The amount of free PHBA excreted never exceeded 0.5% of the dose. The free PHBA level of the plasma increased significantly after paraben administration, reaching a peak in approximately 6 hours and dropping back to zero within 48 hours. The high plasma and urine levels of free PHBA and conjugated products indicate hydrolysis of the ester linkage. No accumulation of methyl or propyl paraben or their metabolic products was noted in dogs which had been given 1.0 g/kg/day for one year. On the contrary, the rate of urinary excretion increased (to 96% of the dose after 24 hrs). Oral ingestion of 70 mg/kg/ by a fasted man revealed that methyl paraben is handled similarly in human metabolism. No detectable ester was found in the plasma or urine. After 12 hours, 50% of the administered dose was recovered from the urine of which 11% was accounted for as free PHBA (136).

Sabalitschka and Neufeld-Crzellitzer gave a human subject 2 g of propyl paraben daily for 5 days. They found that 17.4% of the administered dose was excreted in the form of PHBA, 13.7% free and 3.7% paired with glycine. They computed that about 55% of the propyl paraben was excreted paired with sulfuric acid. No propyl paraben could be found in the urine. The authors were unable to account for all of the administered paraben and, therefore, concluded that breaking of the benzene ring must occur to some extent (249).

IV. Effects on Enzymes and Other Biochemical Parameters

When 10-day old chick embryo femora were cultured for two days in medium containing methyl or propyl paraben, the result was increase in dry weight as compared to femora cultured in control medium. The methyl paraben concentrations found to stimulate growth were 10^{-5} and 10^{-6} M, propyl paraben concentrations were 10^{-5} to 10^{-7} M. It was suggested that these compounds may stabilize lysosomes (314).

In vitro studies have shown that methyl and propyl paraben are bound by protein and that this decreases their antimicrobial activity (1, 196). It is possible that protein-binding has similar effects on other aspects of paraben activity.

The action of methyl paraben on conduction in nerve fiber was studied in the cat. When applied directly to the spinal roots or to the vagus and sympathetic bundle in the neck as an 0.1 or 0.2% solution, conduction in nerve fibers of all sizes, myelinated and unmyelinated, was blocked, with smaller fibers being blocked first. Although the conduction block was reversible upon washing the nerve fiber, it is possible that some degradation occurred among the fibers. This could account for the prolonged or permanent relief, of pain, that is sometimes observed when a local anesthetic containing methyl paraben is injected in various painful states.

V. Drug Interaction

The antimicrobial effects of parabens and sodium benzoate are additive (50). It is possible that other aspects of the preservatives are similarly related.

Bubnoff et al. reported that i.v. and orally administered methyl paraben exhibits an anticonvulsive effect in rats with cocaine-induced cramps (39).

VI. Consumer Exposure Information

The major uses of the parabens in the food industry are cakes, pie crusts, pastries, icings, toppings and fillings (0.03-0.06% of a combination of 3:1 methyl and propyl parabens); soft drinks (0.03-0.05% of a 2:1 ratio of methyl and propyl parabens); creams and pastes (0.1% of a combination of parabens); jams, jellies, and preserves (0.07% of a 2:1 ratio of methyl and propyl parabens); olives and pickles (0.1% of a combination of parabens); and syrups (0.07%).

Under current legislation, the total addition of parabens as chemical preservatives is limited to 0.1% (50).

According to information compiled by the National Research Council the total amount of methyl paraben used in the United States in 1970 was 9,042 pounds and of propyl paraben, 4,031 pounds.

METHYL and PROPYL PARABEN

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p-Hydroxybenzoic Acid Esters as Preservatives

I. Uses, Antibacterial and Antifungal Studies, Properties and Determination*

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The history of the esters of *p*-hydroxybenzoic acid and their uses as preservatives in pharmaceuticals, cosmetics, foods, and industrial products are discussed. Antimicrobial studies show that the methyl, ethyl, propyl, and butyl esters are effective in low concentrations against fungi and Gram-positive bacteria, but less effective against Gram-negative bacteria. The esters are more fungistatic than fungicidal. The *p*-hydroxybenzoates were also tested against microorganisms in the presence of humans serum, and against the growth of *A. niger* in procaine penicillin suspensions. The physical and chemical properties of the *p*-hydroxybenzoates are extensively described, as well as methods of incorporation and a method of determination by ultraviolet absorption.

THE HISTORY of the practical application of the esters of *p*-hydroxybenzoic acid goes back to 1924, when Sabalitschka gave the first of his many reports on their use as antibacterial and antifungal agents (1). In the following years there appeared a great number of articles, mostly in European journals, on the properties and various uses of the esters, particularly as preservatives for pharmaceuticals, cosmetics, and foods. Interest spread to the United States, and commercial production was begun in the early 1930s.[1] The methyl, ethyl, propyl, and butyl esters have been studied most extensively, although benzyl-*p*-hydroxybenzoate and other esters and salts have also been available commercially.

The first official recognition of the parahydroxybenzoates came with the adoption of the methyl ester in the fifth edition (1934) of the *Swiss Pharmacopeia*, which now also includes the propyl ester. These two esters are currently listed in the supplement to the sixth edition of the *German Pharmacopeia*, the *British Pharmaceutical Codex*, which also includes the sodium salts, and in numerous other compendia. In the United States, the methyl ester was first adopted in the National Formulary VII and was subsequently admitted to the U. S. P. XIII, together with the propyl ester, identified, respectively, as Methylparaben and Propylparaben.

A wide variety of applications has been reported for these colorless, neutral preservatives. They have the additional attributes of being essentially nontoxic, nonvolatile, stable, and ef-

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[1] Methyl Parasept, Propyl Parasept, etc., trade mark of the Heyden Chemical Corporation.

fective in low concentrations against diverse microorganisms in acid, neutral, and alkaline solutions. The abundant literature has been reviewed from time to time, notably by MacDonald (2), Neidig and Burrell (3), Kossodo (4), and Sabalitschka (5). It is the purpose of this paper and subsequent articles to describe briefly the more important uses and potential uses of the parahydroxybenzoates, and to report on extensive studies of their properties, antibacterial and antifungal activity, pharmacology, toxicity, and effect on the skin. Some of the preliminary data were presented recently by Sokol (6).

APPLICATIONS

The history of these esters from the practical viewpoint of their usefulness can best be traced by citing the more important and recent literature pertaining to various fields of application. Space does not permit a complete or detailed review, but the investigations mentioned illustrate typical uses.

Pharmaceutical Preparations.—An attempt was made by Boehm (7, 8) and Suess (9) to reduce to table form the concentrations of the parahydroxybenzoic acid esters required to preserve many different pharmaceuticals and cosmetics. In general, higher concentrations were suggested in the case of oily products and emulsions than with aqueous solutions. In view of the greater activity of the higher esters, proportionately lower concentrations of them were recommended. There are so many factors involved, however—such as the physical form of the preparation, the nature of the ingredients, the *p*H, the method of manufacture, and the manner of use—that it is generally advisable to carry out suitable tests to determine optimum amounts.

Gershenfeld and Perlstein (11) investigated various preservatives for acid and basic type gelatins, which are useful as edible emulsifiers. The addition of 0.15% ethyl *p*-hydroxybenzoate effectively inhibited growth of typical resistant organisms in both acid (*p*H 3–4) and alkaline (*p*H 7–8) gelatin, illustrating the preservative's relative independence from *p*H. The same concentration of the propyl and butyl esters was tested with the acid gelatin and found effective, while the methyl ester was partially effective. Since all of the 0.15% of the ethyl, propyl, and butyl esters did not dissolve, as indicated by precipitation, the investigators concluded that preservation is actually attained with lower concentrations.

Preservatives for nasal jellies (1.5% tragacanth in water) were evaluated by Gershenfeld and Tomkin (12). Similar tests were run with ophthalmic solutions (2% boric acid, 1% paredrine hydrobromide). Subcultures were made after six, twenty, and thirty-five days. The ethyl (0.05%) and propyl (0.05%) esters, dissolved as the sodium salts, and the butyl ester (0.02%) exerted bactericidal action after twenty days. The methyl ester (0.15%), as the sodium salt, killed after thirty-five days. Efficient preservation, without objectionable color, appearance, or effect on collapsible tubes, was obtained with 0.05% ethyl, 0.03% propyl, or 0.02% butyl *p*-hydroxybenzoate, dissolved as the sodium salt in the case of the first two.

The value of combinations of esters, from the viewpoint of effectiveness and also solubility, was recognized by early investigators. In practice, two esters were generally used where increased activity and higher concentrations were needed. More recently, Borrini (10) compared the bactericidal efficiencies of phenol, methyl *p*-hydroxybenzoate, and propyl *p*-hydroxybenzoate with three different combinations of three or more esters. He inoculated aqueous solutions containing 0.05 to 0.25% bactericide with *S. aureus* and then seeded meat broth agar plates with these solutions after exposure times of five, ten, thirty and sixty minutes, and twenty-four hours. The growth on these plates diminished with increasing concentration of bactericide and time of exposure. Only five minutes were required with 0.25% of two different combinations to completely eliminate growth. The results showed that combinations of three or more esters were more active than an equal amount of a lower ester alone and also were more soluble. It has been demonstrated that the esters do not diminish each other's solubility in water.

In an investigation of therapeutic bases and the *p*H of the human skin, Harry (13) found a combination of 0.15% methyl, 0.05% propyl, and 0.02% butyl *p*-hydroxybenzoate in creams and lotions highly effective against a wide variety of microorganisms. The relatively low concentrations of 0.025% Methylparaben and 0.015% Propylparaben are prescribed in the U. S. Pharmacopeia for hydrophilic ointment (14). Darlington and Guth (15), in describing the properties of bentonite as a component of ointment bases, stated that mold growth in such bases is prevented by the incorporation of 0.15% Methylparaben and 0.05% Propylparaben. Sander, in a patent on a spermicidal composition (16), suggested the use of propyl *p*-hydroxybenzoate in combination with one or more antiseptic agents as an antimold ingredient.

The stability and neutral reaction of the *p*-hydroxybenzoates have been found advantageous in the preservation of vitamin C solutions, for which a combination of 0.18% methyl ester and 0.02% propyl ester has been recommended (17). A diabetic syrup containing gums, saccharin, and methyl *p*-hydroxybenzoate as a preservative was found to be stable on storage by Woo and Huyck (18). H. Berry (19) suggested 0.01% of a 65:35 mixture of methyl and propyl esters to preserve ephedrine eye drops.

An interesting recent use of the *p*-hydroxybenzoates as antifungal agents in aqueous antibiotic preparations deserves mention. Johnson and Lerrigo (20) found that the methyl and propyl esters did not adversely affect the stability of crystalline sodium penicillin G in saline solution. This compatibility has been confirmed with similar studies (21) made with aqueous solutions of potassium penicillin G and aqueous suspensions of procaine penicillin. Samples containing 0.2% methyl or 0.01% propyl ester were compared with controls over several weeks storage at 25, 38, and 50°. Further tests, described later, with suspensions of procaine penicillin G for aqueous injection showed that 0.02% propyl ester in the water of dilution prevented the growth of *A. niger* over a

period of sixty-one days at 25°. A combination of 0.18% methyl ester with 0.02% propyl ester is now used in this antibiotic preparation. In this connection Dietmann (22) suggested the use of combination of 0.07% methyl ester and 0.03% propyl ester for the maintenance of sterility in pharmaceuticals which cannot be heated. This combination of esters dissolved in water has long been familiar in Germany as *aqua conservans* (23).

Cosmetic Products.—Preservatives suitable for use in cosmetics must meet much the same requirements as those used in pharmaceuticals. Color, odor, stability, effectiveness in slightly alkaline media, and reaction on the skin are important considerations. The esters of *p*-hydroxybenzoic acid possess the desired properties to a high degree and are widely used in cosmetics. The applications include shampoos, creams, lotions, oils, mucilages, pastes, jellies, gum solutions, and others. The antibacterial properties of *p*-hydroxybenzoates have also found application in deodorant creams and powders.

According to Boehm (7, 8) concentrations up to 1% methyl *p*-hydroxybenzoate (or alternatively 0.3% ethyl, 0.2% propyl, or 0.15% butyl) may be required in creams of high oil content. It is advantageous to use the higher esters, propyl and butyl, as the oil content increases, particularly in oils or oil solutions. In emulsions and aqueous preparations, however, combinations of the methyl or ethyl ester with the higher esters are advisable. Such combinations also make it possible to readily incorporate higher concentrations of preservatives, which may occasionally be needed, without exceeding the solubilities of the esters. Experience indicates that better distribution is obtained by incorporating at least a part of the preservative in the aqueous phase.

Many papers have been published, some general and some specific, on the use of *p*-hydroxybenzoates in cosmetics (8, 24). Their use in dentifrices is frequently mentioned (25). Burlinson (26) found 0.05% of the propyl ester to be a most suitable preservative for a gum tragacanth mucilage. Deakers (27) recommended the methyl ester for gums used in facial masks. For hand lotions (28), he proposed a combination of 0.12% methyl ester dissolved in the aqueous phase and 0.10% propyl ester in the oil phase.

Of interest in the manufacture of oily products is the recommendation by Gorchoff (29) that the propyl ester be used as an antioxidant for vegetable oils in hand lotions. Recent tests with lard, by the active oxygen method, have confirmed the fact that this ester has appreciable antioxidant activity. Prevention of rancidity and preservation of a cold cream were described by Gottfried (30), who suggested 0.25% of the propyl ester or, alternatively, 0.1% propyl ester plus 0.12% methyl ester. Similar combinations were suggested for hair oils, skin ointments, aqueous starch pastes, and gum mucilages.

According to McLachlan (31), *p*-hydroxybenzoates have largely superseded older preservatives such as benzoic and salicylic acids. For the microbiological testing of cosmetics, he suggested microscopic examination of samples as well as inspection of incubated samples seeded with two bacteria, a yeast and a mold.

The effectiveness of various concentrations of *p*-hydroxybenzoates as preservatives for typical cosmetic products has been investigated (32). Samples of lotions and creams containing 0.02 and 0.10% of each ester (0.05 and 0.20% in the case of methyl ester) were inoculated with pure cultures of four bacteria, a yeast, and five fungi, and incubated at 28° for two weeks, or three months in the case of the fungi. The tests included a combination of equal parts of the methyl and propyl esters. The creams proved to have a natural resistance to the growth of the microorganisms used, and the growth of the fungi in a pancake make-up was inhibited by 0.5% of any of the esters. The results with the Karaya gum wave set lotion and mineral oil and stearic acid hand lotions indicated that good protection against the organisms tested is achieved with 0.1 to 0.2% of the preservative, particularly with combinations of the lower and higher esters. Products with different compositions may require higher concentrations, which are readily obtained with combinations of two or more esters.

Food Products.—The use of the esters of *p*-hydroxybenzoic acid as food preservatives was one of their most important early applications in Europe. Regulations permitting their use in specified foods were enacted in the years 1932 to 1938 in Germany, Hungary, Italy, Norway, Yugoslavia, Rumania, and Czechoslovakia (1). In Germany, where the *p*-hydroxybenzoates are currently in wide use, the regulations of 1932 permitted the ethyl and propyl esters or their sodium salts (in mixture or in combination with certain other materials such as salt or citric acid) in prescribed amounts in many different foods (33). Permissible concentrations included 0.08% in margarine, 0.09% in fruit preparations, 0.05% in nonalcoholic drinks, and 0.12% in confectionery.

Another food use of the *p*-hydroxybenzoates mentioned recently in the literature is in the prevention of mold growth on cheese. Anderson (34) suggested treatment with the methyl ester prior to coating with wax. Tammisto and Peltola (35) inhibited mold growth during curing by dipping the cheeses in a solution of the ester. The use of the esters on wrapping materials to prevent mold on the surface of foods has been suggested.

A critical investigation of preservatives for foods has been reported by von Schellhorn (36). He suggested the careful selection of a preservative, or a combination of two, to suit particular conditions of *p*H, composition, solubility, etc., so that a minimum concentration could be used. For instance, acids such as salicylic, benzoic, *p*-chlorobenzoic, formic, and propionic showed strong inhibition of certain microorganisms in strongly acid solutions, in which they were undissociated. In more neutral solutions, however, amounts up to 1 or 8% were necessary to achieve inhibition. His tests with various yeasts, fungi, and bacteria showed that ethyl *p*-hydroxybenzoate, on the other hand, was active in both acid and neutral media.

In discussing problems arising from the use of chemicals in food in England, Banfield (37) considered the *p*-hydroxybenzoic acid esters to hold out considerable promise as preservatives, in view of their effectiveness at neutral *p*H. The data available on their toxicity have been favorable, and if substantiated, there appeared to be potential benefits to both the manufacturer and the consumer in the use of these preservatives.

In the United States, the use of the *p*-hydroxybenzoates in foods, up to the present time, has been limited to a few minor specialty applications. Wider use was not encouraged because, in the opinion of Lehman (38), further data were necessary before their safety could be evaluated. The investigations designed to furnish additional data will be reported in succeeding articles.

Medicinal Uses.—The esters of *p*-hydroxybenzoic acid have been suggested for use in antimycotic therapy. Earlier work in this field includes the use by Lomholt (39) of a 5% solution of the methyl ester in alcohol in successfully treating epidermophytosis of the feet. Subsequently, Bang (40) used a similar solution in 70% alcohol and a salve containing 5% of the methyl ester to treat trichophytosis of the scalp. According to Sabalitschka (5), the official Danish list of galenicals includes a solution, a powder, and two ointments containing *p*-hydroxybenzoates, which are used in the treatment of mycoses.

In an investigation of epidermophytosis of the feet and hands, Loos (41) found that 0.01% of the propyl ester and 0.04% of the methyl and ethyl esters inhibited the growth of *Epidermophyton interdigitale*, and that 1% of these esters killed the fungus in thirty minutes. The fungistatic concentration for the sodium salts of the methyl and propyl esters was 0.02% with *Trichophyton gypseum asteroides*, and 0.02 and 0.01%, respectively, with *Microsporon audonini*. In clinical use, a salve containing 5% propyl ester and 10% salicylic acid successfully controlled infections due to these fungi. A powder containing 2 to 4% methyl ester and talc was recommended for prophylactic use.

More recently, Delpiana and Ugazio (42) reported on the treatment of numerous cases of mycoses of the hands and feet with ointments and solutions of *p*-hydroxybenzoates. Favorable results were obtained with 6% solution of the butyl ester in peanut oil, an ointment containing 10% of a mixture of esters and a 20% solution in alcohol of a mixture. The solution form (in alcohol or propylene glycol) was found to be most effective, and could be reduced in concentration in case of irritation.

A new and particularly interesting use of the *p*-hydroxybenzoates was described by McVay and Sprunt (43) in reporting on recent studies of moniliasis in broad spectrum antibiotic therapy. The *in vitro* tests with an 80:20 mixture of the methyl and propyl esters established their inhibitory action against five strains of yeast, including *C. albicans* and *C. krusei*, isolated from patients. Studies made with 186 patients being treated with aureomycin showed that the mixture of esters was of value in preventing the development of moniliasis. This was particularly true in the case of oral administration, even with the relatively small dose of 0.8 to 1.42 Gm. of *p*-hydroxybenzoates per day. Similar results were obtained with vaginal and rectal administration, the mixture of esters preventing the overgrowth of *C. albicans*. No toxic effects of the *p*-hydroxybenzoates were observed in these studies.

Miscellaneous Uses. A number of industrial applications of *p*-hydroxybenzoates have been described in the literature, some of them involving combinations with other antimicrobial agents. Gelatin for photographic use can be preserved with a combination of the methyl and propyl esters, as can various types of emulsions. The esters have long found use in shoe dressings and in cutting oils. They have been found well suited for use as preservatives in pastes and glues that come into contact with the skin or with food products, and have aroused interest as antimold agents in food wrappings. The ethyl ester, in particular, has been employed as an intermediate in the synthesis of pharmaceuticals.

ANTIBACTERIAL AND ANTIFUNGAL STUDIES

Sabalitschka (5) recently reported the activity of the *p*-hydroxybenzoates in inhibition of a yeast fermentation as methyl 3.7, ethyl 5.3, propyl 25, butyl 40, and benzyl 69, when compared with phenol as 1. A study of the data compiled from various sources shows that in general the esters are more active against fungi than against bacteria and that higher esters are more active than the lower ones, although many exceptions to the latter statement are noted in the literature. For example Back (44) stated that, generally speaking, the methyl ester is more effective against mold, whereas the propyl ester is more effective against yeast.

In view of the discrepancies in the values reported for the antimicrobial activities of the various esters of *p*-hydroxybenzoic acid, it was concluded that it would be of value to determine the activities of a number of the commonly used esters, each against a variety of organisms, using standardized conditions. The results provide an antimicrobial spectrum of the esters. It is, of course, possible that appreciably different values might be obtained through variations in technique and strain of organism. By way of example, Gialdi and Ponci (45) reported inhibition of *S. aureus* and *E. coli* by 0.023% butyl *p*-hydroxybenzoate, values not in close agreement with those obtained in the present study.

Vincent and his coworkers (46-48) studied the inhibitory action of a number of esters of 4-hydroxybenzoic acid and related compounds against three fungi: *Aspergillus niger*, *Byssochlamys nivea*, and *Penicillium roqueforti*. They grew the fungi on agar plates made up with Czapek Dox medium with sucrose and adjusted to *p*H 4.4 to 4.8. The diameter of the colony was measured at appropriate time intervals to provide for calculation of the dosage response relationship. Values for log I_{50} (−log millimolar concentration giving 50% inhibition of growth rate) were reported for the esters methyl through heptyl and for a number of related compounds. Although there was some difference in the effect against the three organisms, there was obtained an approximately straight line relationship when log I_{50} was plotted against chain length of the ester. There were also straight lines when log water solubility and partition coefficient were plotted against chain length. Thus, the increasing inhibitory activities of the esters were closely correlated with decreasing solubility in water. The branched chain esters were slightly less active than the corresponding straight chain esters. The logarithmic dosage response curves for all the esters had the same slope, pointing to a similar mode of action for all the esters.

In the determination of the inhibitory actions of the esters, it was decided to determine the concentration necessary to prevent growth. It is the consensus of those who work with antifungal agents that the concentration required to cause an inhibition of 50% of growth (or to prevent 50% of spores from germinating) can be more reliably determined than the concentration providing 100% inhibition (*cf.* articles by Vincent and coworkers). Nevertheless, it was considered advisable in this study to adhere to the older technique since determination of the absolute amounts required to prevent growth of organisms should be more directly translatable to practice in the pharmaceutical and food industries.

Experimental. In the investigation of the antifungal activity of the *p*-hydroxybenzoates, the samples were dissolved in methanol at such concentrations that, when 0.5 cc. was added to the 25 cc. broth, the flasks contained the concentration shown. Twofold increments were generally used. The medium was made up as follows: honey 85 Gm., peptone 10 Gm., water 1 L. The broth was dispensed into 125-cc. Erlenmeyer flasks, and the flasks were plugged and autoclaved for twenty minutes at 20 lb. pressure. A blank of inoculated medium and a control of 0.5-cc. methanol were run. Each flask was inoculated with one drop of spore suspension made by adding 25 cc. of sterile water to a well grown out slant of the organism. Each concentration of each compound was run in triplicate. The flasks were allowed to stand for seven days. At the end of this time the quantity of growth was read, calling the growth in the blank flask 4+.

In making antibacterial tests, the plate streak method was used. The dilutions of the sample were made in water, using sufficient sodium hydroxide for solubility. The medium was so prepared that, when 5.0 cc. of the sample was added to 10 cc. of medium, the proper concentration of *p*-hydroxybenzoates and ingredients was obtained.

The medium was composed of glucose 22.5 Gm., peptone 15.0 Gm., sodium chloride 2.25 Gm., beef extract 4.5 Gm., agar 22.5 Gm., and water 1 L. The *p*H was approximately 6.8. The medium was dispensed into test tubes and sterilized. After addition of the samples, the contents of the tubes were poured into plates. Inoculations were made by dipping a loop in the suspension from 10 cc. of water on a twenty-four-hour slant of the organism and streaking on one sector on the plate. Readings were made after twenty-four hours.

The concentrations of methyl, ethyl, propyl, and butyl esters required to inhibit growth of a number of bacteria and fungi are given in Table I. Under the conditions used, the higher esters particularly are readily inhibitory to fungi in the concentrations which are feasible in pharmaceutical products. Some of the Gram-negative bacteria, such as *Pseudomonas aeruginosa* ATCC 9027 and *Pseudomonas fluorescens* ATCC 9721, are much more difficult to inhibit. Their growth was stopped by 0.2 to 0.4% of the methyl or ethyl ester, but 0.4 to 0.8%, far in excess of the solubility, was required with the propyl and butyl esters. The reason for this behavior has not been determined. It was found, however, that a combination of the four esters in the ratio of their solubilities in water at 25° inhibited the growth of these organisms at concentrations of 0.25 to 0.49%, which are below the saturation values.

TABLE I.—ANTIMICROBIAL ACTIVITIES OF *p*-HYDROXYBENZOATES[a]

Microorganisms	Required for Inhibition, %			
	Methyl	Ethyl	Propyl	Butyl
Aspergillus niger ATCC 10254	0.1	0.04	0.02	0.02
Penicillium digitatum ATCC 10030	0.05	0.025	0.0063	<0.0032
Rhizopus nigricans ATCC 6227A	0.05	0.025	0.0125	0.0063
Trichoderma lignorum ATCC 8678	0.025	0.013	0.0125	0.0063
Chaetomium globosum ATCC 6205	0.05	0.025	0.0063	<0.0032
Trichophyton mentagrophytes ATCC 9533	0.016	0.008	0.004	0.002
Trichophyton rubrum ATCC 10218	0.016	0.008	0.004	0.002
Candida albicans ATCC 10231	0.1	0.1	0.0125	0.0125
Saccharomyces cerevisiae ATCC 9763	0.1	0.05	0.0125	0.0063
Saccharomyces pastorianus ATCC 2366	0.1	0.05	0.0125	0.0063
Bacillus subtilis ATCC 6633	0.2	0.1	0.025	0.0125
Bacillus cereus var. *mycoides* ATCC 6462	0.2	0.1	0.0125	0.0063
Staphylococcus aureus (*Micrococcus pyogenes* var. *aureus*) ATCC 6538P	0.4	0.1	0.05	0.0125
Sarcina lutea	0.4	0.1	0.05	0.0125
Klebsiella pneumoniae ATCC 10031	0.1	0.05	0.025	0.0125
Escherichia coli ATCC 9637	0.2	0.1	0.1	0.4
Salmonella typhosa	0.2	0.1	0.1	0.1
Salmonella shottmuelleri	0.2	0.1	0.05	0.1
Proteus vulgaris ATCC 8127	0.2	0.1	0.05	0.05
Aerobacter aerogenes ATCC 8308	0.2	0.1	0.1	0.4

[a] Precipitation was noted in those instances where the concentration used appreciably exceeded the water solubility of the ester.

In Table II are presented the comparative activities of a number of esters of *p*-hydroxybenzoic acid against *Aspergillus niger*. This organism was found to be the most resistant of all the fungi tested to the action of antimicrobial agents. The optimum chain length of the ester seems to be about three or four, as lower esters are more soluble but less active and the higher ones are so insoluble that a sufficient quantity for inhibition is difficult to get into water solution.

TABLE II.—MINIMAL CONCENTRATION OF ESTERS OF *p*-HYDROXYBENZOIC ACID REQUIRED TO INHIBIT *A. niger*

	%
Methyl	0.1
Ethyl	0.04
Propyl	0.02
Isopropyl	0.05
Butyl	0.02
Isoamyl	>0.015 <0.1
Hexyl	>0.015 <0.1
Cyclohexyl	>0.015 <0.1
2-Ethylhexyl	>0.1
o-Chlorobenzyl	>0.1
8-Hydroxyethyl	>0.1
Tetra-hydrofurfuryl	>0.1
p-Chlorobenzyl	Insoluble
Glyceryl	>0.1
Pentaerythrityl	>0.1

Experiments on the effect of mixtures of methyl and propyl *p*-hydroxybenzoates on the growth of *A. niger* showed that the effects are additive. The data in Table III demonstrate that a mixture containing methyl and propyl esters, each at half the level causing complete inhibition, prevents growth of *A. niger*. This confirms the value of combinations where higher concentrations are required.

TABLE III. ADDITIVE EFFECT OF *p*-HYDROXYBENZOATES

Methyl, %	Propyl, %	Amount of Growth
0.05		0
0.025		3+
	0.015	0
	0.01	+
	0.005	3+
0.05	0.005	0
0.025	0.01	0
0.025	0.005	+

In Table IV is shown the relative effectiveness of propyl *p*-hydroxybenzoate, phenol, and sodium benzoate against five bacteria in a medium of *p*H 6.8 and *A. niger* in a medium of *p*H 5.2. It is notable that, whereas the bacteria differ in their resistance to the propyl ester and sodium benzoate, all were inhibited by 0.2% phenol but not by 0.1%.

TABLE IV. COMPARATIVE EFFECTIVENESS OF PROPYL *p*-HYDROXYBENZOATE, PHENOL, AND SODIUM BENZOATE

	Propyl *p*-Hydroxybenzoate, %	Phenol, %	Sodium Benzoate, %
B. subtilis	0.025	0.2	0.2
S. aureus	0.05	0.2	0.4
K. pneumoniae	0.025	0.2	0.2
E. coli	0.1	0.2	0.4
S. typhosa	0.1	0.2	0.2
A. niger	0.02	>0.01	>0.04

Over the *p*H range of 4 to 7 there was no difference in the effectiveness of the four individual *p*-hydroxybenzoates, methyl, ethyl, propyl, and butyl, against *A. niger*. At *p*H 8 there was evidence that growth was slightly greater at subinhibitory levels in the case of the propyl and butyl. It is believed that this has no significance for these two esters as contrasted with the other two. It is logical to expect a diminution in effectiveness of the compounds as the *p*H of the solution approaches the *p*K value of the acids. It is undoubtedly true that the undissociated molecule is more active than the ion. Hence, for example, at a *p*H identical with the *p*K of the *p*-hydroxybenzoate (about 8.5) the concentration of the undissociated acid is only half the total concentration, and some decrease in effectiveness would be expected. The esters of *p*-hydroxybenzoic acid thus differ from benzoic acid in being relatively independent of *p*H. Benzoic acid, with *p*K value of 4.2, cannot be expected to be active much above a *p*H of 4.

In 1911 Boeseken and Waterman (49) showed that *p*-hydroxybenzoic acid was utilized by molds as a nutrient. More recently Davis (50) has reported *p*-hydroxybenzoic acid to be a bacterial vitamin in that a mutant of *E. coli* unable to synthesize aromatic acids responded to addition of *p*-hydroxybenzoic acid. The latter was therefore suggested as an intermediate in the synthesis of aromatic compounds from shikimic acid. Davis also proposed *p*-hydroxybenzoic acid as a model for chemotherapeutic analogs selectively toxic to microorganisms. Accordingly, an experiment was set up to see whether the activity of ethyl *p*-hydroxybenzoate, against *E. coli* would be reversed by the addition of *p*-hydroxybenzoic acid. The organism was inhibited by 0.1% of the ethyl ester, whereas there was growth at 0.05%. The presence of *p*-hydroxybenzoic acid at 0, 0.001, and 0.01% did not alter the inhibitory effect.

The activity of propyl *p*-hydroxybenzoate in the presence of serum was determined by an agar streak test. Human serum was incorporated into the agar at a 10% level. Concentrations were so arranged that the final plate contained the agar at its normal level, serum at 10%, and the ester at 0.8, 0.4, 0.2, 0.1, 0.05, and 0.025%. The results in per cent required to inhibit are given in Table V.

TABLE V. EFFECT OF SERUM ON ACTIVITY OF PROPYL *p*-HYDROXYBENZOATE

	Control	Serum
B. subtilis	0.1	0.2
S. aureus	0.05	0.2
K. pneumoniae	0.025	0.1
E. coli	0.2	0.2
S. typhosa	0.2	0.2

Preservation of Antibiotic Solutions and Suspensions. The esters of *p*-hydroxybenzoic acid were tested for preservative action in aqueous suspensions of procaine penicillin. Vials of procaine penicillin for aqueous injection 300,000 u./vial were injected with 5 cc. of solution of esters of *p*-hydroxybenzoic acid of the concentration shown in Table VI, followed by one drop of a suspension of spores of *A. niger*. The vials were allowed to stand at 25° for the intervals indicated and were examined macroscopically and microscopically for evidence of growth.

TABLE VI.—PRESERVATION OF PENICILLIN SUSPENSIONS WITH *p*-HYDROXYBENZOATES

Days	Methyl, % 0.01	Methyl, % 0.05	Propyl, % 0.01	Propyl, % 0.02	Propyl, % 0.04	No Preservative
12	+*	+	+	0	0	++
21	++	++	+	0	0	++
28	++	++	+	0	0	++
42	++	++	+	0	0	++
61	++	++	+	0	0	++

* + is the smallest amount of growth and ++ the greatest.

Samples of penicillin procaine for aqueous injection in cartridges were made up with a mixture of 0.07% methyl and 0.03% propyl *p*-hydroxybenzoate. Each cartridge was inoculated with one drop of a suspension of spores of *A. niger*. The control without preservatives showed scanty growth of the organism at eight days and abundant growth at fifteen days and subsequent intervals at 25°. There was no growth in the suspension containing the mixture of methyl and propyl *p*-hydroxybenzoate, even after fifty-five days.

PHYSICAL AND CHEMICAL PROPERTIES

The physical properties and solubilities of methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid are given in Table VII.

The methyl ester of *p*-hydroxybenzoic acid has a slight, characteristic odor, but the other esters are practically odorless in the usual, purified form. No particular taste is noticeable in solutions of the esters, but in saturated aqueous solution they exhibit a slight tang or weak burning sensation in the mouth. In terms of equal concentrations, it is most noticeable with the butyl ester. According to taste panel tests, this tang is not noticeable with lower concentrations or in the presence of flavorings or fatty materials.

The esters are stable in air and are resistant to hydrolysis in cold or hot water. According to laboratory studies (51), they show no hydrolysis under the usual conditions of sterilization. Aqueous solutions, buffered at a *p*H of 6, were heated for two hours at 100°, and thirty minutes at 120°, respectively. No hydrolysis could be detected by ultraviolet spectrophotometric analysis with an accuracy of about 2%. The same was true at *p*H 3. These heating periods produced a slight amount of hydrolysis at *p*H 8, amounting to about 6% with the propyl ester. In general, resistance to hydrolysis increased with the size of the alkyl side chain and with branching of the chain. In buffered solutions of *p*H 3, 7, and 8 at 25°, no hydrolysis was noted in six weeks, the duration of the test.

METHODS OF INCORPORATION

The incorporation of the *p*-hydroxybenzoic acid esters into various products requires a familiarity with their solubility characteristics, particularly in water. While their solubility in water is limited, they can be dissolved in aqueous products at room temperature provided good agitation is maintained. The rate of solution is a function of particle size. With the fine powders that are normally available, concentrations of 0.15% methyl, 0.10% ethyl, 0.03% propyl, or 0.015% butyl can be achieved in twenty or thirty minutes, according to laboratory tests. A coarse powder, on the other hand, dissolves only after long, continued stirring.

The esters can be dissolved more rapidly by heating the medium to 70 or 75° and stirring until solution is complete. This temperature should be kept about 20° below the melting point of the ester used, to prevent agglomeration of the particles. Other methods of incorporation involve the preparation of solutions of the esters in solvents. Alcohol is frequently used, and often one of the other ingredients, such as an oil or propylene glycol, will serve as a solvent. Such a solution must be added slowly with stirring to the aqueous phase, in order to prevent precipitation of the ester. The concentration in the solvent should not be over 15% with the methyl ester or 2.5% with the propyl to obtain clear solutions.

The resistance of the *p*-hydroxybenzoates to saponification makes it possible to dissolve them in 5% sodium hydroxide to form the soluble sodium salts, thus providing another means of incorporation. Only about 80% of the stoichiometric quantity of alkali is needed for solubilization, as for example, 120 lb. of 5% NaOH per pound of methyl *p*-hydroxybenzoate. Such solutions should be kept below 30° and used the same day as prepared. When used to give an ester concentration of 0.1%, they raise the *p*H of neutral, unbuffered products to about 9. In order to adjust the *p*H to 7, an amount of suitable acid, equivalent to the alkali used, should be added. This method of incorporation is equivalent to using the dry sodium salts of the esters, which have been prepared but have the disadvantages of caking and hydrolyzing in the presence of moisture.

METHODS OF DETECTION AND DETERMINATION

The literature contains a number of papers on the detection and determination of the *p*-hydroxybenzoates in various media (52-59). One of the simplest methods of detection and estimation is provided by Millon's reagent, which can be used on aqueous solutions in the absence of interfering substances. Prior extraction of the esters with ether or alcohol may be necessary. A more sensitive colorimetric method makes use of the reagent 4-aminoantipyrine, and has been developed for easy manipulation by Ettinger (60).

The *p*-hydroxybenzoates may be readily determined quantitatively in the absence of interfering substances by ultraviolet absorption. The transmission curves of the individual esters are virtually identical, with a broad absorption band at 245-250 mμ. The optical density is measured at 255 mμ, and the concentration of the ester can be calculated from a standard calibration curve. Interference from *p*-hydroxybenzoic acid, which has approximately the same molecular extinction coefficient as the ester in acid solution, may be eliminated by neutralizing the sample to *p*H 7, and then extracting the esters with ether. The ether is evaporated and the residue dissolved in warm water. *p*-Hydroxybenzoic acid and the ester may be determined in the presence of each other by measuring the optical density at *p*H 1 and *p*H 7, after first measuring molecular extinction coefficients of the

TABLE VII. PROPERTIES OF THE *p*-HYDROXYBENZOIC ACID ESTERS

	Methyl	Ethyl	Propyl	Butyl
Molecular weight	152.14	166.17	180.20	194.23
Appearance	White powder	White powder	White powder	White powder
Melting point, ° C.	125-127°	116-118°	96-98°	69-71°
Solubility, Gm. per 100 Gm.				
Water				
25°	0.25	0.17	0.05	0.02
10°	0.20	0.07	0.025	0.005
80°	2	0.86	0.30	0.15
Methanol, 25°	59	115	124	220
Ethanol, 25°	52	70	95	210
Propylene glycol, 25°	22	25	26	110
Glycerine, 25°	1.7	ca. 0.5	0.4	ca. 0.3
Peanut oil, 25°	0.5	1	1.4	5
Acetone, 25°	61	84	105	240
Benzene, 25°	0.7	1.65	3	10
Ether, 25°	23	43	50	150
Carbon tetrachloride, 25°	0.1	0.9	0.8	1
Mineral oil, U. S. P., 25°	0.01	0.025	0.03	0.1

acid and the ester in the same buffer solutions.

p-Hydroxybenzoates in oils may be determined by dissolving the sample in petroleum ether and extracting the esters with three portions of 80% ethanol prior to measurement of the optical density.

The individual esters can be determined by employing a chromatographic separation and spectrophotometric estimation devised by Higuchi (61). This technique provides a means for analyzing mixtures of the esters and determining them in the presence of interfering substances.

SUMMARY

1. The history of the esters of *p*-hydroxybenzoic acid and their uses as preservatives in a wide variety of pharmaceuticals, cosmetics, foods, and industrial products, and also in antimycotic therapy are traced briefly, with references to pertinent literature.

2. Antimicrobial studies with 22 microorganisms show the methyl, ethyl, propyl, and butyl esters to be effective in low concentrations against fungi and Gram-positive bacteria and less effective against Gram-negative bacteria in the media used. The esters are more fungistatic than fungicidal, but their effect is additive, suggesting the use of combinations to achieve higher concentrations in water.

3. The *p*-hydroxybenzoates are substantially equally effective against microorganisms in acid or neutral solutions in the *p*H range of 4 to 8. Their antimicrobial activity is slightly lower in the presence of human serum.

4. The growth of *A. niger* in procaine penicillin suspensions is inhibited by 0.02% propyl ester of *p*-hydroxybenzoic acid. A mixture of 0.07% methyl and 0.03% propyl ester prevents spore growth in penicillin cartridges.

5. An extensive description of the physical and chemical properties of the *p*-hydroxybenzoates is given. They are stable and do not hydrolyze under the conditions of heat sterilization (two hours at 100° or one-half hour at 120°) at *p*H 3 to 8.

6. Methods of incorporation are discussed in detail and a method of determination by ultraviolet absorption is outlined.

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ON THE TOXICITY AND THE LOCALLY-ANALGETIC EFFECT OF p-OXYBENZOIC ACID ESTERS.

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P-oxybenzoic acid esters have long been used in the treatment of various human fungus diseases (trichophytiasis, epidermophytiasis, moniliasis); BANG, LOMHOLT, MCVAY. In view of their minor toxicity, these substances may be administrated externally and internally without adverse side effects (SCHUEBEL). Several esters have been used as local analgetics (such as orthoform, intracain etc.); ADLER & URI.

For the purpose of systematically testing the antimycotic effect, Bekesi at the PHARMAKOLOGISCHE INSTITUT prepared the simple esters of p-oxybenzoic acid up to the C_{12} derivative. Because of the local-analgetic effect of several derivatives, it seemed appropriate that the prepared homologuous series be tested for toxicity as well as for local analgetic effects. This will now be reported.

EQUIPMENT AND METHODOLOGY

Besides p-oxybenzoic acid proper (i), the follwoing simple esters were available esters of (II) methyl, (III) ethyl, (IV) propyl, (V) isopropyl, (VI) butyl, (VII) isobutyl, (VIII) pentyl, (IX) hexyl, (X) heptyl, (XI) octyl, (XII) decyl and (XIII) duodecyl. Without exception, these were used as Na salts.

Toxicity was tested in white mice (20-30 gm body weight) for subcutaneous administration. Each dosis from each derivative was tested in five mice.

The local-analgetic effect was tested in 3 ways:

a) SURFACE ANALGESIA: according to REGNIER at the horny layer of the rabbits (2½-3 kg). Instilled 0.01% pantocain was used for comparison.

b) INFILTRATION ANALGESIA: according to BUELBRING & WAJDA on the back skin of guinea-pigs (250-300 gm body weight); injection (intracutaneous) of 0.25 ml of 1% solution of homologuous ester; control: same amount of 1% procaine solution.

c) CONDUCTION ANESTHESIA: at the muscle nerve preparations of frogs (electrical irritation of N. ischiadicus with right angular DC pulses, with measurement of the potential triggering a muscle contraction). Control: 1% procaine solution. Repetition of electrical stimulation in 1 minute intervals. All substances tested on 5 frogs for each.

RESULTS

A. TOXICITY TESTS

Fig. 1 shows the number of dead animals as a function of dosis. As is seen, the toxicity of the p-oxybenzoic acid derivatives decreases in parallel with the increase in the number of C atoms in the ester. Naturally the parallelism is not perfect; there is a jump-like decrease in toxicity between product V and product VI, while products I and II, III and IV and XII and XII practically are of the same toxicity. The LD_{50} values from fig. 1 are compiled in table 1.

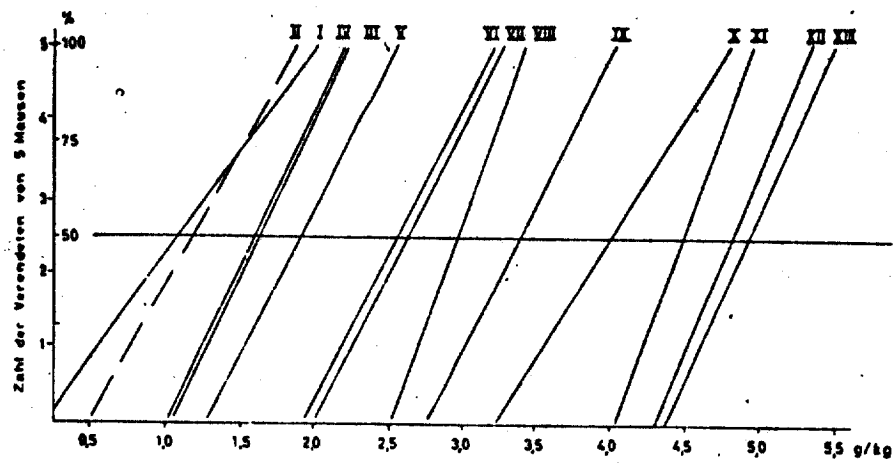


Fig. 1.

Number of dead animals as a function of subcutaneously administered doses of the 13 tested products. Experimental animal : Albino mouse with 20-30 g body weight.

Table 1

LD/50 for the white mouse after subcutaneous administration of various esters of p-hydroxybenzoic acid

No.	Pharmakon	LD_{50} in g/kg
I	p-Oxybenzoesäure	1,05
II	p-Oxybenzoesäuremethylester	1,20
III	" methylester	
IV	" propylester	1,65
V	" isopropylester	1,90
VI	" butylester	
VII	" isobutylester	2,60
VIII	" pentylester	2,95
IX	" hexylester	3,40
X	" heptylester	4,00
XI	" octylester	4,50
XII	" decylester	4,80
XIII	" duodecylester	4,90

B. LOCAL ANALGETIC EFFECTS

a) Surface analgesia. Products I-VII were without effects. Products VIII-XIII show a surface analgetic effect which however is less than that of pantocaine and also shorter in time. Fig. 2 shows the time-dependent surface-analgetic curves for pantocaine and a few selected products from our series. Be it briefly noted that products XI-XIII were superior to product X. In this respect there seems to be an increase in effectiveness with increase in C atoms in the ester.

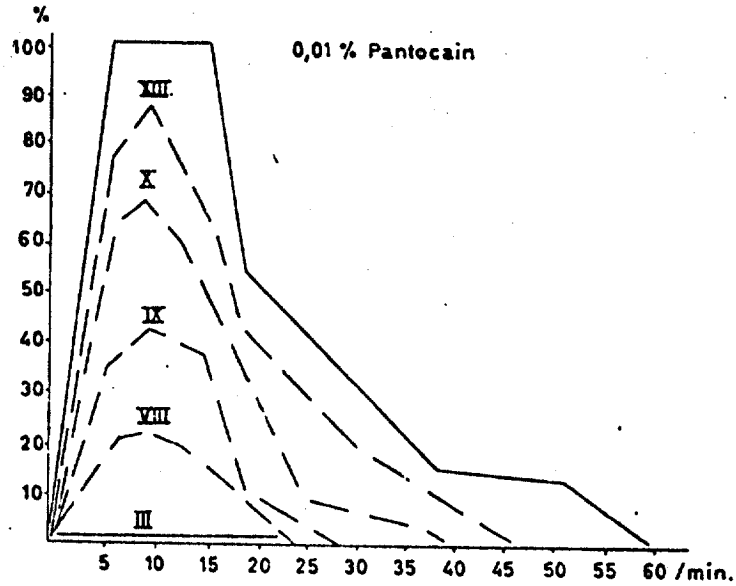


Fig. 2
Surface analgetic effect of single eskers of p-hydroxybenzoic acid in comparison to pantocain. Concentration of all tested substances: 0.01 %.

b) Infiltration analgesia. We recorded the time following intracutaneous injection till the animal will react unambiguously for 3 of the 5 pinpricks. In repeat tests, the same 10 animals were used in such fashion that at 1-week intervals, 3 test products and procaine were injected intracutaneously. Table II shows the results. It will be seen that for some animals the procain reaction differs much, but hardly so for the average of 10 animals. A notable effect can only be observed in products VII-XIII, where again an increase in effect-duration was recorded with an increase of ester C atoms. VII's effect duration is about the same as for procaine, but that of VIII is twice as long. Products XII-XIII last 6 times as long as compared to procaine.

Table II

Duration of analgesia in minutes in the intracutaneous urtica in the back skin of the guinea pig

TIER: animal; DURCHSCHNITT: average

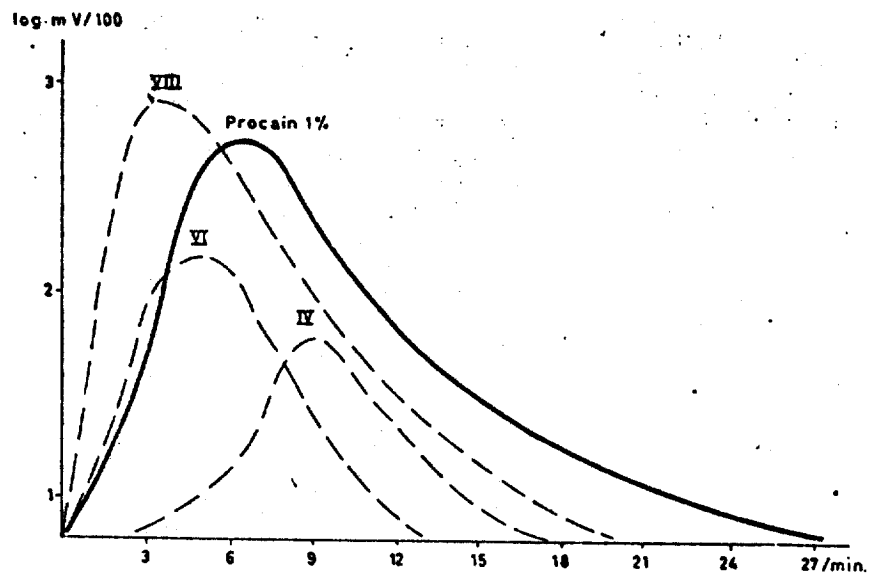
Pharmakon No.	Tier No. 1	2	3	4	5	6	7	8	9	10	Durchschnitt
Procain	40	17	22	38	30	52	43	20	25	32	31,9
I	0	0	0	0	0	0	0	0	0	0	0
II	0	0	0	0	0	0	0	0	0	0	0
III	0	0	0	0	0	0	0	0	0	0	0
Procain	22	43	20	38	32	22	35	38	23	23	29,6
IV	12	10	0	7	10	0	10	10	7	10	7,6
V	8	15	10	5	10	0	5	12	15	10	9,0
VI	18	20	20	15	15	10	7	15	20	20	16,0
Procain	24	40	22	38	25	20	22	38	20	20	26,9
VII	25	52	30	43	30	17	32	35	25	30	31,9
VIII	90	75	92	80	40	90	53	40	80	70	71,0
IX	108	100	45	85	85	115	120	120	70	108	95,6
Procain	32	23	35	22	20	53	30	35	37	23	31,0
X	116	112	100	110	102	112	153	90	95	125	111,5
XI	120	125	180	100	140	100	145	105	125	75	121,5
XII	125	210	196	140	175	150	175	180	180	190	172,1
Procain	20	38	15	22	40	22	20	25	30	37	26,9
XIII	190	235	175	180	225	115	205	185	235	195	194,0

LEGEND: Tests were performed in 5 groups at one-week intervals and with the same animals. All pharmaceuticals were injected in amounts of 0.25 ml of 1% concentration (intracutaneously).

p-oxybenzoic acid and its simple esters are designated by I-XIII, as indicated in text under EQUIPMENT & METHODOLOGY. 1% procaine was used as a control in every test series.

2) conduction analgesia: In contrast to the two above discussed test series, this one product VIII (pentyl ester) was found to be the most effective. Butyl and propyl ester effectiveness could also be unambiguously established, though it was small; the shorter and longer ester substitutions were of minor effectiveness. Fig. 3 shows the time-dependent effectiveness curve of procaine, of the esters from propyl, butyl and pentyl from p-oxybenzoic acid. It will be observed that pentyl ester becomes effective faster and stronger than procaine, while its time of effectiveness is shorter.

fig. 3



The conduction analgetic effect of several p-oxybenzoic acid esters as compared to procaine. All solutions are 1%. Ordinate: potential required for muscle contraction in mv (logarithmic scale in 1/100 mv); abscissa: time in minutes.

DISCUSSION OF RESULTS:

According to our test results, several simple esters from p-oxybenzoic acid display a local analgetic effect that seems to warrant their clinical testing. This can be argued for the more that their toxicity is considerably less than that of procaine. For subcutaneous administration, the LD_{50} of procaine amounts to 800 mg/kg, whereas the LD_{50} of the p-oxybenzoic acid ventyl ester amounts to about 3000 mg/kg; thus this product would be 3-4 times less toxic for mice than procaine.

It is remarkable, as we discovered, that the pharmacological and toxicological properties of the tested esters divided into 3 test series (A,B/a,B/b) show a fairly strong dependency on the number of C atoms in the ester-alkyl, and that in the fourth test series no such was found. The increasing effectiveness with lengthening of the side chain in the benzol ring is a known fact. The larger therefore the attention that must be paid to the contradictory result of the tests concerning the frog muscle-nerve preparation.

We are unable to provide an explanation for this singular phenomenon. Surface activity and distribution coefficient seem to obey TRAUBE's law only up to pentyl ester as regards the homologuous series for alcohols and aldehydes: the pentyl ester's values are surprisingly high when compared to the lower esters. There is no further increase of the above mentioned values for the higher esters. This may explain the better effectiveness of the pentyl ester as compared to the lower homologues, even though there is no good explanation for its superiority with respect to the higher homologues. No hasty conclusions are warranted with respect to this more favorable outcome for pentyl ester in this domain (T/c) with respect to its clinical suitability. Perhaps the more favorable behavior in test T/c is due to the direct application of the preparation on the exposed nerve trunk: if so, for tissue injection, the same considerations would not hold.

SUMMARY

The toxicity of p-oxybenzoic acid and of 12 of its simple esters was tested for subcutaneous injection for white mice, and for local-analgetic effect on rabbit horny layers, and on the back skin of guinea pigs, further for the frog muscle nerve preparation. With increasing ester-alkyl length, toxicity decreases and the local analgetic effectiveness at surfaces and for infiltration anesthesia increases. For conduction analgesia at the frog muscle nerve preparation, pentyl ester proved superior to the other substances tested. The favorable pharmacological and toxicological properties make it appear appropriate to test clinically the individual kinds of this homologuous series.

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MONMOUTH JUNCTION 4351

REPORT

from

APPLIED RESEARCH LABORATORIES, Inc.
DAYTON, N. J.

APPROVED BY THE N. J. STATE BOARD OF HEALTH AS A PUBLIC HEALTH LABORATORY

SUBJECT TOXICITY STUDY

INDEX MX-185
DATE January 8, 1942
PAGE 1

SAMPLE Mixture of 60 Parts of Propyl and 40 Parts of Ethyl Esters of Sodium Para Hydroxy Benzoate.

FROM Heyden Chemical Corporation, New York, N. Y.

RECEIVED

Study of the Chronic Toxicity of a Mixture of 60 Parts of Propyl and 40 Parts of Ethyl Esters of Sodium Para Hydroxy Benzoate.

The following report gives the results of a toxicity study of a mixture of 60 parts of propyl and 40 parts of the ethyl esters of sodium para hydroxy benzoate on rats over a period of eighteen months.

Young albino rats of weaning age were selected for this test and divided into five groups as follows:

Group I - Forty animals were fed the material at a level of 1 milligram per 70 grams which is equivalent to 1 gram of material per 70 kilograms of body weight. The average human would ingest only about one-fifth of this amount daily. At the end of two months ten of these animals were sacrificed for gross pathology and three selected for further histological study of the organs. Likewise, at the end of four months ten additional animals were sacrificed and the organs of three studied histologically.* At the end of the eighteen-month period, the remaining animals were similarly sacrificed for gross pathology and the organs of three animals taken for histological study.

Group II - Twenty animals were fed 10 times the quantity of the material administered in Group I, or 10 milligrams per 70 grams equivalent to 10 grams of material per 70 kilograms of body weight. These animals were continued throughout the eighteen-month period.

Group III - Twenty animals were fed the material at 100 times the quantity fed in Group I, or 100 milligrams per 70 grams equivalent to 100 grams of material per 70 kilograms of body weight. This group served as positive controls and were continued for the eighteen-month period.

* The results of the observations at 2 and 4 months have been previously reported and, therefore, only the results at the end of the eighteen-month period will be included in this report.

Group IV - Twenty animals were fed sodium benzoate at the same level as in G[illegible] I, or 1 milligram per 70 grams. At the end of two months ten of these animals were sacrificed and examined grossly and histological studies made of the organs of three animals. The remaining ten animals were sacrificed at the end of the fourth month and studied in a like manner.*

Group V - Twenty animals were fed a standard stock diet for the period of eighteen months and served as negative controls in order to study the pathology normally present in a rat of advancing age. At the end of the eighteen-month period gross studies of the animals at sacrifice were made together with histological studies of the organs of three rats.

In administering the test material an amount of the propyl and ethyl esters of sodium para hydroxy benzoate was incorporated in the food of Group I so that 1 gram of food would contain 1 milligram of the respective materials. Each morning these rats were given an amount of feed containing the required amount of material as determined by their weight on the previous week. After they had consumed the supplement, the regular stock diet was replaced. The same procedure of feeding the material was followed in Group II, receiving 10 times the amount of material, and Group III, receiving 100 times the amount of material given Group I.

The average monthly weight records, together with the mortality, of Groups I, II, III and V are given in Table I and further charted in Figure I. Gross pathological findings of the organs of individual animals at the end of the eighteen-month period are given in Table II. Table III presents the results of histological studies of the organs of representative animals.

Photomicrographs of representative organs are included.

*The results of this group have been previously reported.

TABLE I

Weights in Grams

Month	Group I	Group II	Group III	Group V
start	38	37	36	39
1st	100	108	96	97
2nd	168	177	162	159
3rd	202	208	180	190
4th	224	232	187	219
5th	242	236	202	226
6th	253	241	209	232
7th	260	240	223	244
8th	270	260	246	266
9th	281	263	232	246
10th	287	273	249	251
11th	296	282	266	263
12th	303	286	272	273
13th	309	292	272	272
14th	305	279	262	263
15th	305	291	263	268
16th	300	298	256	245
17th	301	292	261	256
18th	299	295	259	240
Gain	261	258	223	201
Mortality	1	7	2	8

TABLE II

Gross Pathological Observations
Group I

Rat #7 - Normal.
Rat #9 - Nose bloody. Beginning to lose hair. Lungs contain particles of dust. Small intestines somewhat gaseous.
Rat #10 - Nose bloody. Beginning to lose hair.

TABLE II (Cont)

Rat #11 - [illegible]
Rat #12 - Beginni[illegible] lose hair.
Rat #16 - Poorly nourished and stiff in movements. Loss of hair. Consolidated areas in lungs. Liver dark in color. Very little abdominal fat.
Rat #17 - Minute grey areas on surface of one lung.
Rat #18 - "Crooked neck" from middle ear infection. Greyish areas on surface of lungs.
Rat #19 - Poorly nourished. Audible breathing. Greyish areas on surface of lungs. Liver dark in color and spleen somewhat small. Very little abdominal fat.
Rat #20 - Grey areas on surface of lungs. Spleen small.
Rat #21 - Nose bloody. Minute grey areas on surface. Spleen small and pale.
Rat #23 - Lungs contain particles of dust together with small grey areas. Hydronephrosis.
Rat #25 - Properitoneal hernia. Dust particles in lungs. Spleen slightly pale.
Rat #30 - Movements stiff and animals poorly nourished. Dust particles in lungs surrounded by grey areas. Spleen small and pale.
Rat #31 - Nasal obstruction. Dust particles in lungs surrounded by grey areas.
Rat #34 - Grey areas scattered over surface of lungs. Spleen dark in color.
Rat #35 - Areas of consolidation. Grey areas on surface of lung.
Rat #37 - Loss of hair. Growth on right lung. Dust particles in lungs surrounded by grey areas. Only small amount of abdominal fat.
Rat #40 - Cortex of kidney somewhat pale.

Group II

Rat #41 - Loss of hair. Dust particles in lungs.
Rat #42 - Loss of hair. Hydronephrosis.
Rat #43 - Loss of hair. Cut sections of lungs ooze purulent material. Enlarged mesenteric glands.
Rat #44 - Fleshy area at periphery of one lung. Grey areas on surface of both lungs.
Rat #45 - Dust particles in lungs. One kidney misshapen. Poor contrast between medulla and cortex.
Rat #48 - Surface of kidney pitted.
Rat #50 - Dust particles in lungs.
Rat #51 - Dust particles in lungs.
Rat #53 - Loss of hair.
Rat #54 - Normal.

TABLE II (Cont)

Rat #55 - Dust particles in lungs.
Rat #59 - Grey areas on surface of lungs.
Rat #60 - Dust particles in lungs and grey areas on surface.

Group III

Rat #61 - Loss of hair. Small intestines hemorrhagic. Very little abdominal fat.
Rat #63 - Dust particles in lungs.
Rat #64 - Normal.
Rat #65 - Normal.
Rat #66 - Normal.
Rat #67 - Spleen pale.
Rat #68 - Intestines hyperemic.
Rat #69 - Fleshy area in one lobe of lung.
Rat #70 - Normal.
Rat #71 - Loss of hair. Grey areas on surface of lung.
Rat #72 - Dust particles in lungs.
Rat #73 - Normal.
Rat #74 - Normal.
Rat #75 - Dust particles in lungs. Tip of spleen dark in color. Only a small amount of abdominal fat.
Rat #76 - Fleshy area in one lobe of lung.
Rat #78 - Dust particles in lungs. Tip of spleen dark in color. Large intestine gaseous. Hydronephrosis.
Rat #79 - Emaciated. Fleshy area on one lobe of lung. Small intestines hemorrhagic. Tumor of uterine wall.
Rat #80 - Consolidation in one lung.

Group V

Rat #104 - Loss of hair. Dust particles in lung.
Rat #103 - Loss of hair.
Rat #102 - Grey areas on surface of lungs.
Rat #111 - Bloody nose. Diarrhea. Liver pale with nutmeg appearance. Spleen small and pale. Small intestines distended with gas. Kidney misshapen! Initial hydronephrosis - surface indented and pale. Poor differentiation of medulla and cortex. Only a small amount of abdominal fat.
Rat #108 - Lungs poorly aerated. Very little abdominal fat.
Rat #107 - Poorly nourished and stiff in movements. Spleen small and pale. Very little abdominal fat.

TABLE II (Cont)

Rat #114 - Loss of hair. Small sac-like growth filled with purulent material on lung.
Rat #113 - Fleshy area in one lobe.
Rat #112 - Poorly nourished. Stiff in movements. Small sac-like growth filled with purulent material on lung. Liver and spleen pale. Very little abdominal fat.
Rat #120 - Grey areas on surface of lungs.
Rat #119 - Dust particles in lungs and grey areas on surface. Only small amount of abdominal fat.
Rat #117 - Loss of hair.

TABLE III

Group I

Rat no.	Organs	Observations.
7	Lung	Normal.
	Liver	Nuclei granular. Slight confluence of cytoplasm.
	Spleen	Normal.
	Stomach	Normal.
	Small intestine	Normal.
	Kidney	Cloudy swelling and congestion.
	Adrenal	Normal.
19	Lung	Thickening of alveolar walls and infiltration of leukocytes.
	Liver	Mild cloudy swelling.
	Spleen	Normal.
	Stomach	Normal.
	Small intestine	Normal.
	Kidney	Glomeruli stiff and granular in appearance. Areas of mild cloudy swelling.
	Adrenal	Normal.
34	Lung	Thickening of alveolar wall and infiltration of leukocytes. Dust particles present.
	Liver	Cloudy swelling.
	Spleen	Congested.
	Stomach	Minute area of the mucosa infiltrated with leukocytes.

TABLE III (Cont)

Rat no.	Organs	Observations
34	Kidney	Glomeruli fill capsular spaces. Cloudy swelling of tubules.
	Adrenal	Normal.
	Group II	
41	Lung	Alveolar walls thickened. Dust particles present.
	Liver	Nuclei pyknotic - confluence of cell-cord cytoplasm.
	Spleen	Normal.
	Stomach	Normal.
	Small intestine	Normal.
	Kidney	Glomeruli shrunken and stiff. Mild cloudy swelling of tubules.
	Adrenal	Normal.
45	Lung	Thickening of alveolar walls. Dust particles present.
	Liver	Congested.
	Spleen	Normal.
	Stomach	Normal.
	Small intestine	Normal.
	Kidney	Glomeruli fill capsular spaces. In one area glomeruli have a hyaline appearance.
	Adrenal	Normal.
60	Lung	Alveoli thickened. Dust particles present.
	Liver	Normal.
	Spleen	Normal.
	Stomach	Normal.
	Small intestine	Normal.
	Kidney	Normal.
	Adrenal	Normal.

TABLE III (Cont)

Group III

Rat no.	Organs	Observations
63	Lung	Minute dust particles in alveoli. Few leukocytes present.
	Liver	Normal.
	Spleen	Normal.
	Stomach	Normal.
	Small intestine	Normal.
	Kidney	Normal.
	Adrenal	Normal.
71	Lung	Peripheral alveolar wall thickened and spaces contain many leukocytes. Dust particles scattered throughout.
	Liver	Normal.
	Spleen	Normal.
	Stomach	Normal.
	Small intestine	Normal.
	Kidney	Normal.
	Adrenal	Normal.
75	Lung	Dust particles present in alveolar spaces.
	Liver	Normal.
	Spleen	Congested.
	Stomach	Normal.
	Small intestine	Normal.
	Kidney	Tendency of glomeruli to appear shrunken.
	Adrenal	Normal.

Group V

103	Lung	Normal.
	Liver	Normal.
	Spleen	Normal.
	Stomach	Normal.
	Small intestine	Normal.
	Kidney	Normal.
	Adrenal	Normal.

TABLE III (Cont)

Rat no.	Organs	Observations
111	Lung	Compact areas of leukocytes in alveoli.
	Liver	Nuclei stain faintly. Confluence of cell-cord cytoplasm.
	Spleen	Malpighian bodies poorly defined.
	Stomach	Normal.
	Small intestine	Normal.
	Kidney	Glomeruli fill capsular spaces. Convoluted tubules swollen. Hydronephrosis of one kidney.
	Adrenal	Normal.
113	Lung	Consolidation. One-half of one lobe solid with leukocytes.
	Liver	Congested, mild cloudy swelling.
	Spleen	Slight congestion.
	Stomach	Normal.
	Small intestine	Normal.
	Kidney	Glomeruli shrunken and stiff and stain deeply. Desquamation of tubular epithelium.
	Adrenal	Normal.

Discussion of Results

In applying statistical analyses to the weight curves for the four groups it can be seen that there is a significant difference between Groups I and III and Groups II and III at six months. This difference becomes evident at the fourth month when the weight curve for Group III tends to level off. At the eighth month the average weight of Group III approaches Group V and compares closely with this group for the remainder of the test. At the end of eighteen months Groups I and II differ significantly from Groups III and V in average weight. Since the pathologies of the different groups were similar, it would appear that a stimulation of growth occurred as a result of the small amounts of the material fed in Groups I and II. It is difficult to explain exactly what may have taken place in Group III. There is a retardation of growth between the fourth and eighth month followed by the resumption of normal growth which would indicate a tolerance factor, in that a tolerance to the large amounts of material was established.

However, it is also possible that the effects on the growth rate from large amounts of the material may be related in some way to the age of the rat and the change in metabolic function.

The pathological changes found in each group of animals was essentially the same and typical of advancing age. Advanced hydronephrosis was noted in one or more animals of all groups. This condition, produced by an obstruction in the urinary tract, is not an uncommon finding in old rats since they are frequently subject to diseases of the urinary system. Lung infections and presence of dust particles noted in all groups are not significant findings. Such infections are commonly observed in animals at this age. The inhalation of particles of finely ground food and the resulting disposition of these particles in the lung tissue is not unusual. The mortalities in each group do not differ significantly.

Conclusions

From the above data it is concluded that the propyl and ethyl esters of sodium para hydroxy benzoate, even when fed at a level equivalent to 100 grams per 70 kilograms of human weight, did not produce any pathological changes not found in the control group. In fact these changes are noted in both the control and test groups. However, at this high level a significant decrease in growth rate was evident from the fourth to the eighth months.

Some evidence of growth stimulation was found when small amounts of the material, equivalent to 1 gram per 70 kilograms of body weight and 10 grams per 70 kilograms of body weight respectively, were fed to rats.

Respectfully submitted,
APPLIED RESEARCH LABORATORIES, Inc.
By:

G. R. Durfee
Director

MONMOUTH JUNCTION 4331

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REPORT

from

APPLIED RESEARCH LABORATORIES, Inc.
DAYTON, N. J.

APPROVED BY THE N. J. STATE BOARD OF HEALTH AS A PUBLIC HEALTH LABORATORY

SUBJECT TOXICITY STUDY

INDEX MX-124
DATE January 30, 1939
PAGE 1

SAMPLE
A - Sodium Benzoate
B - Mixture of 40 parts of Sodium Ethyl Para-hydroxy Benzoate plus 60 parts of Sodium Para-hydroxy Benzoate

FROM
Heyden Chemical Corporation
New York, N.Y.

RECEIVED

The comparative toxicity of the above samples was determined by their minimum lethal dose to guinea pigs. Since both of these products are principally employed as preservatives in food, they were orally administered in orange juice. This procedure was therefore adopted to more nearly duplicate the conditions of their use in actual practice. Although animals are customarily starved for 24 hours before administration of the materials tested by this method, this step was likewise omitted as more representative of normal use.

The test materials were fed in graduated doses per kilogram of body weight to group of animals. These guinea pigs were young animals averaging about 275 grams of both sexes selected from our regular stock colony. The animals were maintained in individual cages and were continued on the stock diet customarily fed.

The sodium benzoate was dissolved or suspended in orange juice in a concentration of 1 gram in 5 cc of orange juice for all the various levels used. In the case of the mixture of the esters, the lower levels were given in the same concentration. However, at the higher levels, in order to maintain the volumes within practical limits, it was necessary to reduce the concentration to 1 gram in 2.5 cc. This procedure was further indicated since large amounts could only be given in divided doses [illegible] elapsing thereby, even though always in one day, seemed to increase the degree of toxicity.

The individual animals were weighed immediately before the start of the test and the respective doses calculated on a basis of body weight. These were then fed directly to the animals by means of a feeding syringe graduated in hundreths of a cc. This technique was followed since this method insured each animal receiving the full amount of the exact dose.

Although deaths usually developed within a few days after the administration of the test doses all animals were continued under observation for two weeks. Any death occurring within this period was considered as the result of the treatment and was included in the final tabulation. The M.L.D. for the respective materials was taken as the smallest quantity necessary to cause death in from 60 to 80 per cent of the animals receiving that dose.

A review of the literature states that the M.L.D. for sodium benzoate is approximately 1 to 2.5 grams per kilo (1, 2, 3,) However, the differences in experimental procedure contemplated by us suggested that under our conditions these figures might be too low. On the toxicity of the various individual esters and all of these were done by German workers. Since no experiments appeared to have included combinations of the esters, the toxicity of a mixture of the ethyl and propyl esters could only be inferred as being about twice that for sodium benzoate. (4,5) Furthermore, little information was given in these reports as to the details of the different procedures employed.

In view of these considerations, it appeared desirable to obtain a more definite indication of the probable M.L.D. for each of the two test materials under the actual conditions contemplated by us. Therefore a preliminary test was carried out at various levels but in most cases with a fewer number of animals than customarily used in this type of experiment. The approximate M.L.D. for each material as obtained in this was would then be confirmed by additional groups of from five to ten animals per level. Table I following gives the number of animals used and average mortality at the preliminary levels for both the sodium benzoate and the mixture of the esters.

TABLE I

Material	Dose in g/k	No. of animals	Percent of mortality
Sodium Benzoate	4.5	5	100
	4.0	3	100
	3.0	3	100
	2.0	2	50
Mixture of Esters	4.5	6	50
	4.2	7	43

These results indicated that the M.L.D. for sodium benzoate would fall between 2.0 and 2.5 grams per kilo while that for the mixtures should be about 3.0 grams per kilo. Therefore, as stated above, additional groups of from five to ten animals were placed on test at the more probable of these suggested levels. Table II shows the various doses administered and the mortality together with the fate and changes in weight for the individual animals used in each case.

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APPLIED RESEARCH LABORATORIES, Inc.
DAYTON, N. J.

SUBJECT TOXICITY STUDY

INDEX MX-124

TABLE II

Sodium Benzoate

Guinea pig no.	Dose g/k	Weight in grams At start	At end	Gain or loss	Fate	Percent mortality
580	2.2	228	-	-	-	Died 1st day
581	2.2	247	-	-	-	Died 3rd day
582	2.2	[illegible]	-	-	-	Died 1st day
593	2.2	275	-	-	-	Died 1st day
594	2.2	[illegible]	-	-	-	Died 1st day
595	2.2	303	-	-	-	Died 1st day
596	2.2	274	222	-	52	Survived
597	2.2	319	-	-	-	Died 1st day
598	2.2	272	233	-	39	Survived
599	2.2	310	-	-	-	Died 1st day
Average		264.6			45.5	80.0

Mixture of Esters

583	4.75	237	233	-	4	Survived
584	4.75	192	-	-	-	Died 1st day
585	4.75	266	-	-	-	Died 1st day
586	4.75	270	-	-	-	Died 1st day
587	4.75	187	177	-	10	Survived
588	4.75	[illegible]	257	-	35	Survived
589	4.75	[illegible]	245	-	9	Survived
590	4.75	301	282	-	19	Survived
591	4.75	[illegible]	183	-	92	Survived
592	4.75	[illegible]	238	-	36	Survived
Average		254.8			34.2	30.0
600	5.0	[illegible]	294	-	13	Survived
601	5.0	[illegible]	-	-	-	Died 12th day
602	5.0	285	-	-	-	Died 1st day
603	5.0	272	-	-	-	Died 1st day
604	5.0	300	204	-	96	Survived
Average		[illegible]			54.5	60.0

TABLE II (cont)

Mixture of Esters (cont)

Guinea pig no.	Dose g/k	Weight in grams At start	At end	Gain or loss		Fate	Percent mortality
605	5.3	361	-	-	-	Died 10th day	
606	5.3	270	-	-	-	Died 4th day	
607	5.3	315	257	-	58	Survived	
608	5.3	331	-	-	-	Died 14th day	
609	5.3	241	222	-	19	Survived	
610	5.3	297	232	-	65	Survived	
611	5.3	275	-	-	-	Died 1st day	
612	5.3	271	-	-	-	Died 3rd day	
613	5.3	321	209	-	112	Survived	
Average		298.0			63.5		55.6
614	6.0	280	-	-	-	Died 1st day	
615	6.0	254	-	-	-	Died 1st day	
616	6.0	268	204	-	64	Survived	
617	6.0	303	-	-	-	Died 5th day	
618	6.0	331	260	-	71	Survived	
Average		287.2			67.5		60.0

A comparison of the above data shows that the M.L.D. for sodium benzoate is 2.2 grams per kilogram of body weight when a mortality of from 60% to 80% is used as a criterion. This figure is in close agreement with the values given in the literature and especially in view of the less stringent procedure employed by us. That these modifications in technique were not only warranted by also more representative is amply demonstrated by a consideration of the intended uses of the test materials.

On the other hand, the mixture of the esters produces a much less definite end point in mortality, approximately the same value being obtained whether 5.0, 5.3 or 6.0 grams per kilogram were administered. It is pertinent to note that although the same mortality was produced by each of these doses, the appearance of the surviving animals became progressively worse as the dose was increased. Therefore, the M.L.D. for a mixture consisting of 40 parts of sodium ethyl para-hydroxy benzoate plus 60 parts of sodium propyl para-hydroxy benzoate was found to be between 5.0 and 6.0 gs. per kilogram.

How[illegible] [illegible]ting the minimum figure fulfilling the [illegible]quiremen[illegible] [illegible] this character demonstrates the mixture of the es[illegible] [illegible] somewhat less than one half as toxic as sodium benzoate.

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APPLIED RESEARCH LABORATORIES, Inc.
By:

G. R. Durfee
Director

Arch. Internat. Pharmacodyn. Therap. 34(2): 173-179
1928

From the "Centraal Laboratorium voor de Volksgezondheid" Utrecht, Holland

Solbrol (bi-para-oxybenzoic methyl-ester)

by Lecturer Dr. med. U.G. Bijlsma
pharmacologist of the laboratory

The producer of Solbrol recommends this substance for preservation instead of salicylic acid, because of the statement that smaller amounts of Solbrol are enough and it is even less poisonous than salicylic acid.

There are statements to be found about the antiseptic effect of the meta- and para-oxybenzoic acid. Kolbe (1) found both ineffective, Stockman observed that both had 1/10 of the efectvity of salicylic acid. By the way, nothing could be found in the literature either about the poisonousness of the antiseptic effect of the para-oxybenzoic acid and its ester.

The producer still showed excerpts of opinions of Prof. Joachimoglu (Berlin), which indicated that a dog got a daily 18 mg Solbrol / kg over a period of 28 days without getting ill because of it. The naimal was killed, liver and kidneys did not show any pathologic changes. A human being once took 500 mg Solbrol, a test-person took 200 mg daily over a period of 28 days, later on 500 mg daily over 4 days. Two test-persons took 1 g Solbrol daily over 29 days, later on 2 g daily over 28 days. With none of the test-persons symptoms of intoxication or pathologic findings could be found.

Solbrol is a crystalline product, which dissolves as follows: in water up to roughly 0.4 %; in 50 % glucose-solution up to roughly 0.2 %; in alcohol up to roughly 40 %; in ether up to roughly 17 %; in acetone up to roughly 40 %. It smells a little like anised and has a bitter-acid taste.

The growth retarding effect on micro-organisms was examined:

a. for coli commune bacteria

The test-series showed fully identical results where the growth of B. coli in glucose broth was fully retarded by Solbrol 0.12 %, partially retarded by Solbrol 0.10 to 0.06 % whereas the growth at presence of 0.04 % could not be distinguished from the control tubes. In order to get the same effects with salicylic acid the same concentrations were necessary.

b. for mildew fungi

The mildew-fungi were cultivated on a culture-medium of 5 g dried broad-powder and 15 cm^3 liquid. Solbrol and Salicylic acid were dissolved up to the following concentrations in the liquid.

For the first test-series the culture-media were sowed with a mildew-fungus not defined. It was found that 0.35 % Solbrol fully retarded the

growth. For salcylic acid these concentrations were resp. 0.15 and 0.05 %. So Solbrol in this test reacted half as intensely as salicylic acid.

For the second test-series a mixture of mildew-fungi was vaccinated into the culture-media. Here 0.[illegible] % Solbrol was only a partially retarding concentration, 0.35 % had no effect. No greater concentrations than 0.2 % were examined for the salicylic acid. They had no effect. In this test-series the effect of Solbrol was more than 50 % of that of the salicylic acid.

For a third series Penicillium majusculum was used. It was fully retarded by Solbrol 0.15 %, while salicylic acid 0.4 % did not show any effect. So in this test-series Solbrol reacted more than 2 2/3 more intensely than salicylic acid.

Obviously not all mildew-fungi are equally sensitive to both substnaces, one time Solbrol, and another salicylic acid is more effective. Therefore an objective comparison of the two substances is from this point of view not possible.

c. Stoppage of Fermentation

A suspension of Saccharomyces cerevisiae was put into fermentation-tubes in 10% glucose-solution, to which different concentrations of Solbrol resp. salicylic acid (both up to 0.2 %) were added. The same amount of gas was produced in all tubes; obviously neither 0.2 % Solbrol, nor 0.2 % Salicylic acid retarded the fermentation. Naturally this does not show anything about a possible growth-retardation of the saccharomyces. In order to answer the question about the dangerousness of Solbrol for life and health the following tests were done:

a. deadly dosis for white mice

Mice received increasing amounts of sub-cutaneous injections of Solbrol. Higher dosis up to 165 mg caused a temporary general exhaustion and a slow-down and a recess of the respiration. There was always full recovery. Because of the low solubility greater amounts of subcutaneous injections are not possible. The deadly dosis for white mice thus lies higher than 333 mg/kg for sub-cutaneous administering.

For the salicylic acid there are statements about deadly doses of 150 mg (rats) to 500 mg (rabbits) for different species.

b. a dog weighing 10 kg got twice 1 g Solbrol peroral, each time during 4 subsequent days. Pathologic appearances were not observed. (see table under c)

c. most of the authors who study the effect of salicylic acid on the nitrogen metabolism, come to the conclusion that salicylic acid increases the N-secretion. (Bibliography , Hanzlik (3)). Therefore a test in this direction was also done with Solbrol The nitrogen was determined according to Kjeldahl in the food, urine (caught in a bougie), and faeces (the faeces were dried in hot air, the

combined portions of each period mixed and analysed) of a metabolism-test with a dog. Twice a period of a Solbrol supply was included, one period of salicylic acid supply. Of this substances always 1 g/day was given, with the exception of one day when accidentall 1.5 g Solbrol were added. The results are seen in the following table, and fig. 1 and2. Fig. 1 gives the daily amount of N found in the urine (white column) and the same in the faeces (black column), the latter figured out of the N-contents of the mixed faeces of the whole period.

Out of the table and fig. 1 and 2 it can be concluded, that during the salicylic acid period an unequivocal increase of the nitrogen-secretion in the urine has taken place. You cannot say this for the first Solbrol-period. Even during the second Solbrol-period the amount of N in the urine does not increase, but a considerable simultaneous increase of the N-secretion in the urine, which indeed can be seen. An effect which would lead to an increase of N-secretion in the urine, could be concealed by this. There it cannot be determined with certainty, during the second Solbrol-period whether Solbrol has an effect on the N-secretion in the urine or not. Probably this is not because no such influence could be observed during the first Solbrol-period.

The increase of the secreted amount of N in the faeces of the second Solbrol-period can be based on a hold back of excrement during the preceeding normal period. This cannot explain everything quantitatively, specially not, because the percentage contents of the dry substance of the faeces during the second Solbrol-period is considerably higher concerning N than during the other periods. At least a partially reduced resorption of nitrogen-containing products out of the stomach-intestines tract can be held responsible for this increase of the N-secretion. Whether or not this reduction of resorption is because of the Solbrol is, however, doubtful, as it does not appear during the first Solbrol-period.

d. Finally it was examined whether the growth of young rats is affected by a daily supply of Solbrol (and of salicylic acid) to the food, Each time a litter of young rats was devided into two equal halves: the one group was kept as a check-up, the other group got Solbrol or salicylic acid in the food in amounts of 1/2 or 1/4 mg/g per rat. As one may see out of fig. 3 all animals treated with Solbrol (1/2 mg and 1/4 mg) and salicylic acid (1/2 mg) were retarded in their growth. When it suddenly became cold on September 27 while the central heating did not yet function all treated test-animals got sick and died, even heating on October 1 and the precautions taken in the meantime could not save the animals. The check-up animals withstood all these difficulties. It follows that those animals treated with salicylic acid and Solbrol showed a decreased resistance against harmful, outer conditions. These experiments were

extensively repeated when the temperature situation improved. Now no unfavourable influence of salicylic acid or Solbrol on the growth could be detected.

Opinion:

From statements in the literature, from experiments of Joachimoglu, and from the experiment described above the following is concluded:

1. that the growth retarding effect of the bi-para-oxybenzoic methyl ester for bacteria and mildew-fungi lies within the range of the salicylic acid; that in order to get the same effects equal weights of both substances must be used.
2. that the general (overall) poisonousness of Solbrol is not very high, that consequently there are no dangers of life to the usage of this substance for preservation.
3. that no danger of health have been observed for human beings (Joachimoglu, if the excerpts of his opinion do not conceal any facts), but that at least the taking of large amounts would not be unobjectionable, as could be seen in the last test with young rats, where the chronic supply of Solbrol as well as salicylic acid made the animals less resistant to bad outer conditions. Without these bad outer conditions Solbrol and Salicylic acid were even tolerated up to 4 g/kg per day.

In general there was a great accordance between Solbrol and salicylic acid concerning the growth retarding effect on micro-organisms and its influence on young rats.

In contrast to this the effect of Solbrol on the N-metabolism in of a dog is clearly less than the one of an equal amount of salicylic acid.

For this advantage of Solbrol there is up to now the disadvantage that temporarily one has only little experience with this substance, whereas much more is known about the salicylic acid.

The following fact should be mentioned: If Solbrol were admitted in the same amount as the salicylic acid, then one would have to expect a maximum absorption of 250 mg Solbrol for grown-ups. If this amount were decomposed to para-oxybenzoic acid and methyl alcohol in the body, then in such a way a maximum of 54 mg methylacohol could be supplied to the body. This is a dosis, which most probably would not cause any troubles. And besides this an amount of 250 mg Solbrol per day is an extremely high estimate.

Key to the table

a=date
b= weight of the dog in kg
c=nitrogen in the food
d=addition
e=faeces
e_1=weight (dry)
e_2=nitrogen (average daily amount)
f=urine
f_1=amount
f_2=nitrogen
g=secreted nitrogen
h=nitrogen difference
i= comments
j=average
k=vomiting

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f_2=nitrogen
g secreted nitrogen
h=nitrogen difference
i= comments
j=average
k=vomiting

a Datum	b Gewicht des Hundes Kg.	c Stickstoff im Futter g.	d Zusatz	e Fäces Gewicht (trocken) e_1 in g.	e Fäces Stickstoff (mittl. Tagesmenge) in g. e_2	Urin f Menge cc. f_1	Urin f Stickstoff g. f_2	Ausgeschiedener Stickstoff g. g	Stickstoffbilanz g. h	i Bemerkungen mg. N pro g. Fäces
20/6	19	5,972	—			620	4,826	6,277	— 0,245	
21/6		5,972	—	97,0	1,391	955	4,786	6,177	— 0,205	
22/6		5,972	—			800	4,928	6,319	— 0,347	
23/6		5,972	—			780	4,903	6,294	— 0,322	
Mittel		5,972		24,3	1,391		4,861	6,252	— 0,280	57,2
24/6	19	5,972	1 g. Solbrol			1160	5,132	6,333	— 0,361	
25/6		5,972	1,5 g. Solbrol	81,7	1,201	725	4,872	6,073	— 0,101	
26/6		5,972	1 g. Solbrol			680	4,855	6,056	— 0,084	
27/6		5,972	1 g. Solbrol			590	4,741	5,942	+ 0,030	
Mittel		5,972	1,1 g. Solbrol	20,4	1,201		4,900	6,101	— 0,129	58,9
28/6	19	5,972	—			750	4,862	6,407	— 0,435	
29/6		5,972	—	107,7	1,545	780	5,132	6,677	— 0,705	
30/6		5,972	—			1100	5,082	6,627	— 0,655	
1/7		5,972	—			1195	5,253	6,798	— 0,826	
Mittel		5,972		26,9	1,545		5,082	6,627	— 0,755	57,4
2/7	19	5,972	1 g. Ac. salizyl.			915	5,278	6,645	— 0,673	
3/7		5,972	1 g. Ac. salizyl.	101,0	1,367	910	5,580	6,947	— 0,975	K
4/7		5,972	1 g. Ac. salizyl.			1270	5,725	7,092	— 0,120	Erbrechen.
5/7		5,972	1 g. Ac. salizyl.			875	5,562	6,929	— 0,957	
Mittel		5,972	1 g. Ac. salizyl.	25,3	1,367		5,536	6,903	— 0,931	54,0
6/7	19	5,972	—			1355	5,236	6,010	— 0,038	
7/7		5,972	—	67,0	0,774	845	5,465	6,239	— 0,267	
8/7		5,972	—			1055	4,638	5,412	+ 0,560	
9/7		5,972	—			1170	5,209	5,983	— 0,011	
Mittel		5,972		16,8	0,774		5,137	5,911	+ 0,061	46,1
10/7	19	5,972	1 g. Solbrol			1005	4,584	7,480	— 1,508	
11/7		5,972	1 g. Solbrol	127,3	2,896	925	4,861	7,757	— 1,785	
12/7		5,972	1 g. Solbrol			825	4,366	7,262	— 1,290	
13/7		5,972	1 g. Solbrol			1000	4,424	7,320	— 1,348	
Mittel		5,972	1 g. Solbrol	31,8	2,896		4,559	7,455	— 1,483	91,1
14/7	19	5,972	—			985	4,551	5,595	+ 0,377	
15/7		5,972	—	66,5	1,044	1265	4,994	6,038	— 0,066	
16/7		5,972	—			1150	4,798	5,842	+ 0,130	
Mittel		5,972		22,2	1,044		4,781	5,825	+ 0,147	47,0

G. j

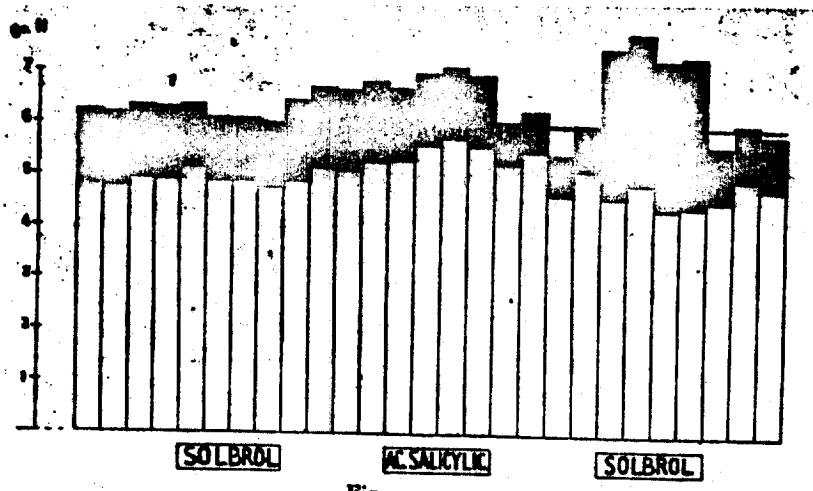

Fig. 1.

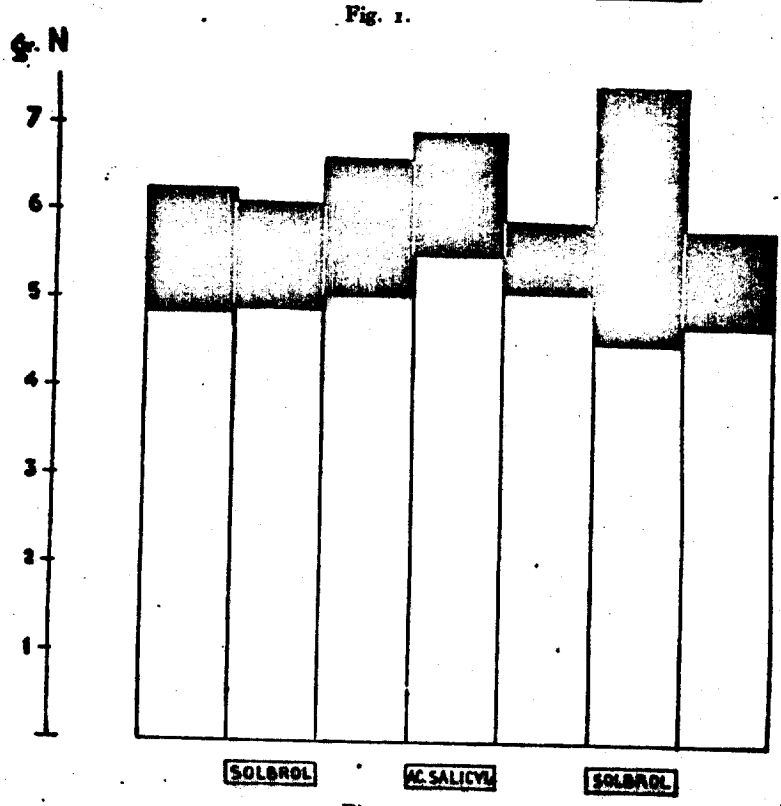

Fig. 2.

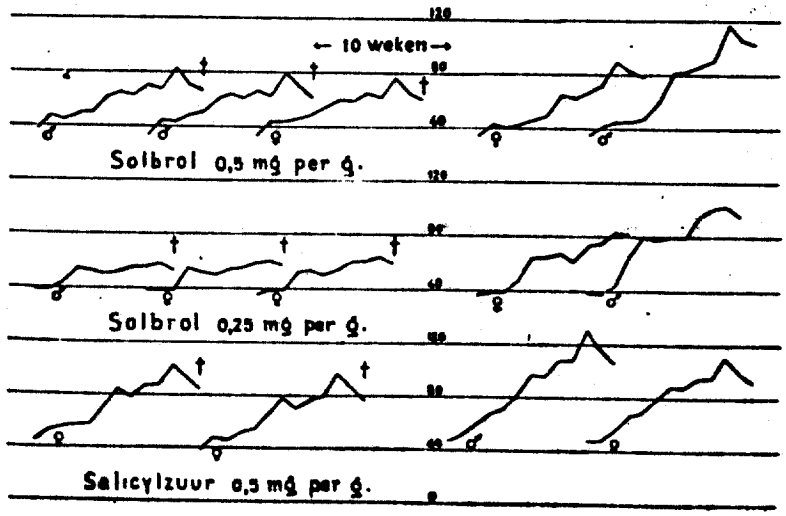

Fig. 3.

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THE INDUCTION OF TUMOURS IN MICE BY INTRAVAGINAL APPLICATION OF CHEMICAL COMPOUNDS

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Many workers have induced carcinoma of the uterine cervix by application of carcinogenic compounds, thus Teutschländer (1926) induced carcinomata of the cervix in rats by application of coal tar. Glucksmann and Cherry (1958) induced sarcomata of the genital tract in rats receiving local application of 9, 10-dimethyl-1, 2-benzanthracene and oestradiol benzoate. Scarpelli and Haam (1960) reviewed the subject of experimental carcinoma of the uterine cervix and consider that oestrogens have a cocarcinogenic or promoting action in the production of tumours in the genital tract.

Tumours have, however, been induced in the genital tract of animals by preparations or substances which are not generally considered to be carcinogenic. Thus Pratt-Thomas, Heins, Latham, Dennis and McIver (1956) produced tumours in mice by intravaginal application of pooled human smegma, and Gardner (1959) induced tumours in BC strain mice by intravaginal application of a mixture of urea, adipic acid and carboxymethyl cellulose. In both these cases the induction period was over 60 weeks.

Although there is no clinical evidence that the chemical contraceptive preparations cause cancer of the cervix or uterus, the spermicides used in such preparations should be tested for toxicity. As carcinogenic agents are generally nuclear poisons, and as spermatozoa consist mainly of nucleus, one might expect good spermicides to be carcinogenic. One of the previously used spermicides, 8-hydroxyquinoline, induced tumours when implanted in the mouse bladder (Allen, Boyland, Dukes, Horning and Watson, 1957). Hoch-Ligeti (1957) described the induction of vaginal tumours by application of two contraceptive preparations (one of which contained 8-hydroxyquinoline) to rats maintained on a low protein diet.

With these observations in mind, tests were carried out with a series of spermicides which are used in contraceptive preparations. Control experiments with the medium alone and with the medium containing known carcinogenic compounds were carried out at the same time. The application of the medium polyethylene glycol (Carbowax 1000) alone, however, induced tumours in quite a high proportion of the treated mice. In the series treated with Carbowax containing 9,10-dimethyl-1,2-dibenzanthracene the tumours appeared much earlier than in the other groups. The results indicate that the repeated instillation of a chemically inert polymer into the vagina of the mouse induces carcinomata.

This action of the polymer may be related to the carcinogenic effect of other inert polymers, injected polyvinylpyrolidone (Hueper, 1956) or carboxymethylcellulose (Lehman, Lusky and Nelson, 1957) in rats.

EXPERIMENTAL

Substances under test were dissolved in polyethylene glycol (Carbowax 1000) and 0·1 ml. introduced twice weekly into the vagina of mice, using a syringe with a blunt needle. Each substance was administered to 20 mice, starting immediately after weaning. The mice were kept in metal boxes with originally 5 animals in each box. All mice were killed 18 months after the first treatment with the substances under test.

RESULTS

The results of the first experiment (Table I) show that although tumours occurred in most of the groups of mice, the highest incidence was generally in groups treated with substances which have induced cancer in other animals, i.e.

TABLE I.—*Incidence of Tumours of Cervix and Vagina in Stock Mice*

Material applied in Carbowax	Number of mice surviving		
	12 months	18 months	With carcinomata
Series 1			
Carbowax only	11	7	5
9,10-Dimethyl-1,2-benzanthracene (0·3 per cent)	10	0	9
2 bis(2-chloroethyl)amino-naphthalene (2 per cent)	9	4	6
Hexylresorcinol (1 per cent)	8	4	4
8-Hydroxyquinoline (1 per cent)	10	2	7
Ricinoleic acid (2 per cent)	10	7	4
Quinine sulphate (0·3 per cent)	8	6	4
Phenylmercuric acetate (0·5 per cent)	12	10	6
Toluquinone (0·3 per cent)	11	10	6
3-Hydroxyanthranilic acid (2 per cent)	12	6	2
Sodium *p* toluene sulphon chloramide (1 per cent)	14	11	3
Boric acid (2 per cent)	13	8	0
Stilboestrol (0·1 per cent)	9	5	1
Methyl *p* hydroxy benzoate (1 per cent)	16	8	0
Triton WR (2 per cent)	9	8	0
HOC 8* (2 per cent)	16	11	1

* HOC 8 is the code name of 1,8,15,22-Tetra (polyoxyethyleneoxy) 4,11,18,25-tetra (1,1,3,3,-tetramethylbutyl)-cyclotetra-*m*-benzylene.

9,10-dimethyl-1,2-benzanthracene (Bachman, Kennaway and Kennaway, 1938), 8-hydroxy quinoline (Allen *et al.*, 1957; Hoch-Ligeti, 1957). Tumours were induced by these two compounds and also by 2-bis(2-chloroethyl)aminonaphthalene much more quickly than by the other substances; all the mice treated with 9,10-dimethyl-1,2-benzanthracene were dead within 9 months of the start of the experiment.

The compounds which did not induce tumours (boric acid, methyl *p* hydroxy benzoate and the non-ionic surface active agent Triton WR) are all mild antiseptics. The low incidence in the stilboestrol group is noteworthy.

The definite incidence of tumours in the group of mice treated with Carbowax 1000 only might have been due to the low osmotic pressure of the polymer. For this reason, another series of experiments starting with 30 mice in each group, was carried out with carbowax containing sodium chloride as shown in Table II.

TABLE II.—*Incidence of Tumours of the Cervix and Vagina in Stock Mice*

Material applied in Carbowax	Number of mice surviving		
	12 months	18 months	With carcinomata
Carbowax alone	24	20	2
Sodium Chloride (0·8 per cent)	22	14	5†
Sodium chloride (30 per cent)	18	14	8

† Including one tumour of anal gland.

The incidence of tumours was lower in the control series of this experiment, and was higher when the carbowax was made isotonic with salt and even higher with 30 per cent salt. This experiment shows that the carcinogenic action of carbowax is unlikely to be due to the effect of the low osmotic pressure.

PATHOLOGICAL OBSERVATIONS

All the tumours developing in this series of animals were carcinomas: no sarcomas were found.

Hyperplastic (? premalignant) changes.—Apart from definite tumour formation, various degrees of epithelial hyperplasia and hyperkeratosis were found in many animals. Blunt epithelial downgrowths often formed part of the hyperplastic process, and occasionally foci of nuclear irregularity and dyskeratosis were found (Fig. 1). There appeared to be all transitions between simple hyperplasia, carcinoma in situ and frank invasive cancer. The subepithelial tissues frequently displayed oedema and inflammatory-cell infiltration.

Tumour formation.—On naked-eye examination the vaginal neoplasms took the form of thick greyish plaques, papillary formations or bulky polypoid masses. The majority of the tumours clearly arose from a very extensive "field" of altered epithelium: the whole vaginal and cervical mucosa were often affected. Three different microscopic patterns could be distinguished, although it must be stressed that these were frequently associated within the same growth:

(1) "Basal cell" tumours, composed of compact groups of small darkly staining

EXPLANATION OF PLATE

FIG. 1. Longitudinal section through vaginal wall of a mouse implanted with HOCS. There is considerable hyperplasia and hyperkeratosis of the stratified squamous epithelium. Some dyskeratosis is seen in the epithelium in the upper part of the field. H. and E. ×112.

FIG. 2. Mouse implanted with phenyl mercuric acetate. Infiltrating vaginal carcinoma composed of small darkly-staining "basal" cells. H. and E. ×112.

FIG. 3. Mouse implanted with 8-hydroxy-quinoline. Poorly-differentiated squamous cell carcinoma infiltrating beneath hyperplastic epithelium of the vaginal wall. Nuclear pleomorphism, hyperchromasia and mitotic activity are all prominent. H. and E. ×112.

FIG. 4. Mouse implanted with phenyl mercuric acetate. Section prepared from the edge of an infiltrating vaginal carcinoma showing "clear", mucin secreting cells at the centres of epithelial downgrowths. H. and E. ×112.

1 2

3 4

Boyland, Charles and Gowing.

cells with hyperchromatic nuclei and scanty cytoplasm. There were no intercellular bridges and keratinisation was minimal (Fig. 2). These neoplasms somewhat resembled basal cell carcinomas of the skin but it is not suggested that they have any close biological kinship with them.

(2) Squamous cell carcinomas, displaying "prickle cell" formation and keratinisation. All grades of malignancy were found, from well differentiated slowly growing tumours to growths which showed nuclear pleomorphism and numerous mitoses (Fig. 3).

(3) Mucin secreting tumours. The mucous cells appeared to arise by differentiation of the proliferating basal cells as they approached the surface. Thus the fronds of a papillary neoplasm were sometimes covered by cells distended with basophilic mucin. On the other hand, where the proliferating cells were arranged as downgrowths, the mucous cell differentiation occurred towards the centres of the groups so that gland-like structures were found (Fig. 4). As might be expected, the tendency to mucin production was usually most marked in tumours occupying the cervix and upper vagina, but in some cases growths in the lower vagina showed extensive mucous cell metaplasia. Mucin secreting and squamous patterns were often combined ("muco-epidermoid" pattern).

DISCUSSION

The experiments indicate that cancer of the female genital tract in mice is induced by non-specific chemically inert materials. They leave open the question of the possible carcinogenic activity of spermicides used in contraceptive preparations. In order to test the possible carcinogenic activity of these spermicides, some medium must be used which does not itself induce tumours. Alternatively, it might be possible that if fewer applications had been made the incidence of cancer in mice treated with the carbowax alone would have been sufficiently low to make an assessment of carcinogenic activity possible.

Although the carbowax used in the experiments may have induced tumours, the material would probably not present a hazard to man. Carbowax is used in cosmetics and as a medium for medicaments.

SUMMARY

Mice were treated by intravaginal application of Carbowax 1000 alone, and containing known carcinogens, and with spermicides twice weekly for one year. Although tumours developed more quickly and in a higher proportion of mice treated with Carbowax containing 9,10-dimethyl-1,2-benzanthracene than in other groups, the incidence of tumours in the control series treated with Carbowax alone was so high that the possible carcinogenic activity of the spermicidal compounds could not be assessed.

We should like to thank Mr. R. O. Rees for help with these experiments. The research was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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RESEARCH ON THE ANTISEPTIC PROPERTIES OF A DERIVATIVE OF SALICYLIC ACID: THE METHYLIC ESTER OF P-HYDROXYBENZOIC ACID

by Dr. Mario Adorni Braccesi

The problem of the preservation of foods by means of chemical substances has become a current one today after the identification of the antiseptic properties in some isomers of salicylic acid without toxic action.

Our health laws, as those of other countries, prohibits the use of chemical preservatives , all of which have a relative toxicity, but these laws could be revised , without impairing their hygienic purposes, by finding a preservative which is active and non-toxic.

Because recently there was prepared in Italy a methylic ester of p-hydroxybenzoic acid (Abiol), identifiable with the Nipagin M prepared overseas and which was the subject of numerous investigations, I studied this compound for two purposes:

1. to establish antiseptic properties,
2. to determine the toxic action on laboratory animals.

The determination of the antiseptic properties was carried out preparing initially liquid and solid culture grounds containing the substance being examined in various proportions and seeding thdse grounds with Schizomycetes and Hyphomycetes . The results were probative. There was an almosg certain antiseptic action on the Schizomycetes with product concentration of 1.5 o/oo. On the anaerobia there was a very evident action with the concentration of 4 o/oo . On the Hyphomycetes the action was certainly active in the concentrations of 1.5 o/oo and 1 o/oo, and in some stocks even in the concentration of 0.5 o/oo.

This compound was also proven to be preservative in the concentration of 1.5 o/oo on a syrup which was maintained at ambient temperature without special precautions. It showed a very distinct antifermentative action without changing the organoleptic properties of the syrup itself.

Proven as a disinfectant, it acted in a time close to that of an equivalent solution of phenol.

These results would have had only a relative value if they had not demonstrated the absolute innocuousness of the product by means of the investigation of the toxicity in laboratory animals: rabbits, guinea pigs, albino rats.

For reasons inherent to the practical application of the product, I followed several ways of administering it. For the part referring to the preservation of food substances I followed the oral administration. For the part referring to the pharmaceutical technique I also used the parenteral and endovenous means.

For the data relative to the posology , since in the literature there is registered the observation carried out on two persons to whom for one month there was administered orally 2 g of methylic ester of the p-hydroxybenzoic acid without the occurrence of either local or general damage, I took this dose as the given basic start , having naturally made the appropriate calculations, with regard to the differences in weight between man and the experimental animals.

I was able to ascertain an innocuousness even with prolonged use and in doses considerably greater than the minimum necessary in its practical application.

From these tests of mine I was able to conclude that Abiolcombines with a very distinct antiseptic action the reward of being practically free of toxic properties.

from the Institute of Hygiene of the
royal university of Florence

Section of Florence- Seating of Feb. 24 1939-XVII.

Arzneimitt.-Forsch. 7(6): 340-345, 1957.

ON THE PHARMACOLOGY OF BENZOIC ACID, OF PARACHLOROBENZOIC ACID AND OF PARAOXYBENZOIC ACID AND ITS ESTERS.

M. v. Bubnoff, D. Schnell and J. Vogt-Moykoff

In a previous communication, pharmacological effects not known before had been reported with respect to several benzoic acid preparations used as preservatives (Bubnoff etal). Besides vascular spasmolytic and ganglioplegic effects, we also were able to show a performance-decreasing effect on the striated musculature pretreated with Prostigmine. Further experiments were supposed to resolve the question whether pharmacological effects can be shown for oral administration of such substances. So far it is only known for benzoic acid that anticonvulsive effects will occur even for oral ingestion, -Zwissler. According to Schuebel & Manger and Metzger etal, the paraoxybenzoic acid esters however are completely reabsorbed from the intestine whether saponified or not. Sokol was able to show non-saponified esters in the plasma (also see Richardson and collaborators) Therefore one had to expect that oral ingestion of paraoxybenzoic acid esters would also show pharmacological effects. The methods we had previously used seemed unsuited for testing orally administered substances which might not become effective until after lengthy reabsorption processes had taken place. Therefore we made use of the method of Eichholtz & Hoppe for testing the anticonvulsive effects in the cocain cramps in rats; this is the method also used by Zwissler for benzoic acid. Following a check of Zwissler's results, such tests were supposed to be carried out also with respect to parachlorobenzoic acid and parachlorobenzoic acid esters.

Furthermore, carrying on with prior research concerning the vascular spasmolytic effects in the kidneys and extremities and also as regards anticonvulsive effectivenss, the influence of benzoic acid and its derivatives on brain blood supply was examined.

METHODOLOGY

We used the method of Eichholtz & Hoppe for testing the anticonvulsive effects of benzoic acid, parachlorobenzoic acid and paraoxybenzoic aicd. The cramp threshold for cocain was determined for 3 tribes of rats (white) each of about 25 members weighing from 100-150 gm, by means of iv. injections in increasing amounts of 1% cocaine solution. The injection rate approximately was 0.1 cc/100gm-30 seconds. Cocaine injections were 48 hours apart. Prior to each, the animals were not fed for 12 hours. The percentage of animals subject to cramps was graphically plotted for each tribe as a function the cocaine dose and the accuracy of these "fundamental cocaine curves" was tested against the determination of unknown cocaine concentrations. The substances to be tested for anticonvulsive effects were ingested 30 seconds prior to cocaine injection if administered iv. and 45-180 minutes before if by pharynx bougie**. Thereafter we increased the cocaine dose until cramps occurred. Each fundamental curve was rechecked prior to testing of each substance; rats with necrotic tails were eliminated and replaced by animals with the same cramp dosis.

** Orally administered substances to be tested were dissolved in aqua dest. Doses with insufficient water solubility were ingested as deposits in water by means of the pharynx bougie.

The concentrations required for iv. administration were satisfied by the given water solubility; merely when dealing with parachlorobenzoic acid was there a requirement for several drops of

For two other tribes we used an adrenaline solution of 1/7-1/8x 10^{-4} added to the cocain in order to induce cramps. This solution too was injected 0.1cc/100 gm in 30 seconds; however we did not increase the amount of liquid administered to determine the cramp dosis, rather we increased the cocain concentration, which was 0.5-0.9%. It was found necessary to keep the adrenaline dosis constant, since for simultaneous increase in cocaine and adrenaline doses, since a high percentage of the animals reached ad exitum following sudden and severe cramps and/or edema of the lungs.

Brain blood supply is measured via the arteria vertebralis in cats weighing from 2.2-3.7 kg under urethane chlorelose anesthesia (250 or 60 mg/kg)-- see Taeschler etal. However we did not prepare the vessel ahead of its entry into the neck vertebra, but only shortly before its orifice into the skull-inside, between the first and second neck vertebra. This eliminates falsifications in blood supply values because of anastomoses with other vessel areas to a large extent. Dye injections into the arteria vertebralis used for recording showed that only a small lateral branchlet which could be neglected was pulling to the neck musculature. In order to freely expose the vertebral artery, skin and musculature between the first and fourth transverse appendices were bilaterally cut; the first neck nerve was used as the reference point for finding the artery, this nerve crossing the vessel shortly before entering the vertebral canal. After carefully freeing the arteries from the connective tissue sheath commonly enveloping vertebral artery and vein, where the latter had to be protected, the right one was ligated and the left one was supplied from a carotis by means of a polyethylene tube. A bubble flow meter had been inserted for measuring the blood supply between carotis and vertebralis. The second carotis was not ligated, in order to afford ample blood supply to the brain. Blood pressure was constantly monitored from the right a. femoralis by means of a Hg manometer. Thrombocide (WZ) was used to prevent clotting. See Bubnoff etal for the solution and concentrations of the substances used. Injections were performed by means of the polyethylene tube leading to the a. vertebralis (0.1-0.3 cc in 50 seconds). In control tests, physiological cooking salt solution and the Tween 80 doses remained without effect on brain blood supply*.

First we checked the effects of benzoic acid, paraoxybenzoic acid and of the methyl, ethyl esters of paraoxybenzoic acid of parachlorobenzoic acid and microbin (WZ) -- the latter a combination of 45% parachlorobenzoic acid sodium and 55% orthochlorobenzoic acid sodium--, on the rats' cocaine-cramps.

Three tribes of white rats of 25 each were used; the mean cramp doses CD_{50} of the three tribes were 12.0, 12.2 and 12.6 mg

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NaOH addition -- up to pH 8-9. The substances were dissolved in physiological cooking salt solution; 0.5 - 1.2 cc/100 gm of rat were injected.

*We are grateful the firm of BENEND in Munich for their kindly making available to us thrombocide. Similarly we thank Atlas Goldschmidt of Essen for Tween 80. Bayer Ag, Leverkusen, made available sodium benzoate, microbine, parachlorobenzoic acid and the ethyl and methyl esters of paraoxybenzoic acid. We also thank Nipa, Berlin, for paraoxybenzoic acid and the propyl, butyl and benzyl esters of paraoxybenzoic acid.

cocaine-kg and therefore the results lent themselves readily to comparison. Each experiment was carried out on 15-18 animals from the same tribe; the remainder served as reserve. First that dose of benzoic acid was determined, which effected a clear increase in the cocaine cramp dosage for oral ingestion. Then we determined the isoeffective doses for the other substances. A comparison was made between the effectivenss of oral and iv. administration for each preparation and for oral ingestion, the time of maximum effect was recorded in every instance (table 1).

Table 1: Time of maximum anti-convulsive effect for oral administration of benzoic acid and its derivatives.

Substance	dosis, mg/kg	maximum effect
benzoic acid	50	45 minutes
"	100	"
parachlorobenzoic acid	50	"
paraoxybenzoic acid	50	"
paraoxybenzoic acid methyl ester	20	"
"	100	180
paraoxybenzoic acid ethyl ester	20	45
"	100	180
microbine	50	30
"	100	90

For oral administration of benzoic acid, 50 mg/kg as a 6% solution introduced with pharynx bougie showed a clear anticonvulsive effect . 45 minutes after benzoic acid ingestion this effect was maximum: CD_{50} at that time had risen from 12.0 to 14.5 mg/kg. For iv. injection of the same dosis the mean cramp dosis was 14.9 mg/kg. Paraoxybenzoic acid had the same effectiveness as benzoic acid: 50 mg/kg for oral ingestion increased CD_{50} from 12.0 to 14.2 (fig. 1).

The subsequently tested ethyl ester of paraoxybenzoic acid showed a stronger cramp-weakening effect than benzoic acid: 20 mg/kg as 0.18% solution sufficed for oral ingestion to achieve the same anticonvulsive effects; CD_{50} was raised from 12.0 to 14.6 mg/kg; see fig. 1. Here again the maximum effect occurred after 45 minutes. For iv. ingestion, already 5mg/kg of ethyl ester raised the mean cramp-dosis from 12.0 to 14.7 mg/kg (fig. 1).

Thus the relationship between the effectiveness of benzoic acid and paraoxybenzoic acid ethyl ester was not the same, neither for oral nor for iv. ingestion. The isoeffective doses of benzoic acid for oral ingestion were 2.5 times higher than for ethyl ester, the relation between the effectivenesses for iv. was 1:10. These differences are due to the fact that the ethyl ester is 4 times more effective for iv. injection, in contrast to benzoic acid.

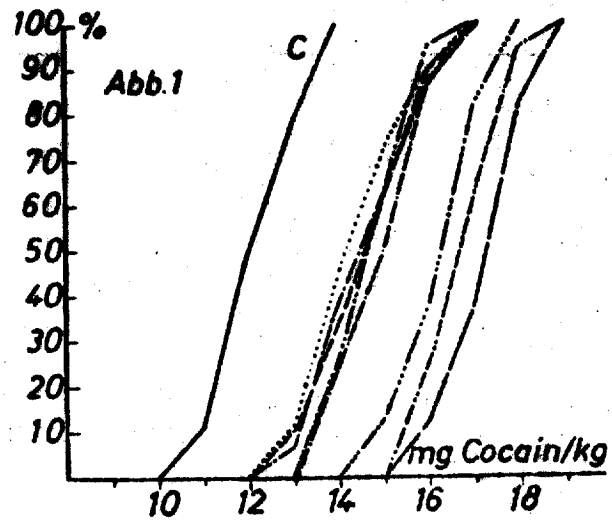
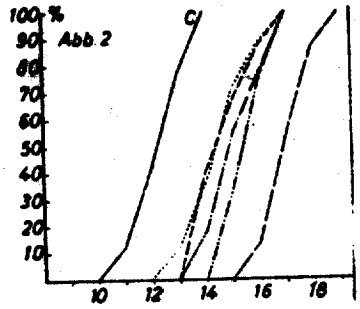


Fig. 1. Anti-convulsive effect of 50 mg paraoxybenzoic acid/kg po. (...); 20 mg paraoxybenzoic acid ethyl ester / kg p. (---); 50 mg of benzoic acid /kg po. (-.-.); 5 mg paraoxybenzoic acid ethyl ester/kg iv. (-..-..); 50 mg benzoic acid/kg, iv. (--.--.); 100 mg benzoic acid / kg po. (-···-); 100 mg paraoxybenzoic acid ethyl ester / kg po. (--..--) and 20 mg paraoxybenzoic acid ethyl ester iv. (— —) for rat's cocaine cramp. C = cocaine basic curve; abscissa: cocaine dose in mg/kg; ordinate: percentage of animals subject to cramps. See text for further details.

For higher oral doses, the anticonvulsive efficiencies of ethyl ester and benzoic acid were comparable. 100 mg of ethyl ester/kg ingested as deposit in water with the pharynx bougie did have an appreciably stronger effect than 20 mg/kg; 3 hours after administration the CD_{50} had risen from 12.0 to 16.7 mg/kg. However the same benzoic acid dosis caused an increase of 4.3 mg/kg in the average cramp dosis and therefore was only slightly less effective; see fig. 1 "hen increasing the iv. injected amount of ethyl ester, the anti-convulsive effect relatively increased more: the iv. injection of 20 mg of ethyl ester /kg led to an increase in CD_{50} from 12.0 to 17.3 mg/kg and thus was more effective than the five-fold dosis (see fig. 1).

Paraoxybenzoic acid methyl ester and parachlorobenzoic acid were tested in the second tribe with a CD_{50}=12.2 mg/kg. Again methyl ester was administered orally and iv. Here again 20 mg/kg per os had the same effect as 5 mg/kg iv, as was already the case for ethyl ester: in both cases the average cramp-dosis rose from 12.2 to 14.4 mg/kg. 100 mg /kg of methyl ester per os corresponded in their effect to about the same dose of benzoic acid or ethyl ester (rise of CD_{50} to 16.9 mg/kg; see fig. 1 and 2). 50 mg/kg of parachlorc benzoic acid per os were about as effective as 50 mg/kg of benzoic acid. Iv. injection of the same dose led only to a slight reinforcing effect with respect to anti-convulsion (fig. 2), as contrasted with methyl and ethyl esters. For oral ingestion CD_{50} was 14.0, and for iv. administration it was 15.5 mg/kg. The strongest effects occurred for oral ingestion of 20 gm of methyl ester and of 50 mg of paraoxybenzoic acid /kg 45 minutes after administration, and for 100 mg of methyl ester/kg 3 hours thereafter.



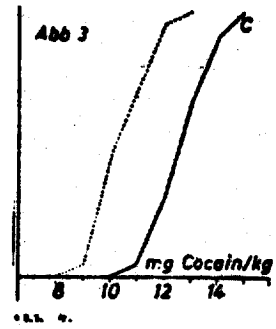


Fig. 2: Anticonvulsive effect of 20 mg/kg po. of paraoxybenzoic acid methyl ester (...); 5 mg/kg iv. of paraoxybenzoic acid methyl ester (———); 50 mg/kg of po. of parachlorobenzoic acid (-.-.); 50 mg/kg iv. of parachlorobenzoic acid (-··-··); and 100 mg/kg of paraoxybenzoic acid methyl ester, po. ((—— ——); rats' cocaine cramps. C= cocaine reference curve; abscissa: cocaine dosis in mg/kg; ordinate: percentage of animals subjected to cramps. See text for details.

Fig. 3: Effect of 100 mg/kg of po. microbine (...) on rats' cocaine cramp. C= cocaine; abscissa: cocaine dosis in mg/kg; ordinate: percentage of animals subjected to cramps. Note the cramp-reinforcing effect of microbine.

We examined the effect of microbine in the third tribe with respect to the rats'cocaine-cramps. We observed to our surprise there was an increase in the cocaine effect beyond 50 mg/kg per os. This effect became clearer yet after oral administration of 100 mg.kg: the mean cramp dosis after 1-1½ hours was reduced from 12.6 to 10.3 mg/kg (fig.3).

In further tests we made use of a combination of cocaine and adrenaline for inducing cramps.

In such cases, ½-1/3 of the usual cocain amounts sufficed to induce cramps; the differences in cocain amounts as required by the individual animals were very slight so that the cramp curves became very steep and so that the curves obtained from testing no longer intersected the reference curve (see fig. 4,5). In order to obtain the cramp-dose, we increased the amount of cocaine from test to test by only ½mg/kg, that is, each time the cocaine concentration was increased only by ⅙. The average cramp dose of the first tribe was 5.1, that of the second 6.1 mg/kg.

First 20 mg/kg of methyl ester, 20 mg/kg of ethyl ester and 50 mg/kg of benzoic acid were tested and compared for oral ingestion. The CD_{50} increased from 5.1 to 7.1 mg/1g (methyl ester), to 7.0 mg/kg for ethyl ester or 6.95 mg/kg for benzoic acid. Therefore the ratio of effectiveness was 2½:2½:1, that is, the same as with cocaine alone. Since already 5.5 mg/kg of cocaine without any pre-treatment led to cramps in all animals, but since 6.0 mg/kg of cocaine after treatment with benzoic acid, methyl and ethyl esters of paraoxybenzoic acid did not induce cramps, such results were particularly impressive (fig.4,5)

Finally 20 and 100 mg/kg of ethyl ester orally administered were tested and compared in the second tribe. The higher dose led to an

increase in the anti-convulsive effect as had been the case for cocaine used alone; however in this instance the effect was not as pronounced; see fig. 1 and 6.

The effects of benzoic, parachlorbenzoic and paraoxybenzoic acids and of methyl, ethyl, propyl, butyl, and benzyl esters of paraoxybenzoic acid on the brain's blood supply was tested in 30 cats (fig. 6,7). Upon injection into the a. vertebralis, all substances led to an improvement of brain blood supply. The magnitude of the vascular enlargement achieved was a function of of the dose and of the tonus of the brain vessels; upon increasing the vascular tonus by superventilation, particularly pronounced vascular spasmolytic effects could be observed. We examined in every animal from 2 to 4 substances that had been injected in alternating sequence into the a. vertebralis in order to determine their efficiencies. Repeated injections in control tests for unchanged blood pressure and same initial blood supply always produced the same vascular enlargement -- see table 2.

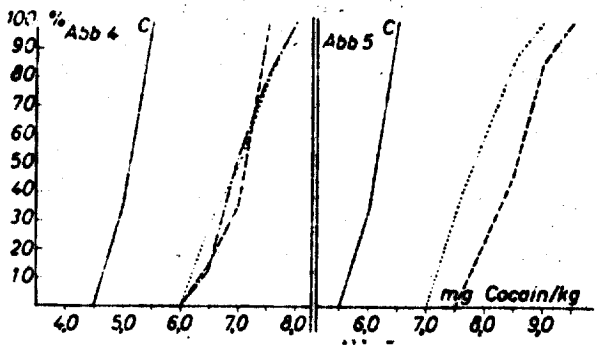


Fig. 4: Effects of 20 mg/kg of paraoxybenzoic acid ethyl ester pO. (...); 20 mg/kg of paraoxybenzoic acid methyl ester po. (---) and of 50 mg/kg of benzoic acid po. (-.-.-) on adrenaline reiforced cocaine cramps. C= cocaine reference curve; abscissa: cocaine dose in mg/kg; ordinate: percentage of animals subjected to cramps. See text for further details.

Fig. 5: Effects of 20 mg/kg (...) and 100 mg/kg (---) of po. paraoxybenzoic acid ethyl ester on adrenaline-reinforced cocaine cramps. C= cocaine reference curve; qbscissa: cocaine dosis in mg/kg; ordinate: percentage of animals seized by cramps. See text for further details.

The iv. injections led to the same difficulties in evaluating vascular width as encountered in the simultaneously executed purine body tests (cf Taugner, Bubnoff, Weth, Waltz, Hochrein, Bartels & Schmid): low doses with little or insufficient effects on blood pressure either left the brain blood supply unaffected or only improved it slightly. But higher doses led to appreciable lowerings in blood pressure which might have left the blood supply unchanged or possibly

slightly, pressure-passively decreased*. After administrating benzoic acid and microbine (WZ) there were occasional increases in blood pressure which made evaluation of vessel musculature condition also difficult (cf Bubnoff, Schnell & Schmid). However neither iv. nor ia. (intra-arterial) administration of benzoic acid and of its derivatives led to a decrease in brain blood supply that was independent of blood pressure.

Abb. 6:
Wirkung von Benzoesäure, Paraoxybenzoesäure und Parachlorbenzoesäure auf die Durchblutung des Gehirns d. Katze. Oben: Blutdruck; unten: Gehirndurchblutung (Abfall der Kurve bedeutet Mehrdurchblutung). 1, 3, 5 = 7 mg Natriumbenzoat/kg; 2 = 7 mg paraoxybenzoesaures Natrium / kg. 4 = 3,5 mg parachlorbenzoesaures Natrium/kg. Alle Injektionen erfolgten in die A. vertebralis.

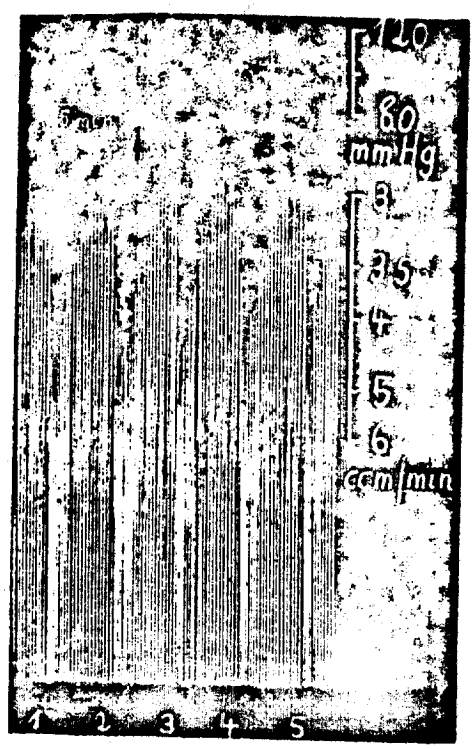


Abb. 7:
Wirkung von Paraoxybenzoesäure-Methyl-, Aethyl- und Propyl-Ester auf die Durchblutung des Gehirns der Katze. Oben: Blutdruck; unten: Gehirndurchblutung (Abfall der Kurve bedeutet Mehrdurchblutung). 1, 3, 5 = 0,3 mg Paraoxybenzoesäure-Aethylester/kg; 2 = 0,3 mg Paraoxybenzoesäure-Methylester/kg; 4 = 0,3 mg Paraoxybenzoesäure-Propylester/kg. Alle Injektionen erfolgten in die A. vertebralis.

Fig. 6: Effects of benzoic, paraoxybenzoic and parachlorobenzoic acids on cats' brains. Top: blodd pressure; bottom: brain blood supply (decrease in curve indicates larger supply). 1,3,5 = 7mg/kg of sodium benzoate; 2= 7 mg/kg of paraoxybenzoic acid sodium; 4= 3.5 mg/kg of parachlorbenzoic acid sodium. All injection into the a. vertebralis.

Fig. 7: Effects of methyl, ethyl and propyl esters of paraoxybenzoic acid on the cat's blood supply. Top: blood pressure; bottom: brain blood supply (drop in curve indicates increase in supply). 1,3,5 = 0.3 mg/kg of paraoxybenzoic acid ethy ester; 2= 0.3 mg/kg of paraoxybenzoic acid methyl ester; 4= 0.3 mg/kg paraoxybenzoic acid propyl ester. All injections into the a. vertebralis.

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*According to the results of Matthews etal, the lowering of the blood-pressure following iv. ester injection seem to be due among other causes to the effect on the cardiac muscle ("myocard depression").

sodium benzoate	
p-chlorobenzoic acid sodium	
p-oxybenzoic acid sodium	
methyl ester	of para-oxybenzoic acid
ethyl ester	
propyl ester	
butyl ester	
benzyl ester	
salicylic acid sodium	
acetate salicylic acid Na *	
ratio	

*) Die beim Aspirin (WZ) angegebenen Dosen sind auf dem Sali-
cyl-Gehalt umgerechnet.

Table 2. Tests on cats' brain vessels. Doses causing equal vessel expansion, sodium benzoate, parachlorobenzoic acid sodium, paraoxybenzoic acid sodium, methyl, ethyl, propyl and butyl esters of paraoxybenzoic aicd, sodium salicylate and acetyl salicylic acid sodium (aspirine-Na, WZ), in mg/kg of injection into a. vertebralis.

For a dose of 5 mg.kg ia., zodium benzoate showed clear vascular spasmolytic properties; amounts of 6-17 mg/kg led to strong vasodilatations that were convenient for comparison tests. The effectiveness of paraoxybenzoic acid was about the same as benzoic acid's: for 8-16 mg/kg the same effects could be achieved as by means of the above described injections of benzoate doses. Microbine's (WZ) vascular spasmolytic effects were not tested for in the cat's brain (see further below). Though parachlorobenzoic acid (4-12 mg/kg) also was somewhat stronger in effectiveness in the cat's brain vessels than benzoic acid (ratio of sodium benzoate to parachlorbenzoic acid sodium = 1:1½-2), such differences were not as clear as they had been in the kidney vessels (see Bubnoff etal). Methyl, ethyl, propyl, butyl and benzyl esters of paraoxybenzoic acid were used in doses of 0.5-0.6 mg/kg. The effectiveness of the esters was comparison-tested in 15 experiments and found to be nearly the same; only for benzyl ester did the isoeffective doses lie at a level 1½-2 as high as for the other esters. Again all esters were appreciably more effective than sodium benzoate: the ratio of efficiencies in ten tests on the average was 22½:1 (15-30:1). Sodium salicylate and aspirin (8-10 mg/kg) too showed vessel-expanding properties; the effectiveness of these substances corresponded approximately to that of paraoxybenzoic acid.

In our above described tests in the vessels of kidneys and extremities, no changes in blood pressure could be detected for ia. injections in cats. However there were frequent drops following injections into the a. vertebralis, more rarely there were increases. These changes which probably were centrally determined in general amounted no more than 5-10 mm. For blood pressure effects beyond this scope, there was no evaluation for the pertinent injections with respect to comparison tests. On two occasions, microbine (WZ) caused a rise in blood pressure for each injection in the amount of 20-35 mm

and therefore could not be tested for its vessel-expanding properties in the brain vessels.

Notes during proof-reading: Benz in his orientation tests examined the esters of paraoxybenzoic acid for their local-anaesthetic effects. For that purpose aqueous solutions of these esters were placed into the oral cavities of 20 members of the Heidelberg Pharmacological Institute. For positive reactions there followed within a few minutes a numb or furry feeling at the tongue. The following results were obtained:

	methyl ester	ethyl ester	propyl ester	butyl ester
solution concentration	0.1%	0.05%	0.03%	0.02%
positive reaction	17	18	16	5
dubious reaction	1	1	3	13
no effect	2	1	1	2

The individual variability in reaction may be due to the low concentration of the ester solutions used.

DISCUSSION:

According to these results, parachlorobenzoic and paraoxybenzoic acids and the methyl and ethyl esters of the latter, besides benzoic acid, show clear anticonvulsive properties for oral administration. The isoeffective doses of benzoic, parachlorobenzoic, paraoxybenzoic acids and of the methyl and ethyl esters of paraoxybenzoic acid are in ratio of approximately 2½:2½:2½:1:1. The effective benzoic acid dose corresponds (50 mg/kg) to that found by Zwissler. For iv. administration, the differences in efficiencies were even more pronounced, the ratio of benzoic acid to ester being about 1:10; benzoic acid and parachlorobenzoic acid for both kinds of applications were about equally effective. But methyl and ethyl esters were roughly 4 times more effective for iv. administration. Metzger etal, also Schuebel & Manger, found no paraoxybenzoic acid esters in the droppings following feeding, nor any free acids. The research from other authors too indicates that there is complete reabsorbtion of these substances from the intestine (Sokol, Jones etal; also see Sabalitschka etal). However the paraoxybenzoic acids are very rapidly hydrolyzed in the body* (Sokol, Richardson etal): for an iv. injection of 50 mg/kg of esters, these were already present in the plasma and only in the form of free or combined paraoxybenzoic acid after 5-30 minutes. For oral administration of 1 gm/kg too, the concentrations of the esters in the plasma when compared with free paraoxybenzoic acid, was only minor. The lesser oral effectiveness of methyl and ethyl esters in our experiments is likely to be mostly due to the rapid hydrolysis, the largest part immediately after iv. administration being still unsaponified. But since the two esters were still 2½ times as effective for oral ingestion as benzoic and paraoxybenzoic acids, it seems that a part of the ester responsible for the higher effectiveness remains unsaponified in the plasma.

For oral ingestion of ethyl esters or benzoic acid in doses as high as 100 mg/kg, we no longer could clearly distinguish differences in effectiveness. For such doses the time from taking effect till reaching the maximum anti-convulsive effect was about 3 hours. Appa-

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*According to in vitro experiments by Richardson etal, hydrolytic fission of the esters mainly occurs in the liver and kidney.

rently during this very long reabsorption time for such ester quantities, a fairly large part is saponified as compared to the part when there is more rapid reabsorption of smaller doses. This would explain how the differences in efficiencies for ethyl ester with respect to iv. and oral ingestion clearly increased with dosing. Microbine (WZ) was the only benzoic acid derivative that surprisingly caused reinforcement of cocaine cramps. This might be related to the increase in blood pressure observed in cats for iv. and intravertebrate administration; blood-pressure increasing substances may also increase the cramp-effectiveness of cocaine (Keil & Ruehling).

All the benzoic acid derivatives tested by us in the cats' brain vessels clearly were endowed with vascular-widening properties. For reasons advanced earlier, we again preferred the ia. injection. The vessel-widening efficiency of benzoic, paraoxybenzoic and parchlorobenzoic acids and of the methyl, ethyl, propyl, butyl and benzyl esters of paraoxybenzoic acid were in the ratios of 1:1.2:1.7:22.5:22.5:22.5:22.5:15**. The esters' effective doses (0.3 -0.7 mg/kg) approximately corresponded to the simultaneously executed tests with purine for a theophylline derivative(KGG 158) substituted into an 8 position and containing an amino group and for theophyyline ethylene diamine (Euphyylin, WZ), that is, for the values thus obtained, (0.18-0.9 and 0.25-1.5 mg/kg resp.; Taugner etal). As was the case for the purine substances, only slight effects could be achieved for iv. injections. In no test were active vasoconstrictions observed.

Table 3 shows the efficiencies of the tested benzoic acid derivatives for the kidney, extremity and brain vessels of cats, the "lissive" efficiencies for the chewing musculature of rats, and the anti-convulsive efficiencies for iv. and po. administrations.

	benzoic acid	paraoxy-benzoic acid	paraoxy-benzoic acid ester**	para-chloro-benzoic acid	micro-bine*
kidney blood supply	1	1-2	30-100	3-8	½-2
extremities blood supply	1	***	20-30	***	1
brain blood supply	1	0,8-1	15-30	1½-2	***
anticonvulsive effect					
oral ingestion	1	1	2½	1	@
iv. ingestion	1	***	10	1	@
"lissive" effect	1	1-1½	10=100	2-4	1-1.7

Table 3: efficiencies of individual substances compared to benzoic acid.

** : methyl, ethyl, propyl, butyl, and benzyl esters of paraoxybenzoic acid. Benzyl ester was slightly less effective than the other ester. The effect on blood supply of extremities was tested only for the methyl, ethyl and propyl esters, and the anti-convulsive effect only for the methyl and ethyl esters.

*** : not determined

@ : In contrast to the other substances, Microbine for peroral ingestion led to reinforcement of the cocaine cramps.

** When converting the substances injected as sodium salts into the content of free acid, one obtained approximately the same relation. A conversion to equimolar doses too leads to no appreciable change of the above indicated ratio of efficiencies.

As seen from table 3, the paraoxybenzoic acid esters were relatively the most effective in the kidney vessels; the ratio of benzoic acid to ester in the brain and extremity vessels was approximately equal (1:15 -30 and 1:20-30 resp.). The esters' anti-convulsive effectiveness was 10 times stronger for iv. injection than it was for benzoic acid; this ratio too was of the same order of magnitude. The effectiveness ratio obtained in the brain vessels for theophylline, coffeine and theobromine approximately corresponded to the ratio found by Eichholtz etal for the rat cocaine-cramp, (1:0.9:0.7 and 2:2:1 resp.) Eichholtz etal attributed the anticonvulsive effect of these substances to the widening of the vessels at the central nervous system. Possibly there is a relation between vessel widening and anticonvulsive effect in benzoic acid and its derivatives too.

The preparations of the benzoic acid series also show vascular spasmolytic, "lissive", ganglioplegic and anticonvulsive effects. The latter could also be demonstrated for oral administration. For all test procedures, the paraoxybenzoic acid esters were appreciably more effective than benzoic acid itself. As already reported in the first communication, the usefulness of these compounds as preservatives should be discussed in future communications and they should take into account these conditions since it was shown that demonstrable effects arise also for oral ingestion, or may arise. Again the feasibility of therapeutic applications is stressed herewith.

SUMMARY

Continuing previous research, the effects from benzoic, parachlorobenzoic and paraoxybenzoic acids and of their esters on the cocaine-cramp of rats and on the brain blood supply of cats were tested.

Benzoic, parachlorbenzoic and paraoxybenzoic acids and the methyl and ethyl esters of paraoxybenzoic acid showed clear anticonvulsive properties for iv. and oral administration. Both esters were 10 times more effective for iv. ingestion than benzoic acid. the ratios of the anti-convulsive efficiencies of benzoic, parachlorobenzoic and paraoxybenzoic acids and of the methyl and ethyl esters of paraoxybenzoic acid for oral administration are 1:1:1:2½:2½. The iv. effective dose of the esters was 5 mg/kg, and that orally effective was 20 mg/kg.

Besides the previously observed vascular spasmolytic effects in kidney and at the extremities, such were also observed this time in the brain vessels. The efficiencies determined with respect to the vascular spasmolytic of benzoic, paraoxybenzoic and parachlorobenzoic acids and the methyl,ethyl, propyl, butyl and benzyl esters of paraoxybenzoic acid for a. vertebralis injections were in the ratios of 1:1.2:1.7: 22.5:22.5:22.5:22.5:15.

Again we thank Prof Eichholtz for his multi-faceted encouragements and many suggestions.

Summary:

Studies on the Pharmacology of Benzoic Acid, Parachlorbenzoic Acid, Parahydroxybenzoic Acid, and its Esters

In continuance of former investigations the action of benzoic acid, parachlorbenzoic acid, and parahydroxybenzoic acid, and its esters on cocaine convulsions in rats and on cerebral blood flow in cats have been investigated. Benzoic acid, parachlorbenzoic acid, parahydroxybenzoic acid, parahydroxybenzoic acid methyl- and ethyl esters showed marked anticonvulsive action following intravenous and oral application. Following intravenous injection, the two esters were about 10 times more effective than benzoic acid; following oral application the relation of anticonvulsive action in benzoic acid, parachlorbenzoic acid, parahydroxybenzoic acid, parahydroxybenzoic acid methyl and -ethyl ester was 1:1:1:2,5:2,5. In the esters the effective intravenous dose was 5 mgs, the oral effective dose was 20 mgs kg body weight. In addition to the vascular spasmolytic action formerly observed in the kidneys and extremeties, spasmolytic action was also observed in cerebral vessel. The proportion of spasmolytic action following injection into the a. vertebralis, in benzoic acid, parachlorbenzoic acid, parahydroxybenzoic acid, parahydroxybenzoic acid methyl-, ethyl-, propyl-, butyl-, and benzyl esters was found to be 1:1,2:1,7:22,5:24,5:22,5:15.

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Polyamide–silica gel thin-layer chromatography of food preservatives

The chromatography of food preservatives has been studied by numerous investigators. The separation of these preservatives on thin layers of cellulose acetate-polyamide[1], cellulose[2] and silica gel[3] has been reported, but there is no report on separation by polyamide–silica-gel layers. In a previous report[4], better separation of red food dyes was obtained with polyamide–silica gel layers; therefore, this method was further applied to separate ten preservatives. For comparison, the thin-layer chromatography of only polyamide and of only silica gel is also described.

Experimental

Preparation of polyamide–silica gel mixed layer. Ten grams of polyamide (ε-polycaprolactam CM 1007S of Toyo Rayon Co., Tokyo, Japan) were dissolved in 80 ml of 90% formic acid; then 20 ml of distilled water were added. After gentle warming (below 40°) and stirring, a homogeneous solution was obtained. It was then cooled to room temperature, and 52 g of Silica Gel G (E. Merck) were added. Of the previous solution 200 ml were poured into a dish (14.5 × 19.5 × 2.5 cm) into which a glass plate (12 × 14 × 0.1 cm) was dipped. Both sides of the glass were covered homogeneously.

The glass was hung for 2 min over the dish to let the excess solution drain off. It was then air dried for 3 h and heated at 100° for 30 min. These layers can be stored for a long period.

Preparation of polyamide layer. Instead of 10, 20 g of polyamide were dissolved before proceeding as described in the previous method, but without adding Silica Gel G.

Preparation of silica-gel layer. Dilute slurries of Silica Gel G (45 g to 100 ml of water) were sprayed at 2 kg/cm² pressure from a distance of 20 cm onto 8 horizontal glass plates (12 × 14 cm) which were then dried at 100° for 30 min. The thickness of the layers was about 250 μ.

Chromatographic procedure. A 0.2% alcoholic solution of samples was applied to the starting line 1.5 cm from the bottom of the layer, and the plate was developed by ascending techniques. The chamber had been equilibrated with the respective solvent for 30 min before use.

Visualization. The layers were sprayed with a 0.07% alcoholic solution of Rhodamine B, and deep-violet spots could be observed under UV light at 366 mμ.

Results and discussion

R_F values obtained with two solvent systems are given in Table I. It is interesting to note that the R_F values of the *p*-hydroxybenzoic acid ester in the two solvent systems are reversed. In the nonaqueous system (solvent I), the R_F values increase with an increase in the molecular weight of the esters, *i.e.* the order of R_F values is the same as that obtained by previous workers[1–3]. However, in the aqueous system (solvent II), the R_F values decrease with an increase in the molecular weight.

In both solvent systems, when using polyamide–silica gel mixed layers the R_F values are lower and separation is better than when polyamide and silica gel layers are employed. A 10-cm ascent from the origin is more rapid using the mixed layers

TABLE I

CHROMATOGRAPHIC DATA

Solvent I: *n*-hexane–benzene–glacial acetic acid (1:1:1); solvent II: water–28% ammonia solution (20:5). a, R_F value obtained on polyamide-silica gel layer; b, silica gel layer; c, polyamide layer.

No.	Substance	Solvent I			II		
		a	b	c	a	b	c
1	Methyl *p*-hydroxybenzoate	0.30	0.73	0.61	0.55	0.91	0.78
2	Ethyl *p*-hydroxybenzoate	0.35	0.77	0.69	0.46	0.88	0.70
3	Propyl *p*-hydroxybenzoate	0.41	0.78	0.74	0.34	0.79	0.59
4	Isopropyl *p*-hydroxybenzoate	0.44	0.78	0.74	0.32	0.77	0.60
5	Butyl *p*-hydroxybenzoate	0.49	0.79	0.78	0.24	0.60	0.49
6	Isobutyl *p*-hydroxylbenzoate	0.53	0.80	0.81	0.21	0.69	0.50
7	Sorbic acid	0.88	0.87	0.98	0.73	0.95	0.94
8	Benzoic acid	0.84	0.89	0.98	0.64	0.90	0.88
9	Salicylic acid	0.66	0.84	0.62	0.49	0.90	0.74
10	Dehydroacetic acid	0.74	0.96	0.80	0.62	0.95	0.85
Time required (min)[a]		90	25	240	130	15	180

[a] Time required to ascend 10 cm from origin.

than using polyamide layers. The content (16%) of polyamide in this polyamide–silica gel mixed layer is higher than that in the previous report (12%)[4] for getting a more stable layer.

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METABOLISM OF A FOOD PRESERVATIVE: PARAHYDROXYBENZOIC ACID AND ITS ESTERS

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Summary - We made a study of the form in which p-hydroxybenzoic acid and its methyl, ethyl and propyl esters are eliminated by the rat, and of the rate at which phenolic acid and its metabolites appear in the urine. We were able, by means of paper chromatography and the use of Millon's reagent, to show the presence in the urine of p-hydroxybenzoic acid, p-hydroxyhippuric acid, an ester and ether glucuronides, an ethereal sulphate, and an unidentified compound. Study of the kinetic process of the appearance of the phenolic acid and its metabolites in the urine showed that free p-hydroxybenzoic acid appeared first, followed by the glucuronide and p-hydroxyhippuric acid, the concentration of which increased as that of the free p-hydroxybenzoic acid fell off. During the experiment the concentration of phenolic acid in the blood remained extremely low.

INTRODUCTION

The study of the metabolism, pharmacology or toxicity of p-hydroxybenzoic acid and its esters has been dealt with in a small number of papers. However, such a study is of as great an interest as that of salicylates, since the esters of p-hydroxybenzoic acid are commonly employed in nonnegligible amounts in the preservation of pharmaceuticals for foodstuffs. In France, the regulations in

force prohibit the use of antiseptics in general for the preservation of foodstuffs, but esters of p-hydroxybenzoic acid would offer some interesting uses in ensuring the metabolic orientation of certain fermentations.

The chief advantage of these compounds is their high activity towards microorganisms and their weak toxicity towards higher organisms (Sabalitschka and Bohm, 1927; Sabalitschka, 1929). Thus, according to Lutwak-Mann (1943) and our personal experience, amounts of 1 g/kg of p-hydroxybenzoic acid or its esters in the rat do not appear to modify the behavior of the animal. However, some experiments that we are now conducting seem to indicate that much smaller doses have a definite effect on the growth of the animal.

In man, most of p-hydroxybenzoic acid is thought to be excreted in the free form or in the form of p-hydroxyhippuric acid, the excretion of glucuronides being extremely low (Quick, 1932; Quilley and Smith, 1952; Williams, 1938). In the rabbit, 6-8% are excreted in the form of ethereal sulfate (Williams, 1938); in the dog, two glucuronide derivatives appear (Quilley and Smith, 1952; Sabalitschka and Bohm, 1927), one attached to the aromatic ring by a glycosidic bond and the other by an ester bond; the dog also eliminates p-hydroxybenzoic acid in the form of p-hydroxyhippuric acid.

According to Lederer and Polonski (1948), p-hydroxybenzoic acid is a normal constituent of urine and is thought to originate from the metabolism of tyrosine.

In this study, it was our objective to determine in the rat the forms under which p-hydroxybenzoic acid and its esters are eliminated, the order of appearance of the different metabolites in the urine, and the rate of this elimination in relation to the concentration in the blood.

EXPERIMENTAL METHODS

The experimental methods were as follows. To rats of Wistar W. A. G. strain weighing an average of 200 g, 100 mg of p-hydroxybenzoic acid or its esters is administered by oral probing.

The rats are kept in metabolic cages for longer periods, and in restraining cages permitting the collection of urine and the withdrawal of blood from the tail, for shorter periods of 6 hr. The urine is collected every half hour; the collection is facilitated by the fact that the withdrawal of blood at the tip of the tail causes the emission of urine by the animal.

Qualitative analysis of urine

Paper chromatography. An ascending chromatography on Arches no. 302 paper is developed by using as solvent a mixture of n-butanol-acetic acid-water (40:4:56 by vol.) in an atmosphere of 0.1 N ammonia. The solvents are identical to those used by Bray, Thorpe and White (1950) and Quilley and Smith (1952). The urine samples (15 to 20 μl) are usually spread without purification. The chromatograms are revealed by reagents similar to those described by Bray et al. (1950): diazotized p-nitroaniline or diazotized sulfanilic acid; 2,6-dichloroquinone imide; ferric chloride (0.2%) in water. However, we prefer the use of Millon's reagent, which gives a cherry-red color with p-hydroxybenzoic acid or its derivatives. The fluorescent compounds are localized by exposing the paper to a UV Hanovia Chromatolite lamp giving an emission at 350 mμ. Moreover, the chromatograms are sprayed with 2N HCl and heated in an oven at 110°C between two plates of glass to hydrolyze the conjugated compounds before the latter are revealed with the above-described reagents.

Isolation and identification of metabolites. Cellulose columns are used to separate macroquantities of metabolites. Cellulose powder is purified by

boiling with 5% acetic acid, washed with water and acetone, and finally with ether. It is mixed with the stationary phase of the above-described solvents and finally introduced into a column measuring 2 cm × 30 cm. The column is then washed with the mobile phase. The 24-hr urines of a group of 20 rats that received a total of 2 g of the derivatives studied are concentrated to dryness at low temperature, dissolved in a few ml of the mobile phase, and introduced into the column, which is then fractionated with the mobile phase.

As soon as p-hydroxybenzoic acid has appeared at the bottom of the column, the eluates are examined by depositing a spot on paper and spraying with Millon's reagent.

Quantitative determination of p-hydroxybenzoic acid in the urine and blood. The quantitative analyses are carried out by using paper chromatography; the substances are eluted, their R_f being known from a control chromatogram revealed with Millon's reagent. The cut paper strips are treated with 10 ml of absolute ethanol, and the p-hydroxybenzoic acid concentration is determined by spectrophotometry at 257 mμ.

The blood taken from the tail vein is collected in a microtube and centrifuged in a Beckmann microcentrifuge; then, 80 μl of 2N alcoholic ammonia is added to 20 μl of plasma; after centrifuging and dilution with absolute ethanol, the absorption is measured on the spectrophotometer at 257 mμ against a reference from the blood of an untreated rat.

Paper chromatography of glucose. We used chromatography on Arches no. 302 paper and the mixture of n-butanol-acetic acid-water described above to detect glucose. Each chromatogram was sprayed with aniline phthalate reagent (0.9 ml of redistilled aniline dissolved in 100 ml of 0.1N phthalic acid). A brown spot is obtained with glucose.

Determination of methyl p-hydroxybenzoate, glucuronates and sulfuric esters in the urine. The determination of glucuronides in the urine was made according to the method described by Deichmann (1943), and that of urinary ethereal sulfates, by the method of Sperber (1948).

RESULTS

Identification of metabolites in the urine

After half an hour, substances giving a positive reaction to Millon's reagent, free p-hydroxybenzoic acid and different metabolites appear in the urine, after the ingestion of either the acid or methyl, ethyl or propyl ester. We were not able to detect the esters in the urine. Given below are different values of R_f that we found for the substances which appeared and were identified (Table 1).

Table 1. R_f Values of Different Metabolites of p-Hydroxybenzoic Acid Which Appeared in the Urine

Substance	R_f	UV Fluorescence	Millon's Reagent
Free p-hydroxybenzoic acid	0.84	0	+ + +
p-Hydroxybenzoylglycocoll acid	0.68	0	+ + +
Ethereal sulfate	0.30	0	+
Compound A	0.21	+	+
Glucuronide derivatives	0.13	0	+ +
Glucuronyl derivatives	0.06	0	+

The glycocoll amide of p-hydroxybenzoic acid was determined by separation on a cellulose column. It passes over after free p-hydroxybenzoic acid. We were able to obtain crystals in the form of needles with a melting point of 135°C. By hydrolysis in 6N HCl for 6 hr in a sealed tube, we were able to detect glycocoll

by paper chromatography by the reaction to ninhydrin, and free p-hydroxybenzoic acid by Millon's reagent.

The spot with R_f of 0.13 is p-hydroxybenzoic acid glucuronide; by hydrolysis of the eluate with 6N HCl, it gives glucuronic acid, identified by the Tollens reaction and contains a free phenol group, a faint green color being given by ferric chloride. The compound with R_f 0.06 is formed by p-hydroxybenzoic acid and glucuronic acid, the conjugation being produced by an ether bond between the phenol group and glucuronic acid. It gives no color with ferric chloride. Hydrolysis with 2N HCl of the substance with R_f 0.34 yields free p-hydroxybenzoic acid and sulfur. This is in all probability an ethereal sulfate. The compound with R_f 0.21 could not be identified; in view of its strong fluorescence, this is thought to be a diphenolic compound, but it is neither homogentisic acid nor 3,4-dihydroxybenzoic acid.

Quantitative Determination of p-Hydroxybenzoic Acid and its Metabolites in the Blood and Urine.

It can be seen that free p-hydroxybenzoic acid is excreted almost immediately following its absorption; after 90 min, the excretion is maximum, then decreases.

p-Hydroxyhippuric acid begins to appear in the urine after 30 min, and its concentration rises uniformly during the 4 hr following the ingestion. Glucuronides, ethereal sulfates, compound A and glucuronyl derivatives appear in the urine only between 30 min and 75 min after the ingestion of the product; the excretion of glucuronides continues during the next 4 hr, a time at the end of which the excretion reaches its maximum (Fig. 1). It is mainly at that time that p-hydroxybenzoic acid is eliminated in the form of gluco- and glucurono conjugates.

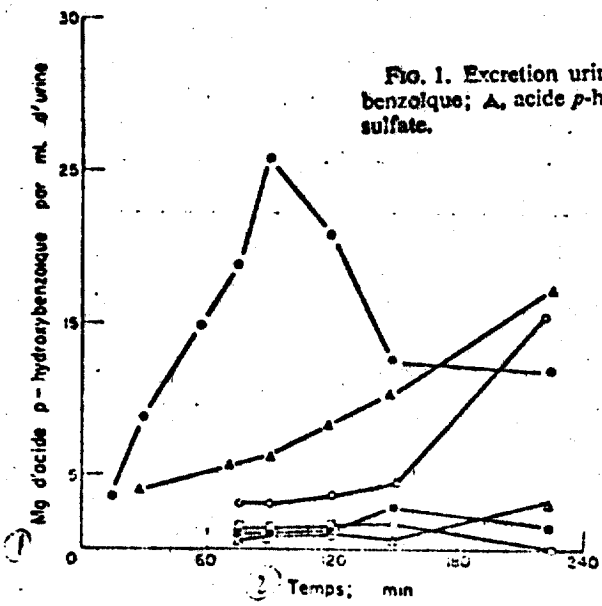

FIG. 1. Excretion urinaire de l'acide *p*-hydroxybenzoïque et de ses métabolites. ●, Acide *p*-hydroxybenzoïque; ▲, acide *p*-hydroxyhippurique; ○, glycuronides; ▣, glycuronyle; □, composé A; △, éthérosulfate.

Fig. 1. Urinary excretion of p-hydroxybenzoic acid and its metabolites. ●, p-hydroxybenzoic acid; ▲, p-hydroxyhippuric acid; o, glucuronides; ▣, glucuronyl; □, compound A; △, ethereal sulfate.

Key: 1 mg of p-hydroxybenzoic acid per ml of urine 2 Time, min

If the excretion of p-hydroxybenzoic acid and its detoxication derivatives is compared to the concentration of the acid in the blood, one finds a continuous rise of the concentration in the blood in the first hour following the absorption of the phenolic acid; then the concentration decreases and reaches a kind of plateau between 1 and 2 hr following absorption (Fig. 2).

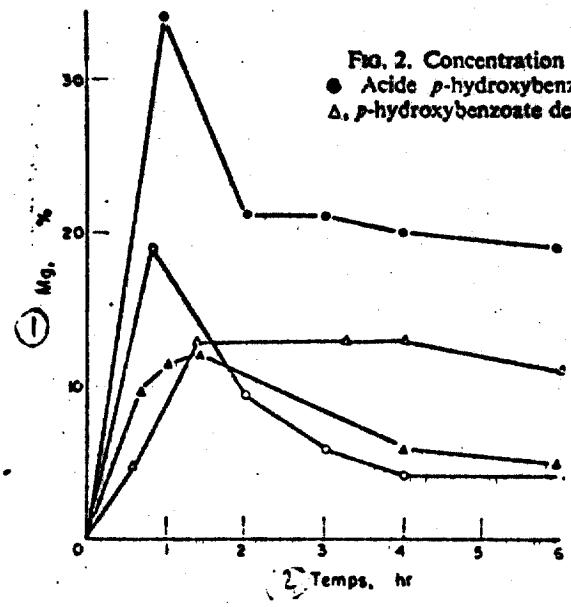

FIG. 2. Concentration de l'acide *p*-hydroxybenzoïque et de ses esters dans le sang au cours du temps. ● Acide *p*-hydroxybenzoïque; ○, *p*-hydroxybenzoate de propyle; ▲, *p*-hydroxybenzoate d'ethyle; △, *p*-hydroxybenzoate de methyle.

Fig. 2._ Concentration of p-hydroxybenzoic acid and its esters in the blood versus time.

●, p-hydroxybenzoic acid; ○, propyl p-hydroxybenzoate; ▲, ethyl p-hydroxybenzoate; △, methyl p-hydroxybenzoate.

Key: 1 mg, % 2 Time, hr

The maximum concentration of free p-hydroxybenzoic acid observed in the urine thus corresponds closely in time to that of the blood. On the contrary, after 2 hr, the concentration of glucuronides and that of p-hydroxyhippuric acid are completely deesterified in the organism; we could not detect them in the urine.

Table 2 shows the concentration of p-hydroxybenzoic acid and its metabolites in the 24-hr urine together with the percentage of the different substances eliminated in relation to the total excretion of p-hydroxybenzoic acid. It can be seen that in contrast to the result obtained by Quilley and Smith (1952) with salicylic acid, the glucurono conjugation and glycino conjugation are important in the processes of detoxication of p-hydroxybenzoic acid. It should be noted that these results are in good agreement with those obtained by direct determination of esterified sulfates and conjugated glucuronic acid; indeed, we noted an increase from 1.8 to 2.6 mg of esterified sulfate in the treated animals in comparison with the

Table 2. Elimination of p-Hydroxybenzoic Acid and its Metabolites in 24 hr in the Urine

Substance	Total urinary elimination in 24 hr in mg of p-hydroxybenzoic acid	Average percentage of eliminated substances relative to total p-hydroxybenzoic acid
p-Hydroxybenzoic acid	18-24	40
p-Hydroxybenzoylglycocoll acid	12.8-13.6	23.5
Ethereal sulfate	2.4-3.2	5
Compound A	1.8-3.2	4.3
Glucuronide derivatives	12.2-13.4	23
Glucuronyl derivatives	0.8-2.8	1.2

control animals and an increase from 10.2 to 16 mg of glucuronides in the same animals.

Identification of reductants

We studied urinary glucose on 24-hr urine samples. The urine of rat treated with p-hydroxybenzoic acid or its esters is weakly positive when the urine is treated directly with Fehling's reagent. But, by using paper chromatography, we were able to detect glucose by spraying with aniline phthalate reagent. Concentrations of reference glucose showed that the latter was present at a concentration of at least 10 μg per 0.05 ml of urine. Thus, a rat excreting approximately 10 to 12 ml of urine in 24 hr would eliminate nearly 2 mg of glucose per 24 hr. These results, similar to those obtained by Quilley and Smith (1952) with salicylic acid, thus attest to an important disturbance of glucide metabolism.

DISCUSSION

The metabolism of p-hydroxybenzoic acid in the rat differs from that of salicylic acid. Indeed, Quilley and Smith (1952) showed that 41 to 63% of salicylic acid was excreted in the free form, less than 1% is excreted in the state of salicyluric acid, 4 to 5% in the form of gentisic acid, and only 1 to 3% in the form of salicylglucuronides in the state of ether, the esterified form being absent.

Our results show that in such a study, it is interesting, from a physiological standpoint, to investigate the kinetics of appearance of the phenolic acid and its metabolites in the urine. Indeed, after 90 min, 67 to 75% of the acid is excreted in the free form, 10 to 12.5% in the form of p-hydroxyhippuric acid, and 8 to 10% in the form of compounds A and glucuronyl derivatives. Let us note that the latter four compounds appear only 90 min after the ingestion of the acid, and p-hydroxyhippuric acid appears only after 30 min (Fig. 1).

On the other hand, after 3.5 hr, 23% of the total excreted p-hydroxybenzoic acid is excreted in the free form, 30% in the form of glucuronides, 35% in the form of p-hydroxybenzoic acid, ethereal sulfates form approximately 6%, and compound A and glucuronyl derivatives, 4% (Fig. 1).

Moreover, it should be noted that the concentration of free p-hydroxybenzoic acid in the blood remains extremely low during the experiment. To explain this rather surprising fact, we must therefore assume that there is a very strong bond between the phenolic acid and proteins, since treatment with trichloracetic acid or ammoniacal alcohol or even with chloroform did not liberate the combined p-hydroxybenzoic acid; in addition, it is possible that an enterohepatic cycle can regulate the concentration of the phenolic acid in the blood within the range necessary for detoxication to be effected. This tends to be supported by current experiments.

Thus, our experiments show that it is necessary to distinguish two stages in the phenomenon of detoxication of a phenolic acid.

1. In a first stage, after a massive absorption of the toxic substance, the renal barrier is thought to be "forced," so to speak, by this substance, which then appears in the urine, chiefly in the free form. This is thought to be a kind of "mechanical" detoxication.

2. In a second state, the detoxication is thought to be chiefly metabolic; the classical processes of detoxication: glucurono- and sulfoconjugation and glycinoconjugation tend to substitute for detoxication by diffusion.

3. In the course of these two processes, it should be noted that the concentration of free p-hydroxybenzoic acid in the blood remains extremely low over the course of the experiment, suggesting that the substance is either detoxicated by the proteins themselves by strong adsorption, or perhaps there is an enterohepatic cycle that would permit the organism to detoxicate the substance at a rate with detoxication functions.

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Metabolism of a Food Preservative: *P*-Hydroxybenzoic Acid and its Esters

Abstract—We made a study of the form in which *p*-hydroxybenzoic acid and its methyl, ethyl and propyl esters are eliminated by the rat, and of the rate at which the phenolic acid and its metabolites appear in the urine. We were able, by means of paper chromatography and the use of Millon's reagent, to show the presence in the urine of *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid, an ester and ether glucuronides, an ethereal sulphate, and an unidentified compound. Study of the kinetic process of the appearance of the phenolic acid and its metabolites in the urine showed that free *p*-hydroxybenzoic acid appeared first, followed by the glucuronide and *p*-hydroxyhippuric acid, the concentration of which increased as that of the free *p*-hydroxybenzoic acid fell off. During the experiment the concentration of the phenolic acid in the blood remained extremely low.

Ann. Allerg. 26: 185-189, 1968

Parabens are preservatives used in numerous pharmaceutical and cosmetic preparations[1,2] and foods.[3] Although in general these chemicals are very innocuous, allergy to them causes a contact dermatitis that often is insidious and unrecognized. This paper discusses the recognition and treatment of paraben sensitivity and presents three case reports illustrating this entity.

PARABEN SENSITIVITY: SUBTLE TROUBLE

STEPHAN EPSTEIN, M.D., F.A.C.A.

"PARABEN" is the term applied to the alkyl esters (butyl, ethyl, methyl and propyl) of p-hydroxy benzoic acid. The parabens are quite soluble in fats, but only slightly soluble in water. Methyl paraben shows the greatest solubility in water (about 0.25 percent). Sabalitschka[4] discovered the antimicrobial activity of the parabens in 1924.

Parabens are known throughout the world as effective preservatives. In the United States, methyl, propyl and butyl parabens are used almost exclusively and often in combination, the total concentration usually amounting to not more than 0.1 to 0.3 percent. Chlortetracycline HCl (Aureomycin®) and tetracycline HCl (Achromycin®) ointments are exceptions,[1,2] as up to this writing they contain a total of 3 percent parabens.

In addition to their use in pharmaceuticals, such as creams, ointments, lotions and some oral preparations, the parabens are used in a concentration of 0.01 to 0.1 percent as preservatives in skin creams, hair lotions, sun tan preparations, face powder, soaps, lipsticks and toothpastes, as well as in syrups, milk preparations, soft drinks, candies and jellies.[3]

Incidence of Paraben Sensitivity

In 1940 Bonnevie,[5] from Denmark, first reported contact dermatitis caused by paraben. An ointment containing 5 percent ethyl paraben had been introduced as a fungicidal preparation in that country in 1938. In 1960, Sarkany,[1] from London, first showed that parabens used as preservatives in medicaments also may cause contact dermatitis. Schorr and Mohajerin[6] reported the first case of paraben contact dermatitis in the United States in 1966. Since then Schamberg[2] and Fisher[7] have published a few isolated reports, and Wuepper[8] has reported four cases. Schorr[9] has now reported three additional cases of paraben sensitivity.

Paraben contact dermatitis is common in Europe, in contrast to the low incidence in the United States. By 1963 Hjorth and Trolle-Lassen[10] had diagnosed more than 140 cases of paraben sensitivity at the Finsen Institute in Copenhagen, Denmark. They ascribed this incidence to the fact that fungicidal ointments containing 5 percent ethyl paraben are apparently potent sensitizers and are widely used in Denmark. In the United States parabens

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are used only in much lower concentrations and presumably therefore sensitization is less frequent.[4]

Detection of Paraben Sensitivity

There are several reasons why sensitivity to paraben used as a preservative has been recognized only recently and still is, as Schorr[9] suggests, a "hidden cause of intractable contact dermatitis." Not only are physicians unaware of this sensitizer, but also, as Hjorth and Trolle-Lassen[10] and Schorr[9] have pointed out, paraben contact dermatitis may present an insidious pattern (see case 1). In such instances, especially when the paraben is an ingredient of a steroid cream, treatment may lead to a protracted dermatitis without acute exacerbation so that neither the patient nor the physician suspects paraben as a possible cause of the dermatitis. Furthermore, patch tests with the causative paraben-containing preparations may be completely negative or only faintly positive and therefore still more confusing to the physician. The situation is similar to the common experience in neomycin sensitivity,[11,12] which so often is unrecognized and shows negative patch tests to preparations containing neomycin in low concentration.

The patient also may show strong contact allergy to other chemicals, thus throwing the investigator off the track because these agents seem to explain the existing dermatitis (see cases 1 and 3). It was for this reason that Sarkany[1] incorrectly suspected tetracycline as the cause of the contact dermatitis in his reported case and I was misled in one case into suspecting gentamycin (Garamycin®) as the cause of contact dermatitis (see case 3).

Therefore, one should suspect paraben sensitivity whenever patients do not improve satisfactorily after the apparently offending chemicals have been eliminated. This is especially important in those instances in which the patient claims that almost every cream or ointment he has used is irritating. These patients are not necessarily neurotics or endowed with an "irritable skin."

Testing for Paraben Sensitivity

Since Sarkany's[1] report it has been realized that patch tests with parabens must be carried out with a fairly high concentration in order to get dependable results. Studies with serial dilutions[1,10] have clearly proved this point. Some recommend a 5 percent concentration of parabens. Wuepper[8] actually applied a 15 percent concentration, as his test material contained 5 percent each of methyl, propyl and butyl parabens. I use a mixture containing 5 percent each of methyl and propyl parabens in petrolatum. These higher concentrations are not primary irritants. Sokol[13] has used test concentrations up to 12 percent without causing irritation.

I have never seen a primary irritation even when testing patients with ointments containing 20 percent of either methyl or propyl parabens. I do not recommend such high concentrations for routine patch tests because they carry a greater risk of sensitization. Such a possibility must be considered in patch testing with chemicals as widely used as the parabens. The evidence thus far suggests that 5 percent concentrations are adequate to demonstrate paraben sensitivity. Judging from Wuepper's[8] and my meager experience, intradermal tests with parabens are not helpful, in contrast to testing for neomycin sensitivity. Perhaps this is due to the low solubility of the compounds.

Cross Sensitivity to Parabens

Most patients who are allergic to one paraben cross react with other

esters. Hjorth and Trolle-Lassen[10] have reported that all of 19 patients who reacted to an ointment containing a mixture of paraben esters reacted to one or several esters when a 5 percent concentration was used. The degree of sensitivity to the various esters may differ in patients who are sensitive to several. However, only about two thirds will react when tested with only one ester.

Treatment of Paraben Sensitivity

The first step in treating paraben sensitivity is to instruct the patient to avoid all preparations, cosmetics, etc., containing parabens. The number of pharmaceutical preparations containing parabens is legion; however, eliminating offending products is fairly easy because the manufacturer must indicate on the label the presence and concentration of the parabens. Schamberg[2,11] has presented a good although incomplete list. Steroid preparations free from paraben are flurandrenolone (Cordran®) ointment and hydrocortisone (Hytone®) cream. Paraben-free antibiotic preparations include polymyxin B-bacitracin-neomycin (Neosporin®) ointment, polymyxin B-bacitracin (Polysporin®) ointment, erythromycin (Ilotycin®) ointment, and oxytetracycline and hydrocortisone (Terra-Cortril®) ointment. Parabens are usually used in the creams because preservatives are necessary for preparations containing water.[1] Those in a petrolatum base do not necessarily contain preservatives.

Elimination of paraben-containing cosmetics is a more difficult problem. There is no law to impel a manufacturer of cosmetics, toothpaste, etc., to indicate the presence of preservatives in his product. Therefore, it is advisable for the patient with a suspected paraben sensitivity to stop using all cosmetics until he can be sure that they do not contain parabens.

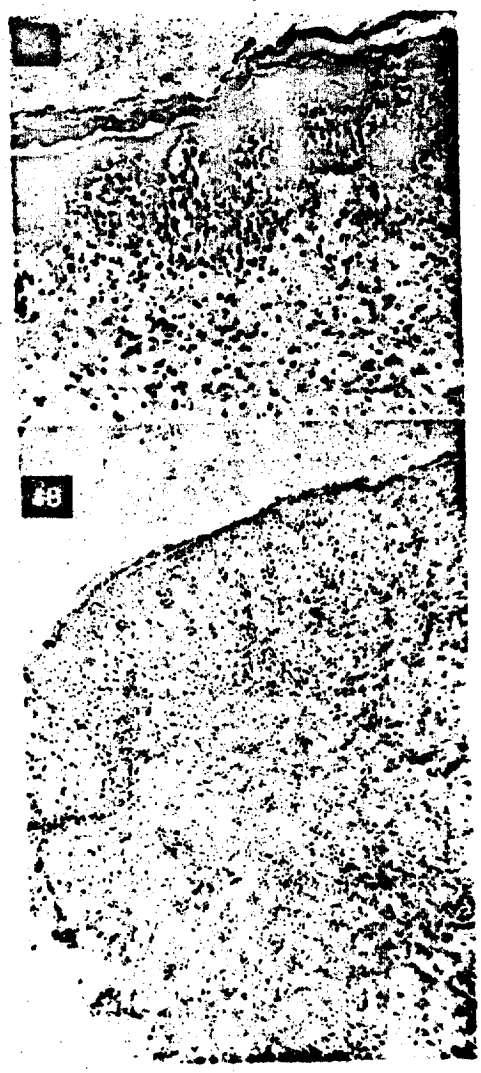

Figure 1. Patch test with a mixture of 5 percent methyl and 5 percent ethyl paraben in petrolatum, biopsied 48 hours after application of test. Swelling, erythema and tiny vesicles were present clinically. (A) Marked spongiosis, vesiculation and transmigration of lymphocytes. (B) Chiefly dermal infiltrate largely around sweat glands.

There is no evidence that parabens in food and in oral and injectable pharmaceutical preparations have caused a paraben dermatitis. However,

in patients with contact sensitivity to paraben, the subcutaneous and especially the intracutaneous injection of paraben-containing preparations such as lidocaine U.S.P. may be expected to produce local reactions similar to those caused by solutions containing thimerosal (Merthiolate®).[16]

Case Reports

Case 1. An 80-year-old white man was referred to me on October 4, 1967, because of an almost generalized itchy, dry, scaly dermatitis of about three months' duration. Two years previously he had had a contact dermatitis that had been correctly attributed to sensitivity to house paint. Another episode of acute dermatitis had followed the use of Tegrin®. The patient had not been completely free from dermatitis since that time, and had used several medications. Gradually the dermatitis, with itching, had spread over larger areas of his body. Physicians consulted previously had considered this insidious dry dermatitis essentially due to the patient's senile dry, irritated skin. Although the patient had used several prescribed creams, the dermatitis had continued to spread.

Paraben sensitivity was suspected. Treatment with a mixture of erythromycin and fluoandrenolone ointments, neither of which contains paraben, dramatically relieved the itching, and the dermatitis subsided within three weeks.

Results of patch tests confirmed the suspicion of paraben sensitivity. Application of an ointment containing methyl and propyl paraben (5 percent each) in petrolatum produced a strong reaction that appeared after 48 hours and persisted for two weeks. Patch tests gave negative or only minor and questionable reactions to several medications and creams containing parabens in low concentrations. Results of patch tests with ointments not containing parabens were uniformly negative. However, within 24 hours, patch tests with Tegrin and turpentine produced very strong allergic reactions, more pronounced than those caused by the parabens. An intradermal test with 0.05 cc of a 1:1000 solution of methyl paraben was negative.

These findings showed that the patient's previous episodes of acute contact dermatitis had been caused by turpentine and by the use of Tegrin. This case represents the insidious, often overlooked form of paraben sensitivity.

Case 2. A 66-year-old white housewife was seen early in October 1967 because of an ulcer of her right leg complicated by a chronic eczema and a subacute contact dermatitis. An itching eczema and leg ulcer, which had developed in 1937, had persisted until 1954, at which time the patient underwent stripping of the veins in the leg. The ulcer healed and the eczema was brought under fairly good control but never cleared completely. In February 1967 the patient apparently bumped her leg and an acute dermatitis developed; exacerbation of the ulcer and the eczema occurred following the use of paraben-containing steroid creams. The dermatitis cleared when the offending medications were discontinued.

Patch tests with two of the steroid creams containing low concentrations of parabens were negative; another cream produced only a questionable reaction. However, patch tests with 5 percent methyl and 5 percent propyl paraben produced strong eczematous reactions characterized by swelling and vesiculation. Histologic study confirmed the severity and allergic nature of the reactions (figures 1a and b). Intradermal tests with 0.05 cc of 1:5000 and 1:500 dilutions of methyl paraben were negative.

This and the following case are examples of an acute contact dermatitis caused by paraben sensitivity.

Case 3. A 43-year-old white man consulted me in April 1967 because of an infected ulcer on the lower part of his left leg. Since birth the patient had had a diffuse, partially verrucous hemangioma of the left leg, with a long history of traumatic ulcerations and secondary dermatitis. He was known to be allergic to neomycin.

The recently developed ulcer responded only slowly to treatment with erythromycin and polymyxin B-bacitracin ointments. Because resistance of the causative *Staphylococcus* to erythromycin was suspected (and later verified), gentamycin was substituted in May 1967. After two months the patient discontinued all topical medication because he thought the ulcer had healed. About the middle of August 1967 he injured his leg and again applied gentamycin ointment. Three to four days afterward an acute weeping dermatitis appeared around the treated ulcer; the condition improved following the elimination of the gentamycin ointment, which contains parabens. Patch tests with gentamycin ointment and cream were repeatedly negative, but patch tests with 5 percent methyl and with 5 percent propyl paraben in petrolatum were strongly positive. An intradermal test with 0.05 cc of a 1:1000 dilution of methyl paraben was negative.

Discussion

The three cases presented here are proven instances of paraben sensitivity, corresponding to those reported in the literature. The first case is an example

of the insidious, readily overlooked form that unfortunately is often permitted to develop into a generalized, disabling and miserable dermatitis. Cases 2 and 3 demonstrate acute contact dermatitis caused by parabens. Cases 1 and 3 confirm the previous observations that patients with paraben dermatitis often also show strong contact sensitivity to other agents, especially topical medicaments. This may mislead the investigating physician in regard to the real cause.

In addition to these cases, I have observed during the past year, two other patients with suspected paraben sensitivity. They had an atopic dermatitis that was aggravated by most creams. Patch tests with the offending medications were negative or only slightly positive, and tests with parabens in stronger concentrations were not done. Therefore, the paraben sensitivity in these instances was not proved.

These cases of paraben sensitivity, along with suggestions to facilitate its recognition and treatment, have been presented to increase the physician's awareness of this form of contact dermatitis.

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Procedure for the Qualitative Extraction of Certain Antimicrobial Preservatives from Foods

By CHRISTIANE GENEST and D. G. CHAPMAN (Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Canada)

In 1959 Genest and Chapman (1) reported a paper chromatographic technique for the separation and identification of a number of antimicrobial preservatives. As a continuation of this work, a procedure has been developed for the extraction of some of these preservatives from a variety of foods.

METHODS

A. Catsup, jellies, jams, preserves and marmalades.—Blend 50 g sample with 100 ml water in a Waring Blendor until a fine slurry is obtained. Acidify 60 ml of the mixture with 5 ml HCl (1+3), then subject to liquid-liquid extraction with 100 ml of chloroform for at least 4 hours. Evaporate the chloroform to dryness on a steam bath, dissolve the residue in 0.5 ml of ethanol, and subject an aliquot to paper chromatography.

B. Fruit drinks.—Acidify 60 ml of the sample directly with 5 ml HCl (1+3) and extract as described under *A*.

C. Margarine.—Melt 50 g on a water bath, add 100 ml of 0.1*N* Na_2CO_3 solution, and shake mixture frequently during the next 2 hours. When margarine is solidified, filter mixture and acidify 60 ml of the filtrate with 5 ml of HCl (1+3). Proceed as described under *A*.

D. Cheese.—Cut cheese into small pieces by shredding, weigh 50 g, and add 100 ml of 0.1*N* Na_2CO_3. Proceed as described under *C*.

E. Preserved fish.—Macerate 50 g in a Waring Blendor with 100 ml of 0.1*N* Na_2CO_3 solution. Transfer to a centrifuge bottle, shake frequently during the next 2 hours, and centrifuge. Decant off the center liquid layer, acidify 60 ml with 5 ml HCl (1+3), and proceed as described under *A*.

Discussion

Steam distillation (5–8) and shake-out procedures (9) were examined, in addition to the liquid-liquid extraction technique (2–4). During steam distillation, *p*-hydroxybenzoic acid and its esters apparently undergo some change in molecular structure, as ortho-compounds are found, the presence of which tend to falsify results. In the shake-out procedure, the emulsion layer is always present with the ether, and is inconvenient to handle. It may, however, be broken by the addition of anhydrous sodium sulfate.

The most satisfactory results were obtained with the liquid-liquid extraction procedure. It also required a minimum of labor, thus interfering least with other laboratory duties. If necessary, the low solubility of *p*-hydroxybenzoic acid and its esters in chloroform can be increased by adding a small amount of ethanol (up to 10%) to the chloroform. If benzoic acid is present, the evaporation of the chloroform must be carried out cautiously since, at this stage, it is easily lost.

Food colors extractable with chloroform may be found to interfere with the subsequent paper chromatographic analysis. Because of this, it is advisable to dissolve the residue, when a colored material is present, in 0.1*N* Na_2CO_3 solution after the evaporation of the chloroform, acidify, extract with ether, and evaporate the ether extract to dryness. The residue is then dissolved in ethanol and an aliquot is subjected to paper chromatography.

The liquid-liquid extraction technique described above has proven satisfactory for the extraction of *p*-hydroxybenzoic acid, salicylic acid, benzoic acid, sorbic acid, dehydroacetic acid, and the methyl-, ethyl-, propyl-, butyl-, and benzyl- esters of *p*-hydroxybenzoic acid from a variety of foods.

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FINAL REPORT

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ABSTRACT

HOMBURGER, FREDDY (Bio-Research Consultants, Inc., Cambridge, Mass.) The purpose of this study was to determine: A. Whether seven test compounds (Thimerosal, ethylene chlorohydrin, Methyl Paraben, Phenol Red, ethylene glycol, Benzethonium chloride and pyridine) cause malignant tumors in mice when given subcutaneously and when four pooled injection sites are transferred into one secondary host, a technique used to accelerate the biological effects. --All seven compounds were devoid of demonstrable carcinogenic effects, in contrast to 3, 4, 9, 10-dibenzpyrene which caused fibrosarcomas under the same conditions in from 8 to 17 weeks after site transfer. B. Whether the test compounds when given intravenously to mice in single or repeated injections accelerate the development of spontaneous lung adenomas in CF 1 and in A mice. --No acceleration of lung adenoma formation was observed with any of the test compounds in single or multiple injections in either mouse strain. This is in marked contrast to the acceleration of tumor formation by 3, 4, 9, 10-dibenzpyrene. C. Whether the test compounds are co-carcinogenic in mice when given subcutaneously following a standard carcinogen. --In spite of published evidence that the two-stage concept of carcinogenesis prevails in subcutaneous tissue, these studies were inconclusive, since the positive control (croton oil) failed to show co-carcinogenic activity under the same experimental conditions. An incidental consistent observation was the decoloration of the fur of black mice caused at and near the site of injection by subcutaneous administration of Benzethonium chloride. This same substance also inhibited the formation of tumors (in the co-carcinogen studies) following subcutaneous injection of 3, 4, 9, 10-dibenzpyrene.

--Author

RECAPITULATION OF MISSION
(Quoted from Contract Proposal)

"1. Attempt to Obtain Tumor Formation in Shorter Periods of Time: This protocol follows procedures outlined in the attached manuscript, which explains our new techniques to shorten the latent period between carcinogen injection and tumor formation. One hundred C57BL/6 mice are used per group and receive injections subcutaneously into the groin. At the end of 8 and 12 weeks, the injection sites of each group are excised, pooled and homogenized in Ringer's solution, and the pooled material is transferred into 25 mice (an increase in amount of transferred tissue by a factor of 4) of the same strain and age. The secondary hosts are palpated weekly and any tumors appearing are treated as in a), above.

2. Intravenous Injection into A/Heston Jax Mice of Test Substances and Observation of Lung Adenomas: According to Harold L. Stewart, the lung tissue of A/Heston mice is the most sensitive indicator for carcinogens available. When known carcinogens are injected subcutaneously into A/Heston mice, greatly increased numbers of adenomas can be counted on their lung surfaces before sarcomas form at the injection site. Upon intravenous injection of benzpyrene and other known carcinogens, the number of lung adenomas increases in proportion to the administered dose. We have confirmed in our own laboratory both of these observations and believe with Stewart that a substance that fails to cause an increase of adenomas of A/Heston mice upon intravenous injection is extremely unlikely to be a carcinogen under other conditions.

Fifty A/HeJ mice will be used per compound and for a vehicle control. At three months of age, they will receive a single intravenous injection of the test substances at the highest tolerated dose (dose-finding must first be done by the intravenous route for each compound). Three to six months later the mice will be killed, their lungs inflated with formaldehyde, and the adenomas on the lung surface will be counted under a x 15 dissecting microscope. Spot checks can be made in some animals to determine whether the experiment could be terminated earlier. Histological studies will be made in selected lung specimens.

3. Studies on Co-carcinogenesis (Promotion): Extensive experience has accumulated in our laboratory on the carcinogenic effect of benzo(rst) pentaphene (old nomenclature: 3, 4, 9, 10-dibenzpyrene), a potent carcinogen that has the advantage of largely remaining at the original site of its injection. Fifty C57BL/6 male mice will be used per group for each test material. They will be randomized as in experiment 1a, above, and after

acclimatization, they will receive 12.5 mμ of benzo(rst)pentaphene in the region of the groin. Mice of the control group will receive the vehicle twenty-four hours later into the same site. This will be repeated at weekly intervals on three occasions. In the test groups, these "promoting" injections will be done in the same vehicle at similar intervals using the test compounds at the doses used in 1a, above. An additional control group will receive promoting injections of croton oil in the same vehicle in the maximum tolerated dose. Weekly palpations will be done in these mice and any palpable tumors will be sectioned for histological examination."

Modification of Experiments

We were authorized to add one group of mice in the subcutaneous co-carcinogen study at one-half of the initial dose of Benzethonium chloride (0.35 mg).

Furthermore, in Supplemental Agreement No. 1 of January 2nd, 1968, the following additional studies were authorized:

"a. Inject intravenously 8 groups of 20 CF 1 female mice on days 0, 30 and 60, with examination of the lungs three months after the last injection.

b. Inject intravenously (single dose) 8 groups of 50 A/Jax female mice."

Finally on May 23rd, the duration of this contract was extended to August 22nd, 1968.

SOURCES OF COMPOUNDS; DOSE-FINDING EXPERIMENTS

On June 6th, 1967, the following substances for testing were received from N.I.H.:

Table 1

List of Compounds and Their Sources

Compound	Bio Code	Wt. (gms)	Manufacturer
Thimerosal	1	3.0	Eli Lilly & Co.
Methyl Paraben	2	9.0	Tenneco Chem. Inc. (Heyden Div.)
Benzethonium chloride	3	10.0	Rohm & Haas
Pyridine*	4	30.0	Fisher Scientific Co.
Phenol Red	5	25.0	Allied Chem. (Nat. Aniline Div.)
Ethylene glycol	6	270.0	Fisher Scientific Co.
Ethylene chlorohydrin	7	6.5	Eastman Organic Chemicals

* The first shipment of pyridine was replaced because of corrosion of the stopper in the first bottle.

Determination of Highest Tolerated Subcutaneous Doses

The maximum single tolerated dose of these compounds was estimated by subcutaneous (s.c.) route in C57BL/6 male mice thirteen weeks of age. The mean body weight of 168 mice (8 mice/group) used in preliminary experiments was 20.0 (range: 17 gm to 25.4 gm).

The data given to us by Dr. Singer of N.I.H. for the LD/50's for these substances were used as a basis for dose-finding experiments. The maximum tolerated dose was assumed to be about 50% or less of these LD/50's. In twenty dose-finding experiments using eight mice for each, the following doses were established as the maximum tolerated amount for repeated injections.

Table 2

Maximum Tolerated Doses for Repeated Subcutaneous Injections in C57BL/6 Male Mice

Thimerosal (suspension)	10 mg/kg	(10 mg/kg- 80 mg/kg)*
Methyl Paraben (solution)	125 mg/kg	
Benzethonium chloride (suspension)	35 mg/kg	(17.5 mg/kg- 180 mg/kg)
Phenol Red (saturated suspension at about 600 mg/kg)		
Ethylene glycol (emulsion)	1300 mg/kg	(1,300 mg/kg-7,800 mg/kg)
Ethylene chlorohydrin (solution)	60 mg/kg	(60 mg/kg- 180 mg/kg)
Pyridine (solution)	300 mg/kg	(300 mg/kg- 865 mg/kg)
Croton oil (solution)	0.1 %	(0.1 % - 25 %)

* The figures in parentheses indicate the dosage range tested.

The vehicle for all substances was tricaprylin (Eastman Kodak Co., Rochester, New York).

Observations on Toxicity and Side Effects by Subcutaneous Route Using Maximum Tolerated Doses

Four days after injection of ethylene glycol, seven animals were lost during the weekend. This may have been due to factors other than the test compound. All animals in the other groups survived and appeared in good condition.

The following gross observations were made at the injection sites:

Thimerosal - extensive inflammatory reaction at the injection site with large, semi-solid, palpable cystic masses. Three animals showed slight ulcera-

tions at the injection site.

Methyl Paraben - Small, ill-defined, soft cysts in majority of animals; later on, small ulcerations which healed.

Phenol Red - Cysts similar to those in the Methyl Paraben group; Phenol Red observed to be excreted in the urine.

Croton oil - A few small ulcerations were observed.

Pyridine - Minor ulcerations which healed.

Benzethonium chloride - Some large ulcers which healed within approximately four weeks and the delayed changes described below.

Ethylene glycol, ethylene chlorohydrin and tricaprylin showed no effect.

Observations on Depigmentation of Fur by Subcutaneously-Injected Benzethonium Chloride

In all of eight animals (C57BL/6 mice) that were given 70 mg/kg of Benzethonium chloride subcutaneously in dose-finding experiments, white spotting of the fur was noted thirty-four days later near the site of injection. Five out of eight animals receiving 35 mg/kg at the same time showed a similar spotty depigmentation of their fur. When the bleached fur was plucked and allowed to regrow, the new hair in all plucked animals grew again as white fur.

Groups of three male C57BL/6 mice were painted by means of a Camel's hair brush with the following doses of Benzethonium chloride: 280 mg/kg, 140 mg/kg, 70 mg/kg, 35 mg/kg, 17.5 mg/kg, 8.75 mg/kg and, as a control, tricaprylin alone. There were severe local blistering reactions at the two highest dose levels, less marked local reactions at the 70 and 35 mg/kg levels, and no visible reaction with 17.5 and 0.75 mg/kg. No immediate bleaching occurred. Ten days later, there was spotty depigmentation of fur at the site of painting in two of the mice that had received 140 mg/kg. Hair exposed *in vitro* for forty-eight hours was not bleached by the concentrations of Benzethonium chloride used in the painting experiment.

Doses for Intravenous Injections; Observations on Acute Toxicity

It was found that the maximum tolerated subcutaneous dose of all compounds was adequately tolerated via the intravenous route and; therefore, the doses used in subcutaneous injections were also used for single and multiple intravenous injections. The gross observations made on animals having received

single intravenous injections of the various compounds are shown below.

Table 3

Acute Toxicity of Seven Compounds when Given Intravenously to A/Jax Mice in Groups of Six Animals

The intravenous injection in Ringer's solution of that dose which, when given subcutaneously, was the maximum tolerated dose, caused the following observations:

Ethylene glycol	26 mg/mouse	no effect
Thimerosal	0.2 mg/mouse	no effect
Ethylene chlorohydrin	1.2 mg/mouse	no effect
Benzethonium chloride	0.7 mg/mouse	half of mice died
Benzethonium chloride	0.35 mg/mouse	no effect
Pyridine	6 mg/mouse	palpitations for one minute; shock; recovery after forty-five minutes
Methyl Paraben	2.5 mg/mouse	gasping respiration; shock; recovery after ninety minutes
Phenol Red	12 mg/mouse	two mice died in first group; four recovered in a second group; four out of five died
Phenol Red	6 mg/mouse	two out of nine mice died
Phenol Red	3 mg/mouse	six mice survived after episode of tachypnea; red color of skin disappeared after twenty-four hours

REPORT ON ATTEMPT TO OBTAIN TUMOR FORMATION IN SHORTER PERIODS OF TIME

Method

As outlined in the original proposal, the method used was that described by Homburger and Treger in Cancer Research (27:1205-1213, 1967). One hundred C57BL/6 males, seven weeks old, received injections of the test compounds into the groin in the doses shown in Table 1. The medium used to dissolve or suspend the test compounds was 0.1 ml of tricaprylin (Eastman Kodak Co., Rochester, New York).

Table 4

Doses of Compounds Used in Injection Site Transfer Studies; Numbers of Survivors

Compound	Dose/Mouse	Survivors
Thimerosal	0.2 mg	21/25
Ethylene chlorohydrin	1.2 mg	24/25
Methyl Paraben	2.5 mg	12/25
Phenol Red	12 mg	25/25
Ethylene glycol	26 mg	15/25
Benzethonium chloride	0.7 mg	25/25
Pyridine	6 mg	25/25
Positive Control		
Dibenzpyrene	25 mg	24/25
Negative Control		
Tricaprylin	0.1 mg	23/25

Five weeks after these injections, the injection sites were excised, minced in 6 ml of Ringer's solution and pooled. The resulting tissue brei was divided into twenty-five equal portions, each of which was injected subcutaneously into one secondary host (C57BL/6 male of same age as primary host [twelve weeks]). The injection sites in the secondary hosts were palpated weekly beginning one week after transfer of the pooled injection sites.

In the positive controls injected with dibenzpyrene, the first palpable tumors appeared eight weeks after transfer of the sites (four tumors or 16%). Ninety-six percent of the animals had palpable tumors seventeen weeks after transfer of the sites. This was exactly as in earlier experiments on injection site transfers with dibenzpyrene (Figure 1). Whenever a palpable tumor was found, the animals were killed and the tumor was preserved for histological studies. No palpable tumors were observed in any of the other groups in this experiment.

Twenty-three weeks after the injection of the compounds into the primary hosts and eighteen weeks after transfer of the injection sites into secondary hosts, all animals were sacrificed. All injection sites were excised and preserved for histological studies.

Gross autopsies were made of all animals, and abnormal organs were prepared for histological study. All carcasses were preserved. Table 4 lists the numbers of survivors after site transfer in all experiments.

Comments on Mortality

The only significant mortality observed was in Methyl Paraben and in the ethylene glycol groups. In the Methyl Paraben group eight weeks after the site transfer, six animals in different cages were found dead without any apparent reason. The carcasses were too autolyzed for study. Four weeks later, another six animals were dead under similar circumstances.

In the ethylene glycol group, ten animals in one cage were found dead eight weeks after the transfer of injection sites. These animals had been reported "in poor shape" one week earlier. Again, the carcasses were too far autolyzed for study. There appears to be a cage factor in this particular instance. However, a similar loss of animals had previously occurred in dose-finding experiments on ethylene glycol.

Five injection sites of each of the groups were taken at random and processed for histological examination on hematoxylin-eosin-stained sections. The histological findings were remarkably similar in all of these groups. They showed granulation tissue forming multiple granulomas with numerous giant cells scattered throughout the subcutaneous tissue. There were strands of scar tissue and numerous cysts, either lined by single layers of endothelium-like cells or by granulation tissue with numerous giant cells, often containing cholesterol clefts. Some of the cysts were filled with amorphous, granular or reticular, pink staining material. There were no instances where fibroblasts in the granulation tissue and scar tissue even remotely suggested malignant transformation. In the tricaprylin controls, similar foreign body reactions were seen. All of the palpable tumors of the dibenzpyrene series proved to be fibrosarcomas. (Figures 2 and 3 show photomicrographs of some transferred injection sites. Figure 4 is an example of a pathology report.)

The only groups wherein any pathology was noted outside of the injection sites were:

a) the positive control (dibenzpyrene) - one enlarged liver showing congestion and distention of bile ducts and sinusoids;

b) In the Benzethonium chloride group, there were four instances of enlarged spleens and livers. These were found on histological examination to be aging changes with probable amyloidosis. There was also one congested liver.

c) In one animal of the Methyl Paraben group, there was one enlarged spleen similar to those described above.

These changes do not appear to be significant.

In the animals receiving Benzethonium chloride, all of the animals receiving the original injection showed decoloration of the fur, as described above. In the secondary hosts, no change of color was noted in the fur.

Conclusions

These studies suggest that none of the tested compounds can be demonstrated to be carcinogenic under the conditions of these experiments.

REPORT ON USE OF "THE MOST SENSITIVE SYSTEM" FOR CARCINOGEN DETECTION: INTRAVENOUS INJECTION INTO A/Heston AND CF 1 MICE AND OBSERVATION OF LUNG ADENOMAS

The methods used in this study were those described by Heston and Schneiderman in Science (117:109-111, 1953), and slightly modified by us (Lung Tumours in Animals, Proceedings of the Third Quadrennial International Conference on Cancer held at the University of Perugia, June 24-29, 1965, pp. 527-536).

Three groups of experiments were done. In the first two sets of experiments, single intravenous (tail vein) injections were made into groups of fifty CF 1 female and fifty A/Jax female mice, and in the third group, intravenous injections were made repeatedly (seven injections at monthly intervals) into groups of twenty CF 1 female mice. At the end of twenty-eight weeks, the mice were sacrificed, the lungs inflated with formaldehyde and inspected under a dissecting microscope for lung tumors visible on the lung surfaces. Histological sections were taken of some of the tumors seen (Figures 5-10).

The results are shown in Tables 5, 6 and 7.

The analysis of these data, based on Table II, Publication No. 749, National Academy of Sciences, National Research Council, shows that in this "most sensitive" system, none of the treated substances is carcinogenic for A/Jax or CF 1 mouse lungs. On the other hand, the low dose of 0.05 mg of dibenzpyrene was significantly carcinogenic at the 5% probability level when tested in CF 1 mice, as were the higher doses in the A/Jax mice.

CO-CARCINOGEN STUDY

Groups of fifty C57BL/6 male mice, randomized by weight so that the average body weight of the groups ranged from 22.1 to 22.4 gm, received 12.5γ of benzo(rst)pentaphene (3,4,9,10-dibenzpyrene [DBP]) in tricaprylin subcu-

Table 5

Intravenous Injections and Lung Tumors in Groups of 50 CF 1 Female Mice; Results after 28 Weeks

Date started	Compound	Dose/ mouse (mg)	No. survivors	Animals with tumors (%)	No. tumors/mouse (average among those with tumors)
9/25/67	Thimerosal	0.2	49	10.2	1.0
10/2/67	Ethylene glycol	26.0	48	10.4	1.0
10/10/67	Pyridine	6.0	46	10.9	1.2
10/16/67	Ethylene chlorohydrin	1.2	46	10.9	1.0
10/23/67	Benzethonium chloride	0.35	46	19.5	1.1
10/31/67	Phenol Red	6.0	44	6.8	1.1
10/31/67	Methyl Paraben	2.5	44	21.7	1.4
Negative Control					
10/2/67	Ringer's (0.2 cc)	---	48	14.5	1.0
Positive Controls					
10/17/67	DBP	0.05	44	34.5	3.6
11/28/67	DBP	0.1	49	85.7	11.2
10/19/67	DBP	0.5	43	81.4	38

To be statistically significantly different from the tumor incidence in the negative controls (14.5%), the incidence in a treated group at the 1% probability level would have to be 40%; at the 5% probability level, the incidence would have to be 31%.

Table 6

Intravenous Injections and Lung Tumors in Groups of 50 A/Jax Female Mice; Results after 28 Weeks

Date started	Compound	Dose/ mouse (mg)	No. survivors	Animals with tumors (%)	No. tumors/mouse (average among those with tumors)
10/26/67	Phenol Red	6.0	44	23	1.1
10/26/67	Methyl Paraben	2.5	44	14	1.3
11/3/67	Ethylene glycol	26.0	41	19.5	1.1
11/3/67	Benzethonium chloride	0.35	39	25.6	1.1
11/8/67	Pyridine	6.0	42	19	1.0
11/8/67	Thimerosal	0.2	43	3.3	1.2
11/14/67	Ethylene chlorohydrin	1.2	45	22.2	1.0
Negative Control					
10/24/67	Ringer's	0.2 cc	48	14	1
Positive Controls					
11/17/67	DBP	0.05	47	68.1	8.7
11/16/67	DBP	0.1	48	83.3	19.8

Statistical significance considerations same as in Table 2.

Table 7

Repeated Intravenous Injections and Lung Tumors in Groups of 20 CF 1 Female Mice; Results after 28 Weeks

Date started	Compound	Dose/ mouse (mg)	Total dose (mg)	No. survivors	Animals with tumors (%)	No. tumors/mouse (average among those with tumors)
11/21/67	Methyl Paraben	2.5	17.5	20	20	1.2
11/21/67	Phenol Red	6.0	42	18	11	1.0
11/21/67	Thimerosal	0.2	1.4	19	5.3	1.0
11/21/67	Benzethonium chloride	0.35	2.45	13	23	1.3
11/22/67	Ethylene chlorohydrin	1.2	8.4	18	28	1.2
11/22/67	Ethylene glycol	26.0	182	20	15	1
11/22/67	Pyridine	6.0	42	20	15	1
11/22/67	Ringer's	---	1.4 ml	18	11.1	1

To be statistically significantly different from the Ringer's controls (11%), the tumor incidence in any treated group would have to be 45% at the 5% probability level.

No positive controls were included, since extensive experience has taught us that with multiple injections of known carcinogens, the tumor incidence is 100% long before twenty-eight weeks elapse.

taneously and twenty-four hours later, the test compound in the same site. Additional injections of the test compound were made seven and fourteen days later, except in the case of Benzethonium chloride (Compound #3, Table 8) where only two injections were made. An additional series of Benzethonium chloride at 0.35 mg/mouse was started later after authorization had been obtained. Groups of mice received two injections of 70 mg each, the second group having received three injections of 35 mg each.

The injection sites were palpated weekly and tumors noted when 1 cm in diameter. Table 8 shows the results (also illustrated in Figure 11). The animals were killed at the end of the 29th to 31st weeks of the experiment. Histological examination showed that all tumors studied were the typical fibrosarcomas as usually produced by dibenzpyrene.

The outcome of this study is inconclusive as to co-carcinogenic effects. This is so because croton oil failed to have any co-carcinogenic action under the present experimental conditions. This renders less significant the observation that

Table 8

Co-carcinogen Study - Vehicle: Tricaprylin

Compound	Dose/mouse/ injection (mg)	Date started	Weeks after start	No. Animals		Cumulative no. tumors	Cumulative % tumors	Remarks
				Initial	Present survivors			
Control - DBP	0.025	6/22/67	31	50	48	25	52.0	No ulcerations
Control - (DBP) + Croton Oil	0.025 0.1	6/22/67	31	50	45	22	48.8	No ulcerations
Benzethonium chloride	0.7	7/5/67	29	50	41	14	34.1	All mice 'Pinto'; no ulcerations †
Benzethonium chloride §	0.35	8/17/67	23	50	38	0	0.0	97.4% 'Pinto'
Ethylene chlorohydrin	1.2	7/5/67	29	50	47	20	42.5	No ulcerations
Pyridine	6	7/5/67	29	50	46	19	41.3	No ulcerations
Phenol Red	6	6/21/67	31	50	46	21	45.6	No ulcerations
Thimerosal	0.2	6/21/67	31	50	45	18	40.0	No ulcerations
Methyl Paraben	2.5	6/21/67	31	50	49	26	53.0	No ulcerations
Ethylene glycol	26.0	6/21/67; 6/26/67	31	70*	49	19**	38.9	No ulcerations

0.1 ml of 0.1% solution of croton oil in tricaprylin.
† 'Pinto' refers to decoloration of fur previously reported.
§ Additional group of mice receiving one-half of the maximum tolerated dose of Benzethonium chloride.
* Because ten mice died shortly after the first injection, an additional twenty mice were injected, beginning six days after the first experiment.
** Average of two groups

none of the test compounds were co-carcinogenic.

There is a body of literature which suggests that the two-phase concept of carcinogenesis (initiation and promotion) is valid in the subcutaneous site. The pertinent publications are the following:

1. Sall, R.D. and Shear, M.J., Studies in Carcinogenesis. XII. Effect of the Basic Fraction of Creosote Oil on the Production of Tumors in Mice by Chemical Carcinogens. J. Nat. Cancer Inst. 1(1):45-55, 1940.

2. Cabot, S., Shear, N., Shear, M.J. and Perrault, A., Studies in Carcinogenesis. XI. Development of Skin Tumors in Mice Painted with 3:4-Benzpyrene and Creosote Oil Fractions. Am. J. Pathol. XVI(3):301-312, 1940.

3. Klein, M., The Action of Croton Oil in the Induction of Sarcomas in Mice. J. Nat. Cancer Inst. 11(4):843-848, 1951.

It is possible that the dose of croton oil used in the present experiment (0.1 mg) was too high. Klein observed positive co-carcinogenic effects with 0.004 mg of croton oil.

It is suggested that studies on subcutaneous co-carcinogenesis be repeated with the purified or synthetic phorbol esters now available from the work of Hecker (The Tumor-Promoting Principles from Croton Oil, In Progress in Experimental Tumor Research, F. Homburger, Ed., Vol. 12, S. Karger AG, Basel/New York, 1969) and Van Duuren (Tumor-Promoting Agents in Two-Stage Carcinogenesis, In Progress in Experimental Tumor Research, F. Homburger, Ed., S. Karger AG, Basel/New York, 1968) which, while more potent co-carcinogenic agents, are less irritating and less toxic.

COMMENTS ON BENZETHONIUM CHLORIDE

One interesting observation is the significant inhibiting effect of Benzethonium chloride upon tumor formation. This suggests that Benzethonium may be a cytotoxic compound as could also be deduced from its bleaching effect on hair.

Depigmentation of hair can occur in areas overlaying healing wounds or inflammation. In subsequent growth cycles, such hair will grow again with its natural color.

According to Dr. Herman B. Chase of Brown University, Providence, Rhode Island, who is an expert in this field of hair pigmentation and de-pigmentation

(vide pp. 229-237, In The Biology of Hair Growth, Montagna and Ellis, Eds., Academic Press, 1958), the phenomenon which we have observed with Benzethonium chloride is rare, difficult to explain, and calls for additional research in depth. This could well be a radiomimetic effect such as has been described for nitrogen mustards.

We quote the following two paragraphs from A. Haddow's chapter In The Physiopathology of Cancer, F. Homburger, Ed., Hoeber-Harper, New York, 1959 (Sec. Ed.), p. 604:

"From all the evidence available it appears that the main primary action of the nitrogen mustards is exerted during the "resting stage", that the resulting damage becomes cytologically manifest only during mitosis, and that such damage may accumulate in successive cell divisions, as indicated by the presence of micronuclei of different ages, until the cell is in many cases no longer viable. Both the clinical effects which these substances are capable of producing, and the cytologic changes which form their basis, fully justify the description of such agents as "radiomimetic" (Dustin, 1947), even though there may be profound differences between them and ionizing radiations in the details of their action. The striking similarity in at least their biologic end results is also shown in other phenomena which can be induced by radiation and the mustards equally."

"Examples of this similarity are as follows: (1) local greying or bleaching of hair in mice, described by Hance and Murphy (1926; Hance, 1928) as produced by roentgen irradiation, and extensively studied by Chase and his coworkers (Chase, 1949, 1951; Chase, Quastler and Skaggs, 1947; Chase and Smith, 1949; Chase and Rauch, 1950), and for the case of chemical agents by Boyland and Sargent (1951); (2) characteristic acute and chronic degenerative changes in the bone-marrow and testis."

(emphasis ours)

The evidence so far obtained is insufficient to indict Benzethonium chloride as either a somatic mutagen or a radiomimetic drug. However, the observations made suggest strongly that it is cytotoxic and additional research in depth is indicated to ascertain that this widely used compound is as inocuous as has been hitherto assumed.

LEGENDS

Figure 1: Comparison of subcutaneous tumor induction (25γ DBP) by injection site transfer and without transfer in C57BL/6 mice.

Figure 2: (Path. No. 1303(5)) Hematoxylin-eosin-stained section of injection site transfer material. Original injection, 6 mg of pyridine in tricaprylin 12/7/67; site transfer 1/9/68; sacrificed 5/21/68. Note foreign body reaction with round cell infiltration, giant cells, cholesterol clefts, cysts (magnification x 400).

Figure 3: (Path. No. 1303(3)) Hematoxylin-eosin-stained section of an injection site transfer material from another animal in the same group (pyridine). This shows the same features with more emphasis on giant cells and granulomatous nature of reaction. Figures 2 and 3 are typical of all sections studied in this experiment (except the positive controls which showed fibrosarcomas similar to that shown in Figure 12).

Figure 4: Example of a Pathology Report.

Figure 5: (Path. No. 1531) The following hematoxylin-eosin-stained sections (magnification x 200) show indistinguishable adenomas of the lung, such as seen in both A and CF 1 mice, observed in animals having received the treatments described. Illustrated in Figure 5, effects of 100γ DBP in an A/Jax mouse, sacrificed twenty weeks after injection. These lesions are identical with those observed in untreated animals and illustrated, for example, in H. Stewart's chapter, entitled Experimental Cancer of the Alimentary Tract, In The Physiopathology of Cancer, F. Homburger and W. H. Fishman, Eds., 1st edition, Hoeber-Harper, New York, pp. 3-45, 1953.

Figure 6: (Path. No. 1534) Effects of 100γ DBP in a CF 1 mouse, sacrificed twenty-three weeks after injection.

Figure 7: (Path. No. 1533(2)) Effects of 500γ DBP in a CF 1 mouse, sacrificed twenty-three weeks after injection.

Figure 8: (Path. No. 1536(2)) Effects of ethylene chlorohydrin in a CF 1 mouse, sacrificed twenty-eight weeks after first of seven injections.

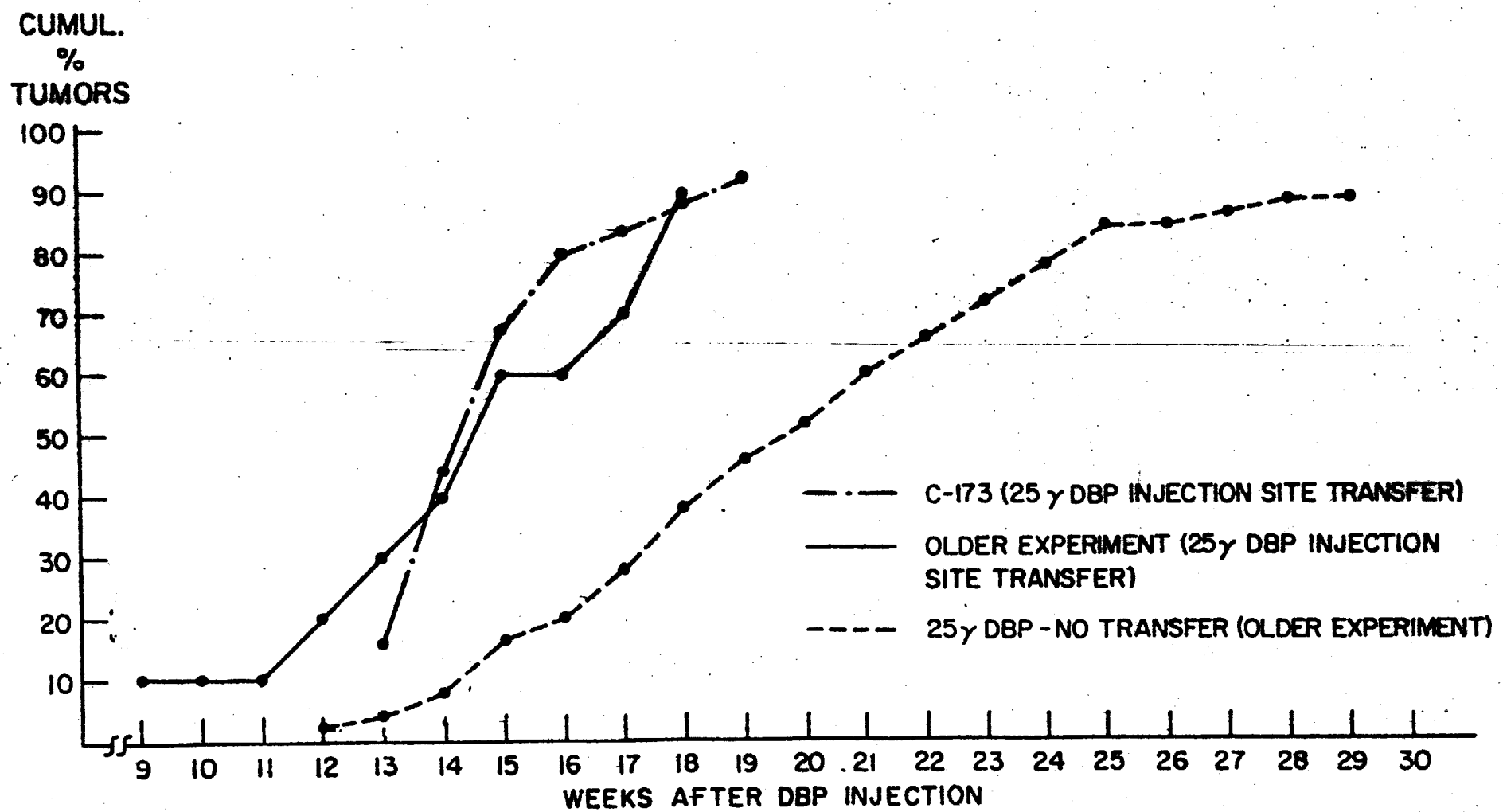
Figure 9: (Path. No. 1538(2)) Effects of Methyl Paraben in a CF 1 mouse, sacrificed twenty-eight weeks after first of seven injections.

Figure 10: (Path. No. 1539(3)) Effects of Benzethonium chloride in a CF 1 mouse, sacrificed twenty-eight weeks after first of seven injections.

Figure 11: Effect of the seven compounds on subcutaneous tumor induction with 12.5γ DBP in 0.1 cc tricaprylin.

Figure 12: (Path. No. 930) Hematoxylin-eosin-stained section (magnification x 400). A typical fibrosarcoma, invading muscle, as usually produced by subcutaneous injection of DBP. The one illustrated here occurred after injection of 500γ DBP followed by ethylene chlorohydrin.

Figure 1



COMPARISON OF SUBCUTANEOUS TUMOR INDUCTION (25γ DBP) BY INJECTION SITE TRANSFER AND WITHOUT TRANSFER IN C57BL/6 MICE

Final Report - Project C-173
Figure 4

REQUEST FOR PATHOLOGY

(Check one)
BIO-RESEARCH INSTITUTE ( )
BIO-RESEARCH CONSULTANTS (✓)

No. 68-1139
Tissue No. 8636 - 8641
Project No. C173

Request for: Histology ✓ Special Stain _____ Frozen Section _____
Cytology _____ Bacteriology _____ Gross Photo _____ Microphoto _____

Date of death/sacrifice 4-24-68 Animal No. 1-6 Sex ♂ Species M Strain C3H/6

Description of Material: Transfer site

History of Animal: Ethylene chlorhydrin (1.0 mg/0.1 cc Tricaprylin) inj 11-8-1967
Site transfer 11-13-67. Sac'd 24 weeks after inj of carcinogen

What specific information is wanted from pathological study?
Formation of cancer cells?
other findings

REPORT

Requested by N. V. Ryan

8636 #1 A piece of dense fibrous tissue throughout which are scattered many cysts of various sizes. In some areas some muscle is recognizable. There are also round cell infiltration and giant cells.

8637 #2 Same description as above. In addition, this section shows one area of a densely blue-staining granular material surrounding some giant cells, which could be a focus of calcification.

8638 #3 This shows the same tissue, but adjoining it a large area of necrosis with round cell and polymorphonuclear infiltration.

8639 #4 This shows the same tissue, but in this section is also contained a lymph node adjoining a granulomatous foreign-body reaction.

8640 #5 Fibrous tissue adjoining an area of fatty tissue which contains numerous small cysts.

8641 #6 This section shows a large cyst measuring 1/2 cm x 3 mm, containing an amorphous and, in places, reticulated pink -staining material surrounded by a thin fibrous capsule and embedded in fatty tissue.

Report by F. Homburger, M.D.
Date May 13, 1968

Figure 2

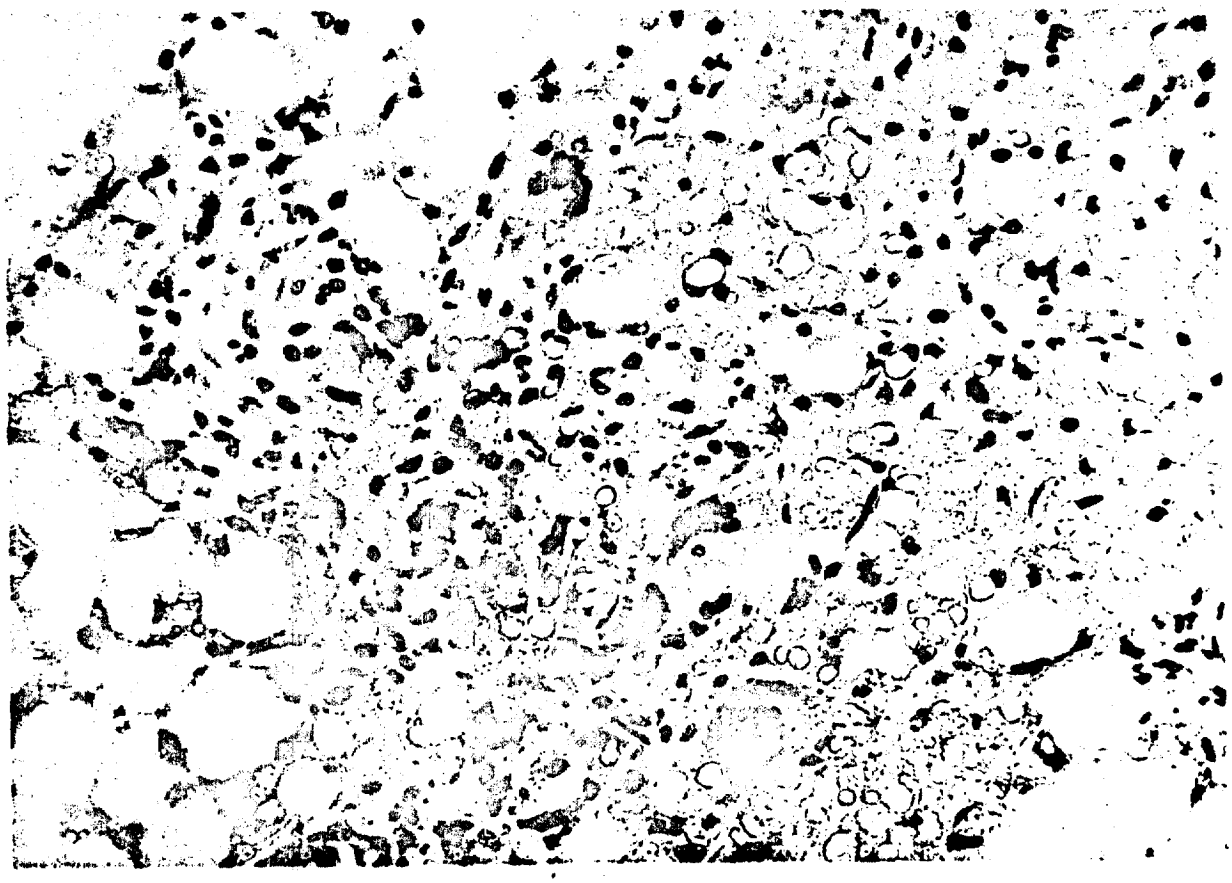


Figure 3

Figure 5

Figure 6

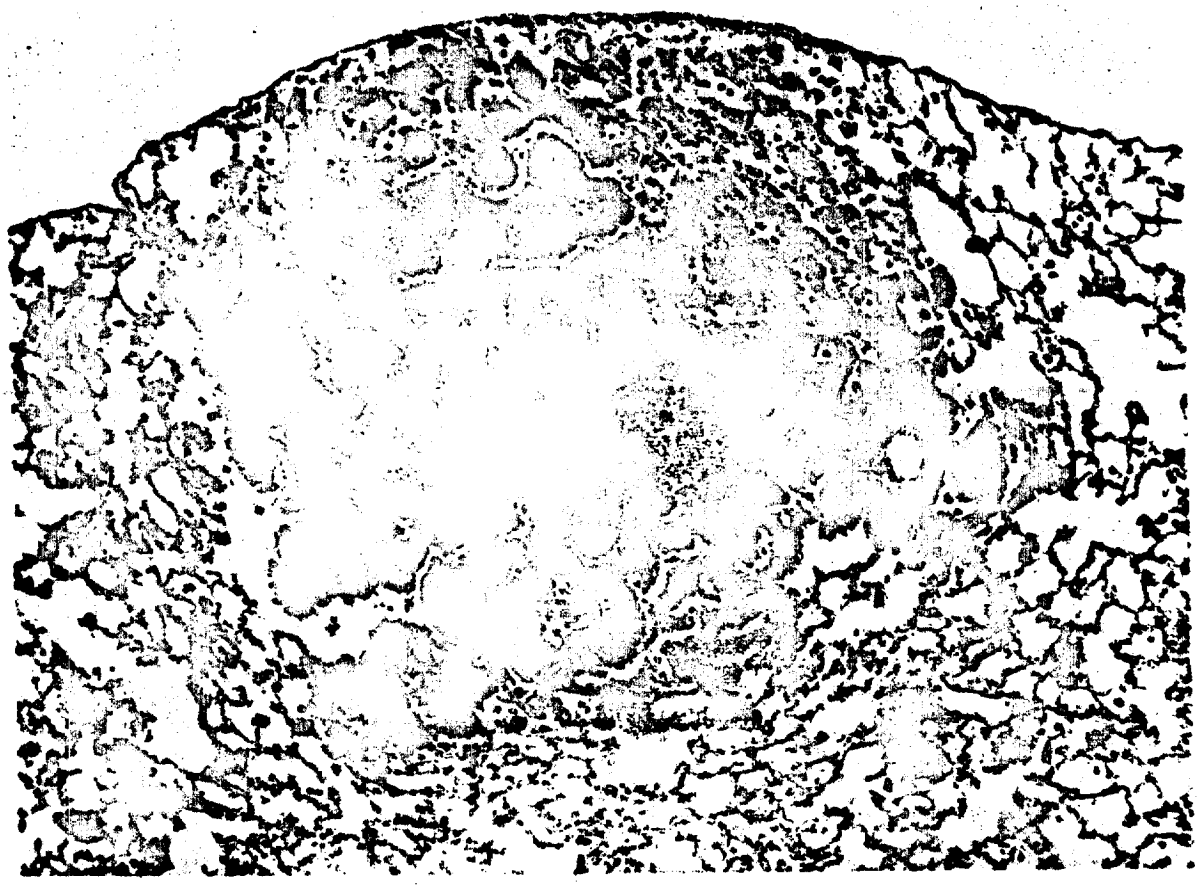
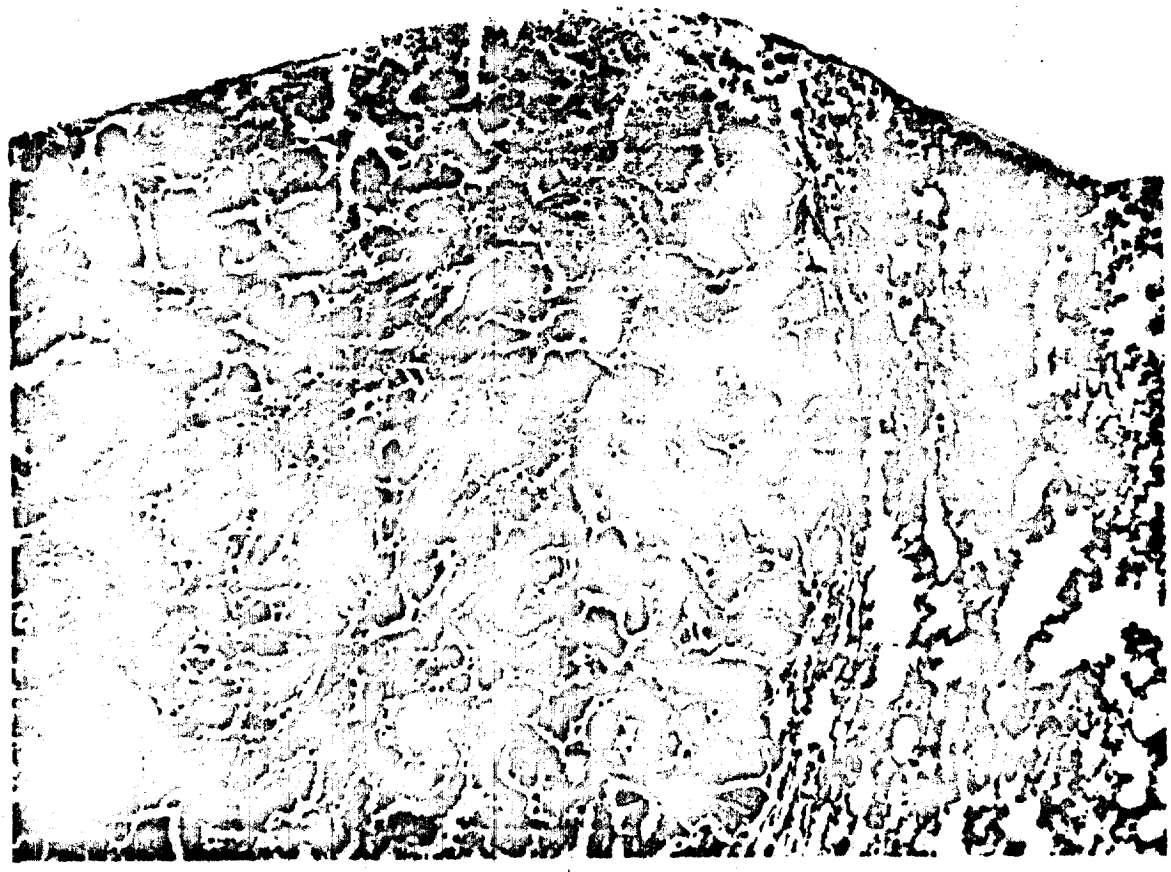


Figure 7



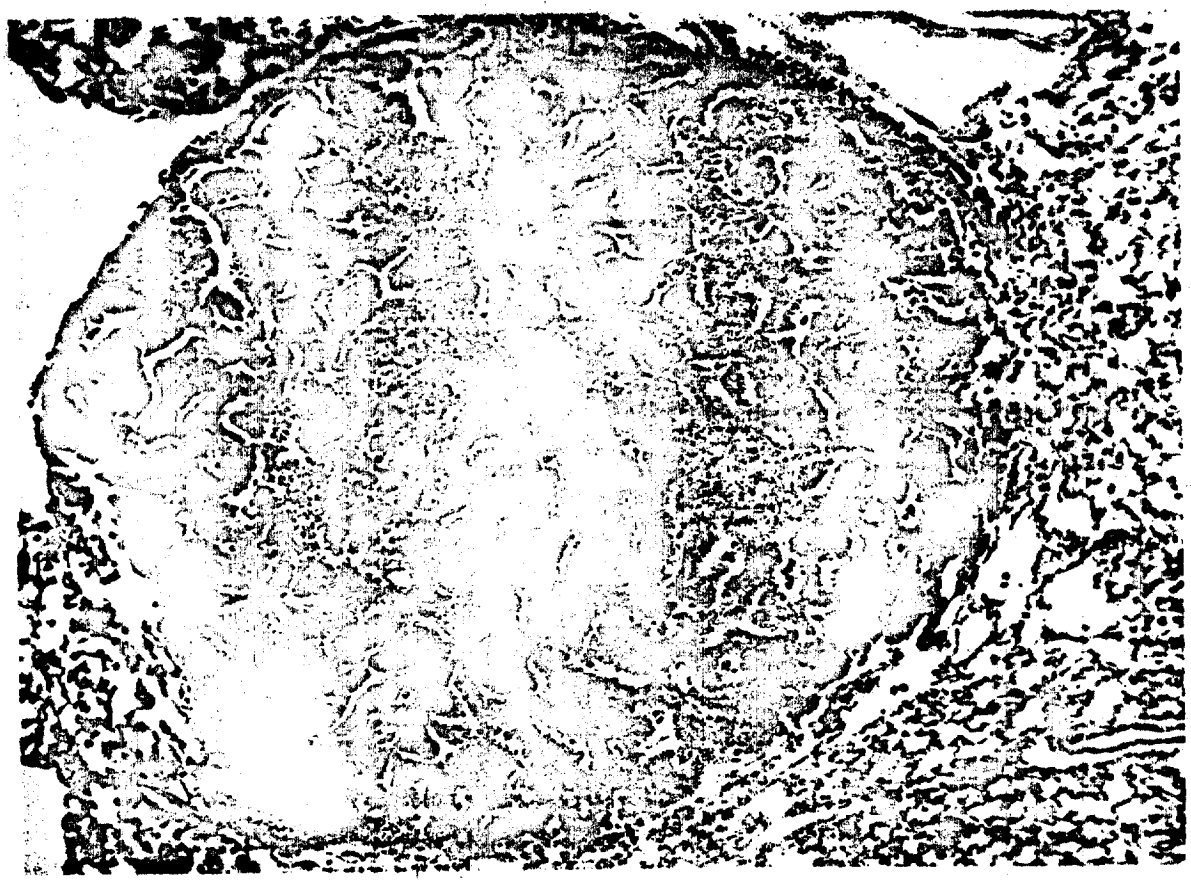
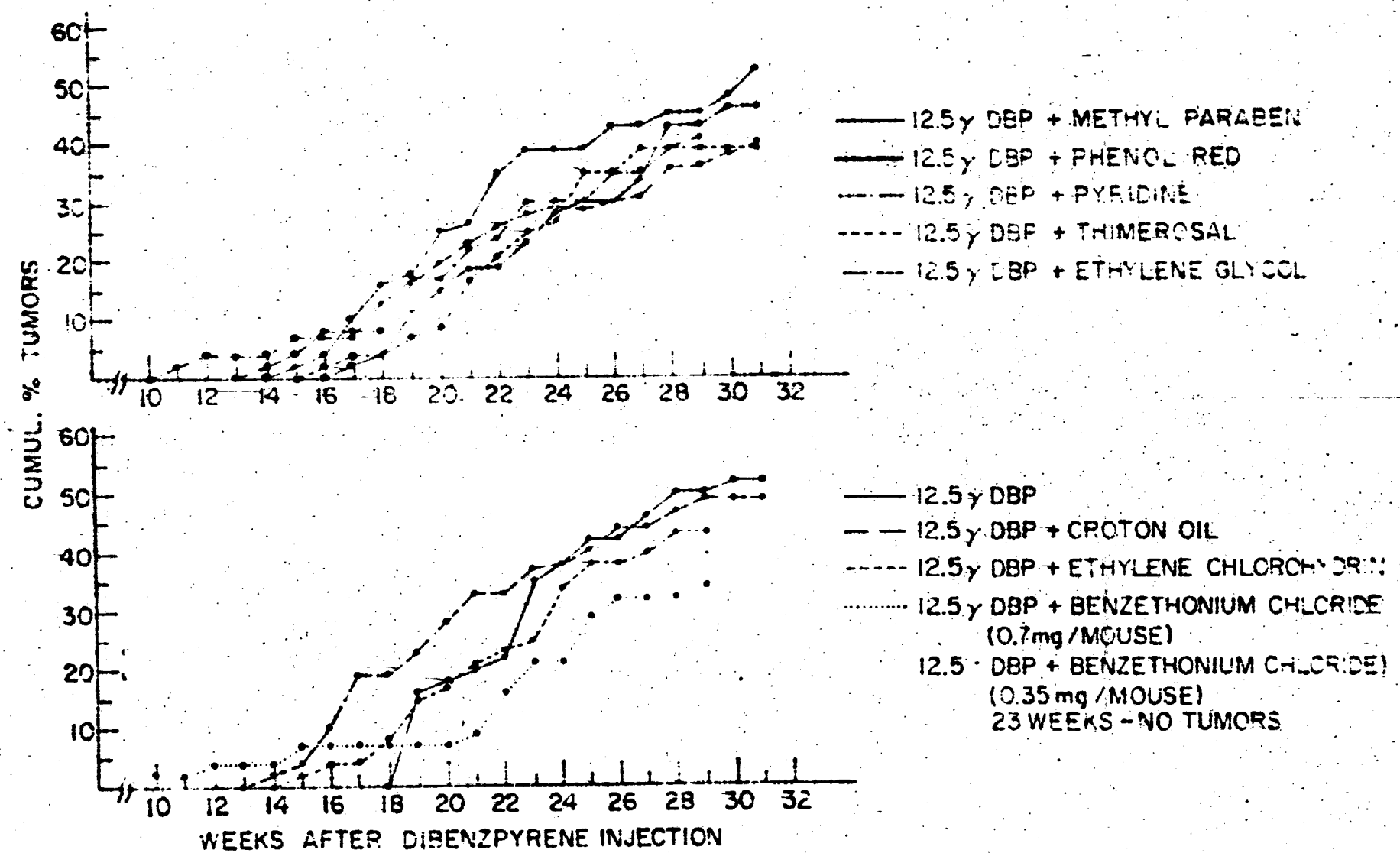


Figure 9

Figure 10

Figure 11



EFFECT OF THE SEVEN COMPOUNDS ON SUBCUT. TUMOR INDUCTION WITH 12.5γ DBP IN 0.1cc TRICAPRYLIN

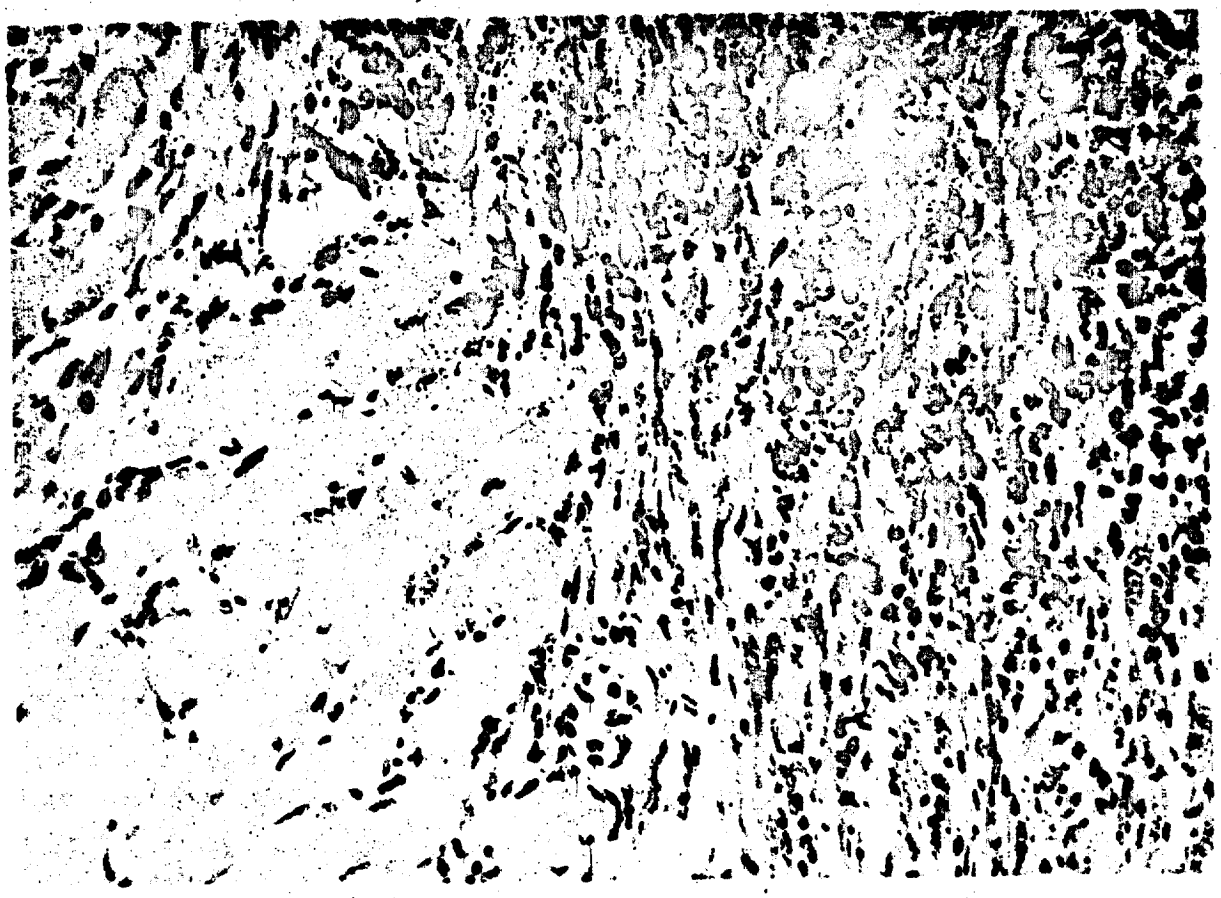


Figure 12

Separation, Identification, and Estimation of Aromatic Food Preservatives and Sorbic Acid by Paper Chromatography and Ultraviolet Spectrophotometry

By TORE HÖYEM (Department of Microbiology and Hygiene, The Veterinary College of Norway, Oslo, Norway)

Methods for the chromatographic separation and quantitative determination of some aromatic food preservatives have been published in *This Journal* (1, 2).

In this paper a method is described which permits the simultaneous separation, identification, and quantitative estimation of benzoic acid, the methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid, and sorbic acid. The separation is made by descending paper chromatography, and the positions of the compounds on the chromatogram are determined by taking contact prints in ultraviolet light. For further identification, and at the same time, quantitative estimation, the spots are eluted and the absorptions of the eluates are measured in a spectrophotometer.

METHOD

Apparatus and Reagents

(a) *Chromatographic tank.* — Rectangular tank, 19 × 29 × 60 cm. Place the solvent vessel 52 cm from the bottom of the tank.

(b) *Chromatographic solvent.*—Shake a mixture of *n*-butanol, ammonia, and water (5:2:3) in a separatory funnel. Place the bottom layer in a beaker in the bottom of the tank; use the top layer as the mobile solvent.

(c) *Filter paper.*—Whatman No. 1.

(d) *Ultraviolet lamp.* Chromatolite Short Wave Lamp No. 2015 (Shandon), fitted with a OX7 filter to provide radiation at 253.7 mμ.

(e) *Photographic paper.*—Kodak Duostat 23, 29.7 × 42 cm sheets.

(f) *Standards.*—0.1 g of each acid or ester (or their sodium salts) in 100 ml of distilled water, ethanol/water (1:1), or 95% ethanol.

Procedure

Place 5–50 μl of the standard solution to be chromatographed along the base of a starting line drawn 7.5 cm from the top edge of the paper. Let the paper equilibrate in the tank for 1 hour; then add the solvent and let the chromatogram develop for 18–20 hours at 20°C. (The solvent front will travel a distance of 40–45 cm within this time.)

Remove the paper from the tank, mark the solvent front, and air-dry the paper in the hood. When dry, take a photographic print of the paper in ultraviolet light as described by Markham and Smith (3). Pin the paper chromatogram over a sheet of photographic paper on a table and expose it to the ultraviolet lamp for a few seconds (the time required to obtain good prints depends upon the distance between the paper and the lamp). On the developed print, the positions of the compounds chromatographed appear as white areas on a dark background, and may easily be traced on the chromatogram itself. Determine the R_f values in the usual way.

For further identification and quantitative determination cut the spot areas out of the paper together with congruent paper blanks positioned in the same areas on the paper as the spots themselves, and place in test tubes. Add measured amounts of 0.01*N* HCl (pH 2), or 0.01*N* NaOH (pH 12), and leave the tubes for at least 2 hours with occasional shaking. Read the absorbances of the eluates against the paper blank eluates in the region 200–300 mμ in a Beckman Model DU spectrophotometer or equivalent, and plot the spectra. (This step may be omitted if the R_f values are conclusive.)

Make a quantitative determination by reading absorbances (*A*) at the wavelengths of maximum absorption and calculate the concentrations of the compounds from their absorptivities, adjusting for dilutions. The molar absorptivity (*a*) is defined as: $a = A/bc$, where *b* is in cm and *c* in g/L.

Results

The R_f values of the compounds chromatographed are given in Table 1. The substances give very satisfactory photographic prints within reasonable concentrations, as shown in Fig. 1. The optimum quantities to be put on the paper were found to be as follows: benzoic acid, 25–250 μg; *p*-hydroxy-

benzoic acid esters, 5–100 μg; and sorbic acid, 2–25 μg (Fig. 1B).

It is well known that the aromatic food preservatives and sorbic acid all exhibit characteristic spectra in the ultraviolet region, owing to the presence of conjugated double bonds in their molecules. The shape and position of the spectra depend upon the pH of the solutions. The spectral properties may serve as a tool to verify the chromatographic identification of the compounds. Figure 2 shows spectra obtained with some food preservatives. As the curves show, there are profound spectral differences between the compounds, making the identification rather easy.

Table 1. R_f values of some food preservatives chromatographed at 22° C on Whatman No. 1 paper; solvent, n-butanol-ammonia-water (5:2:3), top layer

Compound	R_f
Benzoic acid	0.43
p-Hydroxybenzoic acid methyl ester	0.71
p-Hydroxybenzoic acid ethyl ester	0.80
p-Hydroxybenzoic acid propyl ester	0.84
p-Hydroxybenzoic acid butyl ester	0.87
Sorbic acid	0.51

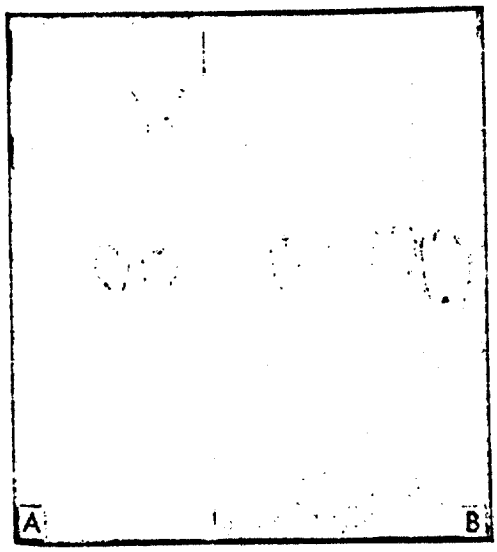


Fig. 1—(A) Ultraviolet contact print of food preservatives; left to right: p-hydroxybenzoic acid methyl ester, the butyl ester, sorbic acid, and a mixture of these three compounds. (B) Ultraviolet contact print of chromatogram with increasing amounts of sorbic acid; left to right: 1, 3, 5, 10, and 25 μg.

Table 2. Spectral characteristics of benzoic acid, its esters, and sorbic acid

Compound	pH 2					pH 12				
	λ max.	λ min.	220/260	250/260	280/260	ε max.	λ max.	λ min.	220/260	250/260	280/260
Sodium benzoate	230, 275	210, 261	11.2	1.84	1.25	11300	225, 269	210, 259	14.7	1.66	0.43
p-Hydroxybenzoic acid methyl ester (Na)	255	223	0.27	0.98	0.25	12800	219, 288	211, 212	0.72	0.43	2.06
p-Hydroxybenzoic acid ethyl ester (Na)	255	223	0.27	0.97	0.25	13650	222, 293	212, 212	0.76	0.41	2.42
p-Hydroxybenzoic acid propyl ester (Na)	257	223	0.21	0.85	0.27	13650	223, 291	213, 212	0.76	0.40	2.22
p-Hydroxybenzoic acid butyl ester	257	224	0.75	0.86	0.27	16800	222, 299	209, 211	2.64	0.21	1.85
Sorbic acid	255	212	0.92	0.98	0.23	26900	216	202	0.67	1.36	0.18

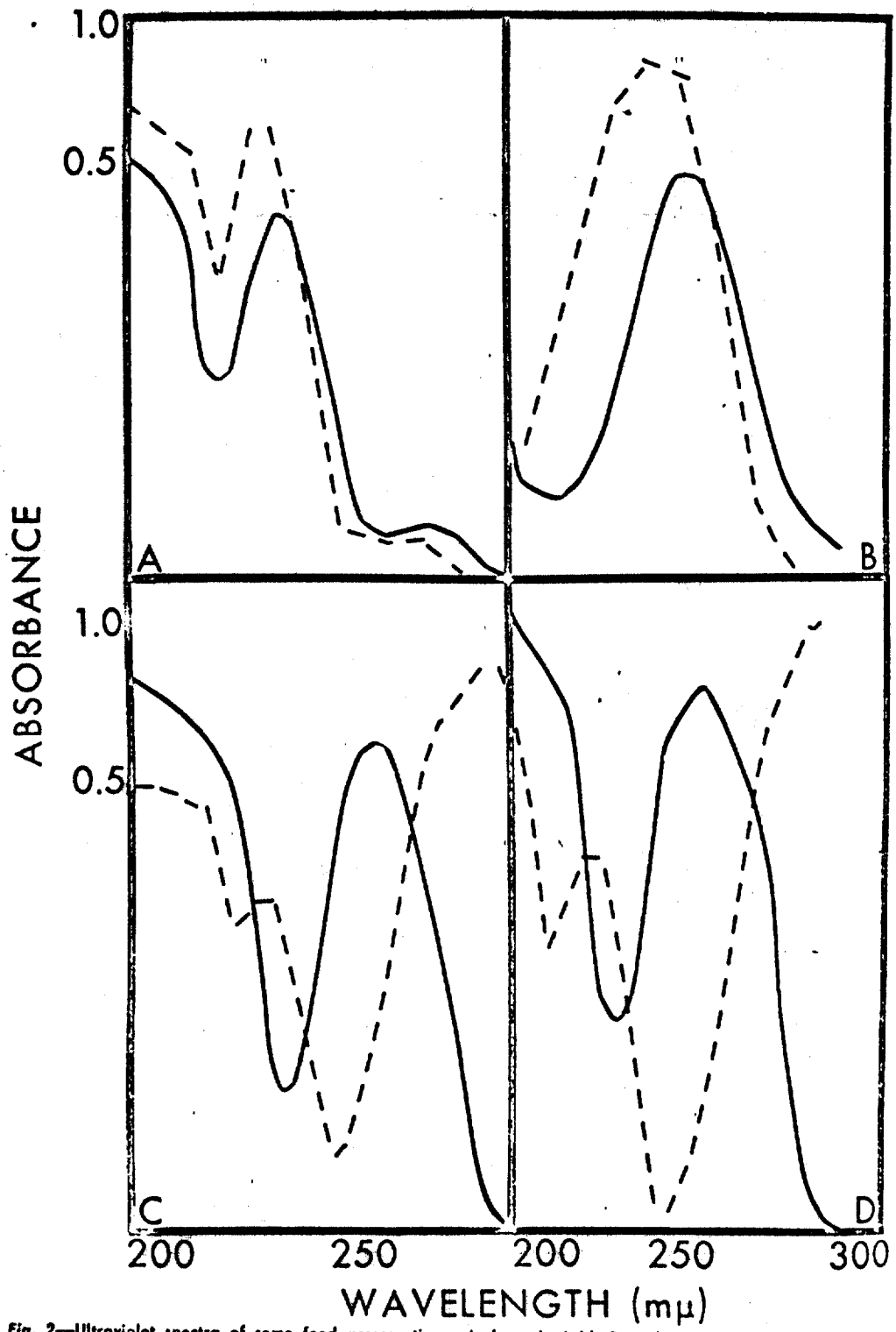


Fig. 2—Ultraviolet spectra of some food preservatives. A, benzoic Acid; B, sorbic acid; C, *p*-hydroxybenzoic acid methyl ester; D, *p*-hydroxybenzoic acid butyl ester. Spectra taken at pH 2 (————) and at pH 12 (- - - - - -) with a Beckman Model DB spectrophotometer.

Spectral characteristics are tentatively given in Table 2. Maxima and minima together with ratios A_{220} / A_{260} (220/260), A_{250} / A_{260} (250/260) and A_{280} / A_{260} (280/260) may give valuable information about the identity of the compounds. The absorpivities at maximum (a_{max}) are calculated for the compounds at pH 2.

Discussion

The chromatographic method described in this paper gives very satisfactory separation between some of the compounds. However, the separation of the four esters is rather poor, so that a mixture of the esters may not be separated sufficiently by this method. This difficulty may be partly overcome by using the smallest amounts of esters which are detectable by the method described here.

It is important not to exceed the optimal amounts of substances to be chromatographed. Too small amounts cannot be detected, while too large amounts overload the paper and cause severe tailing and false R_f values.

The detection of the spots by photographic contact printing is easy and rapid, but the exposure time is, of course, quite critical. Choosing the right exposure time is largely a matter of experience.

The spectra obtained are fairly specific, and are very good criteria of the identity of the compounds. However, they do not differentiate the esters; thus the only criterion of identity is the R_f value. The spectra of all the substances vary with the pH of the solution, and it is necessary to work at definite pH values. This is especially important in the quantitative determinations.

Although the method described here was originally developed to test the purity of commercial formulations of food preservatives, it should be readily applicable to the determination of UV-absorbing acids and esters in food. So far, the method has been found to work satisfactorily in the qualitative identification of the compounds in foods of different types. Its main advantage in this respect is that only small samples of food are required for analysis: 1 ml of liquid or 1 gram of solid food material is sufficient both for the qualitative and quantitative determination.

Further experiments, especially with regard to the quantitative determination, are being carried on in this laboratory, and detailed procedures for various types of food will be presented in a later publication.

References

(1) Mitchell, L. C., *This Journal*, **40**, 592 (1957).
(2) Van Dame, H. C., *ibid.*, **43**, 593 (1960).
(3) Markham, R., and Smith, J. D., *Nature*, **163**, 250 (1949).

J. Pharmac. Soc. Japan (Yakugaku-Zasshi) 83: 721-723, 1963.

Sadao Iguchi, Magobei Yamamoto, and Toshinobu Aoyama:

Studies on Medicinal Preparations by Gas Chromatography. I

Gas Chromatography of Preservatives

(Institute of Pharmaceutical Sciences, Faculty of Medicine, Kyushu University*1)

Separatory detection of preservatives by gas chromatography was examined. A mixture of sorbic acid, benzoic acid, salicylic acid, dehydroacetic acid, menadione, and methyl, ethyl, butyl, and propyl p-hydroxybenzoate was separated completely into the componental compounds without any pretreatment, under the conditions of 30% DC 550 silicone on Celite 545, at 190°, with carrier gas of hydrogen.. Salicylic acid tended to undergo thermal decomposition and phenol peak sometimes appeared in the product but no such decomposition occurred in other compounds.

(Received May 30, 1963)

When analysing medicinal preparations and preservatives we are frequently confronted with extreme obstacles. Separatory detection in analysing various combination of preservatives is difficult since the same analysis method used for a single compound cannot be employed. In order to challenge this problem, there has been an active research for a long time on separatory detection of mixed preservatives. For example, the report has already been published about the detailed examimation by means of Ultra Violet spectra[1], filter paper chromatography, and paper electrophoresis methods.[2] However, these methods are not necessaril satisfactory and more time-saving and yet accurate means are still sought after.

Gas chromatography (GC) which has ostensibly marked its progress recently is thought to be the answer. In recent years, a sequence of examination result of analysis by means of GC has been reported[3,4].. However, so far, none of the report shows a satisfactory separatory detection.

---

*1. Katakatsu, Fukuoka
1. Fujii, Harada: Shokuei-shi, Vol.3, p.172 (1962)
2.. Kusuda,. Takeuchi: Ibid., Vol. 2(4), p.72 (1961); Vol.3, p.374 (1962)
3. E.C. Hennings, Jr., et al.: Anal. Chem., Vol.30, p.1946 (1958)
4. Kawashiro, Ishii: Shokuei-shi, Vol.2(1), p.50; Vol.2(4), p.54 (1961)

Having evaluated the GC analysis conditions, the authors have discovered the ways to separate completely the mixture into the componental compounds without any pretreatment, by using DC 550 as solid solution.

The preservatives that were detected in this experiments are nine: sorbic acid, benzoic acid, salicylic acid, dehydroacetic acid, methyl p-hydroxybenzoate, ethyl p-hydroxybenzoate, menadione, propyl p-hydroxybenzoate, butyl p-hydroxybenzoate. Figure 1 illustrates the GC of these compounds. A sharp peak is noted for each of these thus indicating a complete separation.

Here the possibility of thermal decomposition was taken into consideration especially with SA, SOA, DHA and MNQ. First SA. SA sometimes decomposes under the GC conditions as indicated in Figure 1, and phenol peak appears in its formation (Figure 2). However, with SOA, DHA and MNQ, distillate of corresponding peak was directly collected and its IR spectra was measured. When GC infusion was compared with that of the original, perfect concurrence was noted as Figure 3 shows. Therefore, it was established that there was no decomposition by flashheater.

The above result of GC implies that it is possible to determine each preservative; hence it contributes much to the medicial preparation as well as to the analysis of preservatives in foodstuffs.

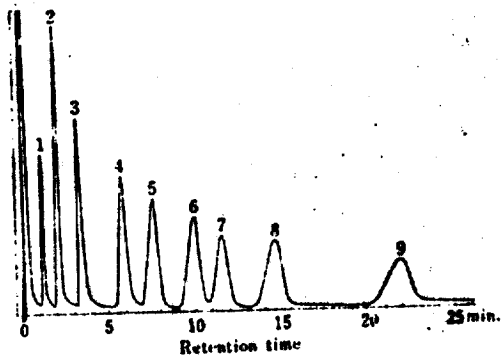
Experiment

GCG Model 2 Thermal Transmission detector was used for GC. For solid phase, infiltrated 30% of DC 550 silicone into Celite 545; particle size 80-100 meshed, 5mm X 1m of U-shaped stainless tube was filled. Aging 24° before use, 10 hours, carrier gas, H_2.

Figure 1 and 2 show the effective analysis conditions. Samples were diluted in 1-2% Me_2CO solution. It was poured by means of micro-syringe. Figure 1 shows the peak of 200-400γ.

In conclusion, our gratitude is due to Professor Kanzo Sasaki of Shionogi Laboratory for his assistance in IR spectra measurement.

Kyushyu University
School of Medicine
Pharmacology Division



1 sorbic acid
2 benzoic acid
3 salicylic acid
4 dehydroacetic acid
5 methyl *p*-hydroxybenzoate
6 ethyl *p*-hydroxybenzoate
7 menadione
8 propyl *p*-hydroxybenzoate
9 butyl *p*-hydroxybenzoate

Conditions: column, 30% DC 550 silicone on Celite 545 1 m. × 5 mm.; temp., 190°; carrier gas, H_2 50 ml./min.; detector, T/C cell.

Fig. 1. Gas Chromatogram of a Mixture of Nine Preservatives

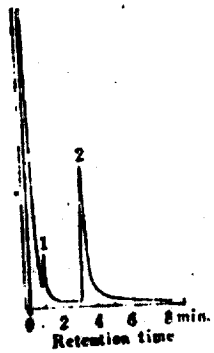


Fig. 2. Gas Chromatogram of Salicylic Acid

1 phenol (produced by thermal decomposition of 2)
2 salicylic acid

Conditions: same as Fig. 1.

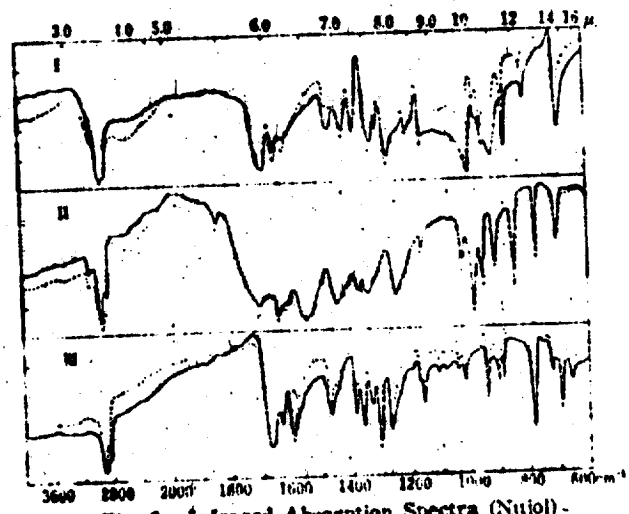


Fig. 3. Infrared Absorption Spectra (Nujol)

I sorbic acid
II dehydroacetic acid
III menadione
—— effluent
····· original

Hakko Kogaku Zasshi 44(2): 97-105, 1966.

STUDIES ON FOOD PRESERVATIVES

(II) Determination of Esters of p-Hydroxy Benzoic Acid

Shigeo Inamine, Atsushi Obatake and Toshio Matsuda

(Ueono Seiyaku, Co. Ltd., Koraibashi, Osaka)

A routine method of analysis used for the determination of esters of p-hydroxy benzoic acid in foodstuffs was studied. mainly using soy sauce.

Recently the 4 aminoantipyrine method (4-AA method) (Fig.1)[1] has been reported and employed as a routine analysis method, but it is too complicated to be employed for routine analysis. Routine analysis requires a quicker method. In this study, the previous 4-AA method was simplified and made more suitable for routine analysis. The procedure of the new method is shown in Figure 9.

Preface

Esters of p-Hydroxy Benzoic Acid is used in soy sauce, vinegar, mineral water as food preservatives. It is widely used especially with soy sauce. Among the determination methods of esters of PHB, i.e. burom method, Millon method, ultraviolet color absorption method, 4-AA method, the last one is the most popular.

PHB esters is insoluble in water, so to dissolve in foodstuffs requires a good technique. In case of soy sauce, it is known that the preservative may lose its effect because of decomposition by PHB esters hydrolysis. Therefore before manufacturing, it is necessary to determine whether it contains a high quantity of PHB esters. In order to determine PHB esters as a routine method, it would have to be expeditious and accurate. This study analyses 4-AA method in every angle in order to establish it as a routine method.

Experiment and Result

Apparatus: Shimazu self-registering spectrophotometer, SV-50A, Tōa cymoscope pH meter, HM Model 5A.

Reagents: Butyl p-Hydroxybenzoate (Ueno Seiyaku)
PHB (Ueno Seiyaku)
4-AA (Katayama Kagaku)
$K_3Fe(CN)_6$ (Katayama Kagaku)
For other chemicals, the best ones available in market were used.

Concerning 4-AA methods, we have reports from authors Matsuda and Shibaraki[1] (Figure 1), Takeuchi and Sugita[2], and Ohara[3] they have established 4-AA method to determine PHB esters. However, the method introduced by Shibasaki and Matsuda, Sugita and Ohara required the following procedures: after 4-AA has emitted colors in buffer solution, it was necessary to extract with chloroform, and then measure the absorption of color since the color was unstable. In Takeuchi and Yoshii method, it eliminate the chloroform extraction procedure and determined the absorption of color in the buffer solution, but it lacked stability. Therefore both methods are just as complex in that they require too much sample quantity.

In this study, in order to simplify the PHB esters determinatic method, we focussed our attention on the analysis of the buffer solution, the effect of thermal reaction, 4-aa, optimum concentratio of $K_3Fe(CN)_6$, and ionic strength of buffer.

1. Basic Examination of the DEtermination Method.

A. Examination of the buffer solution: In order to eliminate chloroform extraction, we examined the stability of 4-AA emission of color by changing the pH of the buffer solution. To obtain accurate results in this experiment, PHBA of best kind was used instead of PHBA that hydrolysised PHB esters. We poured 80 ml of buffer that contains PHBA 600γ into a 100 ml flask, added 4-AA 1% of solution 4ml, mixed well, and added $k_3Fe(CN)_6$ 4% solution 3ml and the mixture was made the volume up to 100ml. The reaction mixture was incubated at 25C (room temperature). We measured the color absorption of the solution at 505 mμ, as compared to the buffer and examined its sensitivity as well as stability. Also the same treatment was done to the buffer without PHBA, and examined the absorption of color. The results are shown in Figs. 2, 3, and 4.

As far as sensitivity is concerned, o.1M of K_2HPO_4-NaOH buffer solution is the highest, then o.1M of H_3BO_3-NaOh, o.o5M of $NaHCO_3$-$NaCO_3$ follow. Other buffer solutions show poor stability, thus are inappropriate as fixed buffer solution.

On the other hand, as far as the absorption of color of blank is concerned, as illustrated in Figure 4, 0.1M of K_2HPO_4-NaOH buffer solution is extremely high, thus inappropriatee as fixed buffer solution, but o.1M of H_3BO_3-NaOH is low in color absorption of blank. It also has no change in absorption of color in a wide range of pH and is considered to be excellent buffer solution. The authors then decided on H_3BO_3-NaOH as a fixed buffer solution. However, since the color absorption of blank tends to be higher when pH becomes acidic, it is difficult to extract PHBA from ester layers. Thus they settled on pH 10 buffer solution. Figure 5 shows 4-AA effect of time on absorption spectra of color produced by H_3BO_3-NaOH. Change of color is noticeable even to the naked eyes

and the absorption spectra of color also changes, but change of peak in absorption of color is not recognized. Therefore there is no effect on the determination.

B. Effect of temperature on the formation of 4-AAdye. According to Shibasaki and Matsuda's[1] report, in order to emit colors by 4-AA, keep the temperature as low as possible in order to stabilize the color. The operation to stabilize the temperature is extra trouble and not very advisable for expeditious and routine method.

Table 1 shows the effect of temperature on absorption of color of 4-AA in 26C in water (same as room temperature) and 5C in water (cooled with ice). As expected, as a result of thermol reaction it showed higher absorption in 5 C, but only with a difference of about 4%, and there was no significant influence by temperature. Therefore, should the temperature of the experiment take place in the room, we can ignore the effect of temperature on determination value.

C. Effect of 4-AA incubation time on color absorption. If $K_3Fe(CN)_6$ is added without stirring at the same time as after adding $K_3Fe(CN)_6$ to 4-AA it shows a significant effect on the absorption of color; The degree of absorption of color was lowered. That is because 4-AA does not react on PHBA, but rather directly on $K_3Fe(CN)$. In order to consider the latent time on $K_3Fe(CN)_6$, after adding 4-AA and thorough stirring, the absorption of color was studied; o, 5, 10, and 30 minutes of incubation in room temperature was observed. As shown in Table 2, there was no effect by the incubation time. From the above observation, after adding 4-AA, if stirred thoroughly, it does not matter when you add $K_3Fe(CN)_6$.

D. Effect of chemical concentration on color. In order to determine the optimum concentration of color emission of reagents, we varied the quantity of 4-AA and $K_3Fe(CN)_6$ and studied the effect of absorption of color. Figure 6 indicates that $K_3Fe(CN)_6$ was stabilized at 0.12% concentration, while 4-AA additives were varied, and studied the absorption of color. As more was added to 4-AA the absorption of color became higher, reached its highest at 0.04% and became stable after that. In case of blank without PHBA, at 0.04%, absorption of color was recognized very slightly. But in actual determination, it is insignificant.'

Figure 7: Fixed the additive volume for 4-AA at 0.04% and varied the volume for $K_3Fe(CN)_6$. The color absorption increased as $K_3Fe(CN)_6$ was increased.and reached its highest at 0.12%. But when it goes down beyond that, the absorption of color decreased. Consequently, 4-AA o.04%, $K_3Fe(CN)_6$ 0.12% seems to be the optimum chemical concentration. Thus the experiment was carried under the above conditions. Of course, according to the quantity of PHBA the optimum concentration of 4-AA and $K_3Fe(CN)_6$ vary. When it contains a large quantity of PHBA, the optimum concentration of reagent is expected to increase. In actual determination, when the absorption of color is read beyond 0.8,the regular spectrophotometer fails to record accurately. When the absorption of color is higher,

in order to obtain an accurate determination, it is necessary to abbreviate the degree of the absorption of color from about 0.3 to 0.7. In case of actual foodstffs there is no chance of additive being limited by permissible quantity as well as solubility of PHB esters becoming incapable of determination on account of higher absorption of color.

E. Effect of ion strength in buffer solution. It is known that the 4-AA emission of color is influenced by ion strength of the buffer.[4] Figure 8 shows the effect of pH and ionic strength on absorption of color produced by PHBA. As the ionic strength increased in the buffer the absorption of color was lowered where pH is high. When ionic strength is 0.1M, relatively stable absorption of color is observed within the range of pH, 9.0-10.0. The result was favorable. When the ionic strength is lower than 0.1M, not enough buffer potential is obtained, so ionic strengh 0.1M was decided on.

2. Study of Determination Procedures.

In order to come up with an effective, time-saving, simplified method, they set up the method as shown in Figure 9. In this case, what differs from that of Shibasaki and Matsuda are: 1) Ester extraction was eliminated by adding the equal amount of saturated $NaHCO_3$ solution to the reagent; 2) Esters was extracted only once; 3) eliminated the washing with $NaHCO_3$ 1% solution; 4) used 0.1M of H_3BO_3-NaOH as a buffer solution; and 5) eliminated extraction of chloroform of 4-AA.

To add saturated $NaHCO_3$-Na_2CO_3 to reagent means to acifidy the reagent, and to prevent the migrating of phenolic organic acid to ester layer. Therefore, after the extraction of esters, the washing with 1% $NaHCO_3$ for the purpose of excluding phenolic organic acid. When the sample itself is acidic (e.g. vinegar), it is necessary to alkalize the $NaHCO_3Na_2CO_3$ by adding more than the equal amount.

Figure 10 shows the recovery of added BPHB from soy sauce. By the first extraction the majority of BPHB has shifted to esters layer and by the second extraction, a peak as a result of 4-AA emission of color was not recognized.

From the above example, when extracting PHB esters from the sample, only one extraction is all that is required and the second extraction is needless. In the case of chloroform extraction, as mentioned above, when H_3BO_3-NaOH is used as buffer solution, the color of 4-AA is stable therefore chloroform extraction is not necessary. In order to emit color from 4-AA, by PHB esters, it is necessary to hyrolysise to get PHBA. Hydrolysis can be done in salfuric acid,[5] but it is faster and most effective to hydrolysise in alkaline NaOH.

The authors studied the time required to hydolysise 1N NaOH at a temperature of 100 C. A cooling tube was used to heat 100 C in water. As shown in Fig. 11, not enough hyrolysis occurs after five minutes of heating, but after 10 minutes absorption of color

stabilizes. Thus ten minutes is concluded to be necessary for hydrolysis.

Table 3 shows the recovery of PHBA from ethyl ester with buffer. Because buffer is alkaline, extraction is easily done. At first extraction is easily done. At first extraction, almost all of PHBA was recovered with buffer. AT second extraction, a little absorption of color was recognized. At third extraction none was recognized. From this experiment, two extractions procedur was adopted.

Normally, the determination of PHB esters by 4-AA method was proceded by hydrolysing the established quantity of PHB esters, emit color from 4-aa, measure the absorption of color, draw a curve and calculate the content. However, in this study, the operation to hydrolysise PHB esters to form standard solution was eliminated. The authors attempted to form the standard solution by disolving the first class PHBA known quantity into 1.1M H_3BO_3-NaOH. To determine the PHB esters as a routine method to produce buffer solution each time is too much trouble. To produce a preservable standard solution, it is necessary to eliminate unnecessary procedures. In order to determine the preservability and stability of the standard solution, we poured the PHBA 100 ml buffer solution and water into erylenmyer flask with top and was incubated at 20°C and then at 37°C. Then 5 ml of PHBA solution was added to buffer solution and reagent, and after the emission of color, made the volume up to 100 mls. Then absorption of color was measured and any change was recorded while being preserved. Figure 12 shows its result.

In buffer solution, absorption of color is comparatively stable, and therefore stable as standard solution. The changing of pH is as follows: after one month of incubation at 20°C, 9.7; at 37°C it lowered to 9.5. In case of water, as compared to buffer solution, it was very stable, and has the tendency to lower the absorption of color. Thus unsuitable as the standard solution.

Figure 13 shows the comparison between the standard curve resulting from hydrolysis of BPHB and standard curve of PHBA. If calculated in molecular weight, the curves of BPHB and PHBA coincide.

From the above result, we can see that o.1M of H_3BO_3-NaOH buffer solution of PHBA is satisfactory as standard solution. The determination hence is performed by using this standard solution. By the following formula, the content of PHB esters was calculated.

CONTENT OF PHB ESTERS (γ/ml)=

$$\frac{\text{(absorption of color of sample)} \cdot \text{(PHBA (γ) in standard solution)}}{\text{(color absorption of standard solution)} \cdot \text{(PHBA molecular weight)}}$$

or

$$\frac{\text{PHB esters molecular weight}}{\text{Extraction volume of the sample(ml)}} \times \text{recovery rate}$$

Table 4 shows the recovery of added BPHB from water. Tables 5 and 6 show recovery of added BPHB from soy sauce and vinegar.

The recovery rate from water is almost 100% but in the case of soy sauce, the recovery rate goes down to 95%, vinegar, 97%, only slight higher than that of soy sauce. Allowing the determination error of 2%, the figure is quite accurate. To determine preservatives directly from foodstuffs, it is difficult to obtain food that does not contain PHB esters. Therefore we have to take into consideration the effect of the food itself on 4-AA absorption of color of PHB esters.

In this study, when the absorption of color was measured in such foodstuffs as those not containing PHB esters, the following was observed; in vinegar, it was hardly below 0.01 and was noticeable; in soy sauce, depending on the kind and manufacturer, the absorption of color of approximately 0.01-0.02 was recognized. The foodstuffs that contains a large quantity of PHB esters, the absorption of color does not have too much effect on determination value, but it has a more effect on foodstuffs with less PHB esters. Therefore, it is necessary to rectify the calculation by subtracting the color absorption of contrasting food from that of the sample food. The color emission of foodstuff itself by 4-AA is considerably low; in vinegar and mineral water hardly any absorption of color is recognizable. In soy sauce, fruit juice, approximately 0.01-0.02 of absorption of color was recognized. But in determination it is sufficient to establish absorption of color of the sample. In this report, in case of soy sauce, we used 0.2 as contrasing color absorption and disregarded it in the case of vinegar.

In this report, the experiment was conducted only on vinegar and soy sauce in recovery of PHB esters from foodstuffs. However, the same thing can be done with the foofstuffs in which PHB esters is permitted as preservatives, such as, fruit juice and mineral water.

Summary

The determination of PHB esters by 4-AA method has already been reported and established. But the method is rather complex and not a very desirable one for routine analysis. The authors reevaluated the method from all angles, improved it and modified it to be suitable for routine procedure, and came up with the steps as shown in Figure 9.

This determination method is much simpler as compared to the ones introduced in the past, and has the advantage of being able to determine the large quantity of sample at the same time.

In conclusion, we express our thanks to Professors Terui and Shibasaki of the Department of Engineering, Osaka University for their guidance. Also we are indebted to Mr. Ueno, Vice President

of Ueno Seiyaku, Ltd. for allowing us to publish this report. The portion of this report was presented at the National Hakkō Engineering Conference in 1963.

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(Received October 2, 1965)

5ml sample

|

Extract with ca. 100ml ethyl ether or ethyl acetate in 2-3 steps

|

Wash with 1% $NaHCO_3$ in 3-4 steps

|

Wash with water

|

Evaporate in vacuo to dryness

|

Residual matter

|

Dissolve with 20ml 1N-NaOH

|

Hydrolyse at 100°C for 15min

|

Make acidic with 3N-H_2SO_4

|

Extract with ca. 70ml ethyl acetate

|

Wash with water

|

Extract with ca. 40ml N/5 $NaHCO_3$-Na_2CO_3 (1:1) buffer in 2 steps

|

Cool below 3°C

|

Form 4-AAdye by adding 2ml of 3% 4-AA aq. soln. and 10ml of 2% $K_3Fe(CN)_6$ aq. soln.

|

Extract with ca. 15ml chloroform in 2 steps

|

Chloroform soln. (40ml) of 4-AAdye

|

Measure optical density at 460mμ with electric photometer

Fig. 1. Determination of esters of p-hydroxybenzoic acid by 4-AA method (Shikasaki, Matsada)[1].

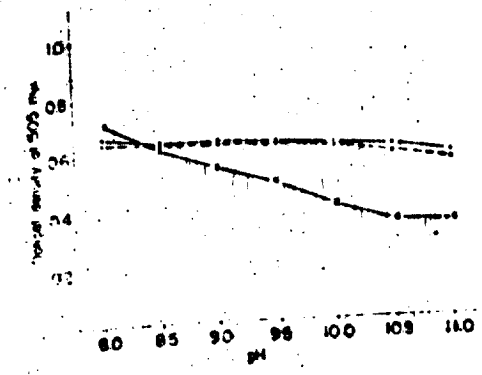

Fig. 2. Effect of buffer, pH and time on absorbance of the color produced by p-hydroxy benzoic acid (PHBA).

— · — 0.1M H_3BO_3-NaOH incubated for 5min
.... 0.1M H_3BO_3-NaOH incubated for 2.5hr
— · — 0.1M NH_4Cl-NH_4OH incubated for 5 min (incubation temp. 25°C)

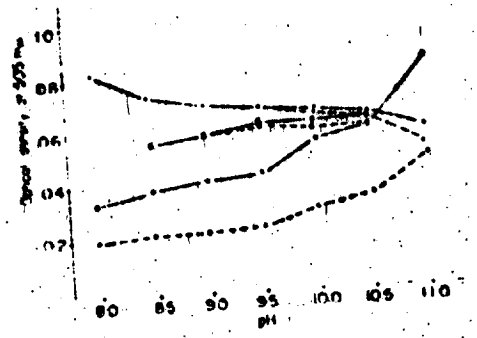

Fig. 3. Effect of buffer, pH and time on absorbance of the color produced by PHBA.

— · — 0.1M K_2HPO_4-NaOH incubated for 5min
.... 0.1M K_2HPO_4-NaOH incubated for 1hr
— · — 0.05M $NaHCO_3$-Na_2CO_3 incubated for 5min
.... 0.05M $NaHCO_3$-Na_2CO_3 incubated for 1hr
— · — 0.1M HCl-NaOH incubated for 5min
.... 0.1M HCl-NaOH incubated for 15min

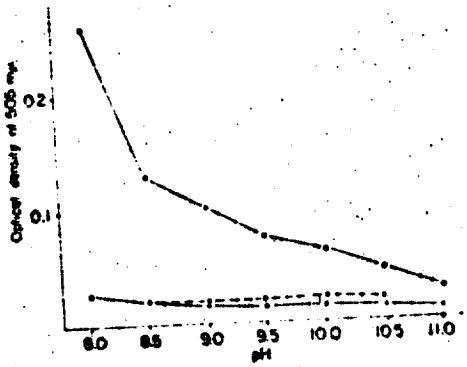

Fig. 4. Effect of buffer and pH on absorbance of blank vs. buffer.

— · — 0.1M K_2HPO_4-NaOH
.... 0.1M Na_2CO_3-$NaHCO_3$
— · — 0.1M H_3BO_3-NaOH

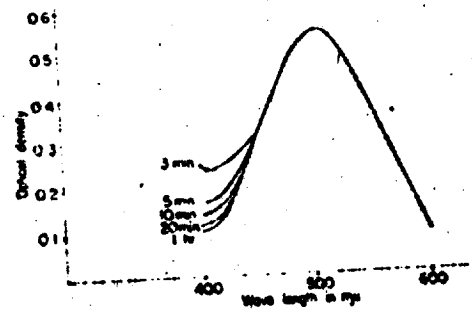

Fig. 5. Effect of time on absorption spectra of color produced by PHBA.

To 80ml of 0.1M H_3BO_3-NaOH (pH 10) buffer containing 600γ PHBA, 4ml of 1% 4-AA aq. soln. and 3ml of 4% $K_3Fe(CN)_6$ were added and the mixture was made the volume up to 100ml with same buffer after formation of 4-AAdye, the reaction mixture was incubated at 25°C (room temp.).

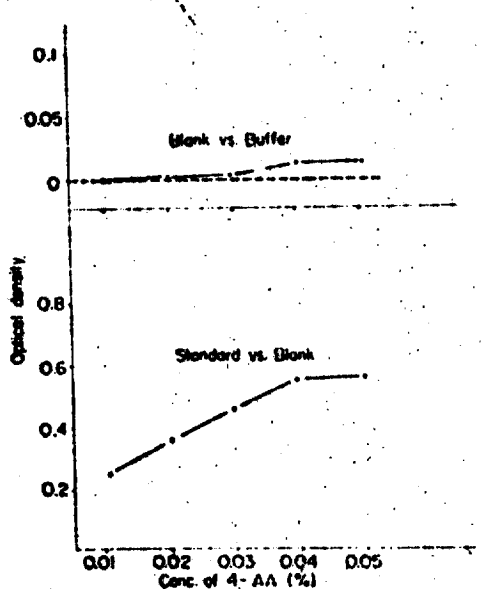

Fig. 6. Effect of the amount of 4-AA on the color produced by PHBA.

PHBA : 600γ
Buffer : 0.1M H_3BO_3-NaOH (pH 10)
$K_3Fe(CN)_6$: 0.12%

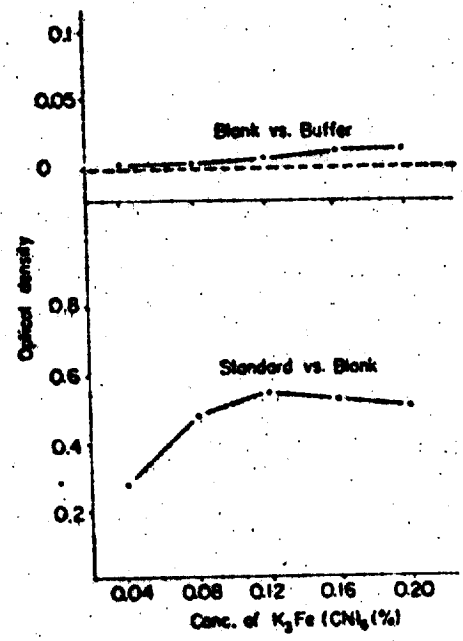

Fig. 7. Effect of the amount of $K_3Fe(CN)_6$ on the color produced by PHBA.

PHBA : 600γ
Buffer : 0.1M H_3BO_3-NaOH (pH 10)
4-AA : 0.04%

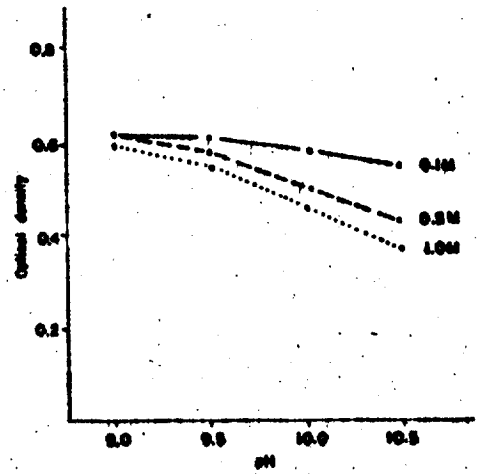

Fig. 8. Effect of pH and ionic strength on absorbance of the color produced by PHBA.

PHBA : 600γ/ml
Buffer : H_3BO_3-NaOH (pH 10)

10ml sample

↓

Add 10ml saturated $NaHCO_3$ soln.

↓

Extract with 50-70ml ethyl ether

↓

Wash with water (10ml, 2 or 3 times)

↓

Evaporate in vacuo to dryness

↓

Dissolve residual matter with 20ml 1N-NaOH

↓

Hydrolyse at 100°C for 15min

↓

Make acidic with 3N-H_2SO_4

↓

Extract with 50-70ml ethyl ether

↓

Wash with water (5ml)

↓

Extract with N/10-H_3BO_3-NaOH (pH 10) buffer (40ml, 10ml 2 times)

↓

Collect buffer with 100ml volumetric flask

↓

Add 4ml 1% 4-AA aq. soln. and shake for mixing

↓

Add 3ml 4% $K_3Fe(CN)_6$ aq. soln. to form 4-AAdye

↓

Make the volume up to 100ml with buffer soln.

↓

Measure optical density at 505mμ by electric photometer

Fig. 9. Determination of esters of p-hydroxybenzoic acid by new 4-AA method.

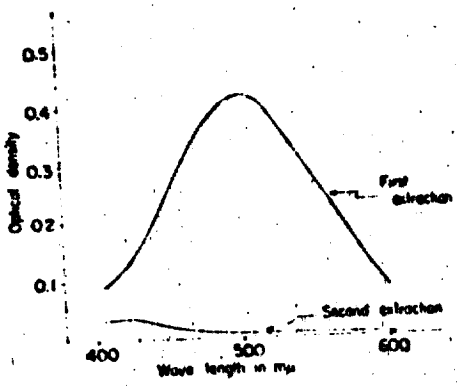

Fig. 10. Recovery of added butyl-p-hydroxy benzoate (BPHB) from soy sauce.

10ml of soy sauce containing 61γ/ml of BPHB was added 10ml saturated $NaHCO_3$ aq. soln. and extracted with 50ml ethyl ether (First extraction). The under layer (soy sauce and saturated $NaHCO_3$ soln.) of the mixture was separated and extracted with 50ml of ethyl ether (Second extraction).

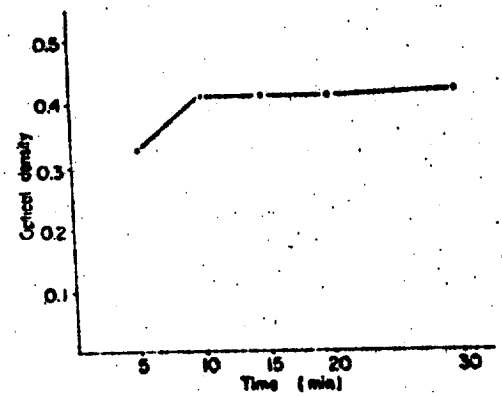

Fig. 11. Time course of the hydrolysis of BPHB.

The reaction mixture of total volume of 20ml containing 600γ BPHB was incubated at 100 °C. After hydrolysis, the reaction mixture was processed by the method shown in Fig. 9.

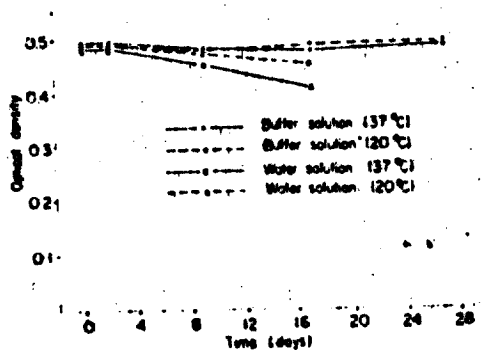

Fig. 12. Change of absorbance at 505mμ by incubation time.

The water and the buffer solution containing 100γ/ml of PHBA was incubated at 20°C and 37°C. After these incubations, 5ml of the solutions were sampled and processed by the method shown in Fig. 5.

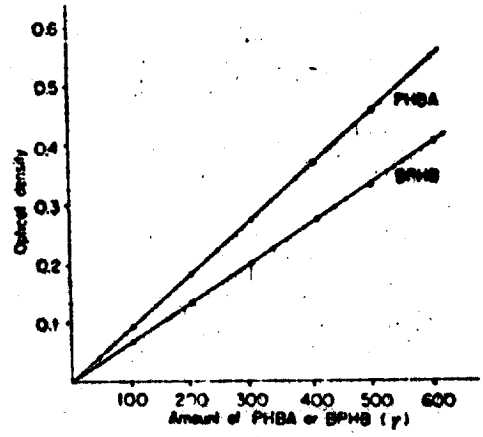

Fig. 13. Optical density at 505mμ and amount of PHBA, and BPHB.

Table 1. Effect of temperature on the formation of 4-AAdye.

The reaction mixture (100ml) contains 0.1M H_3BO_3-NaOH (pH 10) buffer 500γ PHBA, 4ml of 1% 4-AA solution and 3ml of 4% $K_3Fe(CN)_6$.

No.	5℃	26℃
	Optical density at 505mμ	
1	0.503	0.488
2	0.503	0.485
3	0.507	0.486

Table 2. Effect of incubated time after adding 4-AA on formation of 4-AAdye.

To 80ml of 0.1M H_3BO_3-NaOH (pH 10) containing 500γ PHBA 4ml of 1% 4-AA aq. soln was added. After incubation for 0, 5, 10, 30min at 26℃ (room temp.), the mixture was added 3ml of 4% $K_3Fe(CN)_6$ in order to form 4-AAdye and then made the volume up to 100ml with the buffer.

Time (min)	0	5	10	30
Optical density at 505mμ	0.485	0.487	0.490	0.485

Table 3. Recovery of PHBA from ethyl ether with buffer.

70ml of ethyl ether containing 600γ PHBA was extracted with 30ml 0.1M H_3BO_3-NaOH (pH 10) buffer in 3 times.

	1st extraction	2nd extraction	3rd extraction
Optical density	0.510	0.020	0

Table 4. Recovery of added BPHB from water.

BPHB added: 61γ/ml

Extract with ethyl ether in 1 step (70ml)		Extract with ethyl ether in 2 steps (50ml, 50ml)	
BPHB determined	Recovery %	BPHB determined	Recovery %
61.0 γ/ml	100.0	62.9 γ/ml	96.5
61.7	101.0	64.5	100.7
63.3	98.9	64.3	100.3
61.0	100.0	61.3	100.3
61.0	100.0	64.7	101.0
61.0	100.0	64.5	100.7

Table 5. Recovery of added BPHB from soy sauce.

The sample were extracted with 70ml ethyl ether in 1 step.

BPHB added: 61γ/ml

Common soy sauce		Usukuchi soy sauce	
BPHB determined	Recovery %	BPHB determined	Recovery %
61.5 γ/ml	96.2	62.2 γ/ml	97.2
60.8	95.2	61.3	95.2
61.5	96.2	61.7	96.5
61.3	95.9	61.2	95.6
59.7	93.5	61.7	96.5
60.8	95.2	61.7	96.5

J. Am. Pharm. Assoc. Sci. Ed. 45(4) 263-273, 1956.

p-Hydroxybenzoic Acid Esters as Preservatives III.

The Physiological Disposition of *p*-Hydroxybenzoic Acid and Its Esters*

By PASSIE S. JONES, DORIS THIGPEN, JAMES L. MORRISON, and ARTHUR P. RICHARDSON

Free *p*-hydroxybenzoic acid and its metabolic products in which the phenolic group has not been destroyed have been determined in tissues and biological fluids of drugs, tablets, and man following the ingestion of the acid and its esters. Recoveries from the urine of animals, regardless of the method of administration, range between 50 and 95 per cent except in the case of butyl esters where the recoveries were approximately 40 per cent. High plasma levels and urinary output of free and conjugated *p*-hydroxybenzoic acid in dogs indicate that hydrolysis of the ester linkage and metabolic conjugation constitute the chief paths of alteration for all of the esters studied except the butyl derivative. Topical application of ointments containing 10 per cent methyl and 10 per cent propyl *p*-hydroxybenzoate to the shaved backs of white rabbits produced no irritation during forty-eight hours of contact. Under this condition no drug concentration was detected in the kidneys.

THE INCREASING interest in and use of esters of *p*-hydroxybenzoic acid as food, cosmetic, and drug preservatives has created the need for detailed information as to the absorption, distribution, and disposition of this series of compounds. This paper describes certain chemical methods which have been found useful for such studies and results obtained in dogs, rabbits, and man.

A quantitative method of determination of these substances in tissues and biological fluids was devised by utilizing the ability of phenols to couple with 4-aminoantipyrine (1). Under alkaline oxidizing conditions, the reagent combines with the phenolic substances *para* to the hydroxy group to form a highly colored compound. In order for this reaction to take place the phenolic group must be free and the position *para* to it unsubstituted. However, the presence of a free carboxylic acid group in the *para* position does not prohibit coupling, since under the conditions used this group is removed by decarboxylation.

It was felt that this method was particularly suited to the analysis of tissues and biological fluids for *p*-hydroxybenzoic acid (PHBA) and its methyl, ethyl, propyl, and butyl esters, for if, during the course of metabolism of the esters, they should appear in the tissue or fluid simultaneously with the free acid, they would be distinguishable by the restrictive conditions necessary for coupling with aminoantipyrine. Hydroxybenzoates, with ester groups *para* to the hydroxy group, would necessitate hydrolysis before coupling could take place, and thus PHBA could be determined in their presence without interference. The esters, on the other hand, could be analyzed for in the presence of PHBA by making use of differential extraction since before hydrolysis their acidity, and consequently their solubility in sodium bicarbonate, differs markedly from PHBA.

From past studies with similar compounds, it was felt that further detoxication of the esters would be possible in the body (2). The phenolic group of the hydroxybenzoates could link with sulfate and glucuronic acid, and if the ester were first degraded to PHBA, the acid group would be available for glucuronic acid and glycine conjugation. Any of these conjugation products would require hydrolysis before color development could take place, and they could be in turn distinguished from the esters due to the bicarbonate solubility of the conjugates.

Simple hydrolysis of the tissue or fluid sample under study would reduce the esters and their metabolic products to PHBA, and in such a way the total material present could also be determined.

EXPERIMENTAL

Procedure. The procedures as finally adopted for use are as follows: A buffer of *p*H 10-11 was prepared for the medium in which the coupling reaction takes place in order to insure maximum stable color development. It was prepared as follows: 430 cc. of 0.1 *N* NaOH + 500 cc. of 0.1 *M* H_3BO_3 diluted to 100 cc.

Determination of PHBA in Water, Urine, and Plasma. One cc. of plasma or 1 cc. of urine diluted 1:10, 1 cc. of 6 *N* H_2SO_4, and 7 cc. of absolute ether were shaken mechanically for six minutes in a glass-stoppered centrifuge tube. The mixture was then

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centrifuged for one minute at 1500 r. p. m., and a 4-cc. aliquot of the supernatant ether solution was transferred to a test tube and the ether removed by evaporation from a warm water bath. Four cubic centimeters of the buffer were added to the tube and the solution heated for five minutes at 90-100° to insure complete solution of the residue. Upon cooling to room temperature, 1 cc. of amino-antipyrine reagent (1.36 Gm./liter) and 0.5 cc. of potassium ferricyanide solution (8.67 Gm. of $K_3Fe(CN)_6$) and 1.8 cc. of concentrated NH_4OH/L.) were added to the tube. The resulting colored solution was shaken by hand for a few seconds and the transmittance was determined at 490 mμ on the Beckman spectrophotometer. Color develops immediately upon shaking and remains stable for at least an hour.

Determination of *p*-Hydroxybenzoates in Water, Urine and Plasma. One cubic centimeter of plasma or urine, 1 cc. of saturated $NaHCO_3$, and 7 cc. of absolute ether were mechanically shaken for six minutes in a glass stoppered centrifuge tube. The mixture was then centrifuged for one minute at 1500 r. p. m., and a 5-cc. aliquot of the ether phase was evaporated in a glass-stoppered centrifuge tube from a warm water bath. One cubic centimeter of water and 1 cc. of 6 N H_2SO_4 were added to the residue and the tube heated for two hours in a boiling water bath. It had been previously determined that further hydrolysis did not take place after 2 hours. Upon cooling, 7 cc. of absolute ether was added to the tube, and the extraction was carried out by mechanical shaking for six minutes. The remainder of the procedure was identical with the PHBA determination. Calibration curves were run for all esters using this procedure.

Determination of Total Material (PHBA + *p*-Hydroxybenzoates + Metabolic Conjugates) in Water, Plasma, and Urine. This procedure is identical with the PHBA determination except that before the addition of the ether, the acidic phase was heated for two hours in a boiling water bath.

These methods are sensitive to 0.3-0.5 γ/cc. except in those urine determinations where dilution of 1:10 is necessary to remove interfering blank color. In those cases the sensitivity is 3-5 γ/cc. Complete standard curves were run with water, plasma and urine standards carried through all the steps of the procedure, including extraction; all these curves were identical. No standard curves were available for the "Total" material, but values were read from the PHBA curve. By correcting for the aliquots, 75-85% of the added material may be accounted for in the procedures.

Determination of PHBA, *p*-Hydroxybenzoates and "Total" Material in Tissues. Satisfactory [illegible] for these compounds have been made in [illegible] spleen, muscle, kidney, brain, and pancreas. The organ under study was cut up and added to an equal quantity by weight of saline. After preliminary mixing in a Waring Blendor, the resulting mixture was ground in a tissue grinder until it might be drawn through a syringe. One cubic centimeter of the mixture was used for analysis. The procedures for the determination of PHBA, *p*-hydroxybenzoates, and "Total" material in tissues are identical with those in plasma except that the ether aliquot was shaken mechanically in a glass stoppered centrifuge tube for six minutes with 4 cc. of the buffer. The mixture was then centrifuged for 1 minute at 1500 r. p. m. and the supernatant ether phase removed by suction. Color was then developed in the buffer solution. This double extraction was found necessary to remove blank interference.

Standard curves were run in all the tissues with a sensitivity of approximately 1γ/cc. Recoveries were of the same range as those from water, urine, and plasma.

Glucuronic acid determinations were made whenever possible to reveal the extent of conjugation with this compound. The method of Maughan, Browne, and Evelyn (3) was found satisfactory for our use. Specific analysis for the other conjugates was omitted for simplicity; however, the quantity of these metabolites may be determined by subtraction of the Parasept®, free PHBA, and glucuronic acid conjugate fractions from the "Total" material.

ABSORPTION AND EXCRETION

In order to understand more fully how the body handles these drugs, a comparison was made of oral and intravenous administration of the esters and PHBA. Intravenous injections of 50 mg./Kg. of PHBA, methyl, ethyl, propyl, and butyl *p*-hydroxybenzoates were each given to a series of three or more fasted dogs, and blood and urine samples were taken at fixed intervals following the injection until levels fell to zero. Determinations of the compound and its various metabolic products were then made on the samples. A similar study was carried out in which 1.0 Gm./Kg. of the drugs was given orally in capsules to the dogs. Summaries of these experiments are tabulated in Table I, Fig. 1 and 2.

Excellent recoveries of "Total" material were noted from the urine of dogs having received intravenous and oral doses of PHBA, methyl ester, ethyl ester, and propyl ester. These ranged from 60-95% indicating that the major portion of the drug as it is metabolized and excreted by the body retains its phenolic structure. Most of the material is excreted by six hours after injected doses whereas appreciable amounts are still detected in the urine thirty hours after oral ingestion with the greater part of the drug being excreted between six and twenty four hours. These results, along with data obtained from the plasma concentrations after oral feeding, seem to indicate that absorption is essentially complete.

Recoveries of "Total" material after oral and intravenous doses of the butyl ester were only 48% and 40%, respectively. It was felt that this might indicate a relatively less effective hydrolysis of the butyl ester by the body which would allow other degradation processes such as oxidation and reduction to take place, thus, destroying the structure necessary for coupling with amino-antipyrine. This difference in metabolic handling is noticed particularly following intravenous injections where an extremely rapid introduction of the drug into the body takes place.

Detoxication mechanisms seem to proceed efficiently immediately after ingestion of the drugs. The concentration of the *p*-hydroxybenzoates in the plasma only rarely surpasses the lower limit of sensitivity of the determinations even immediately

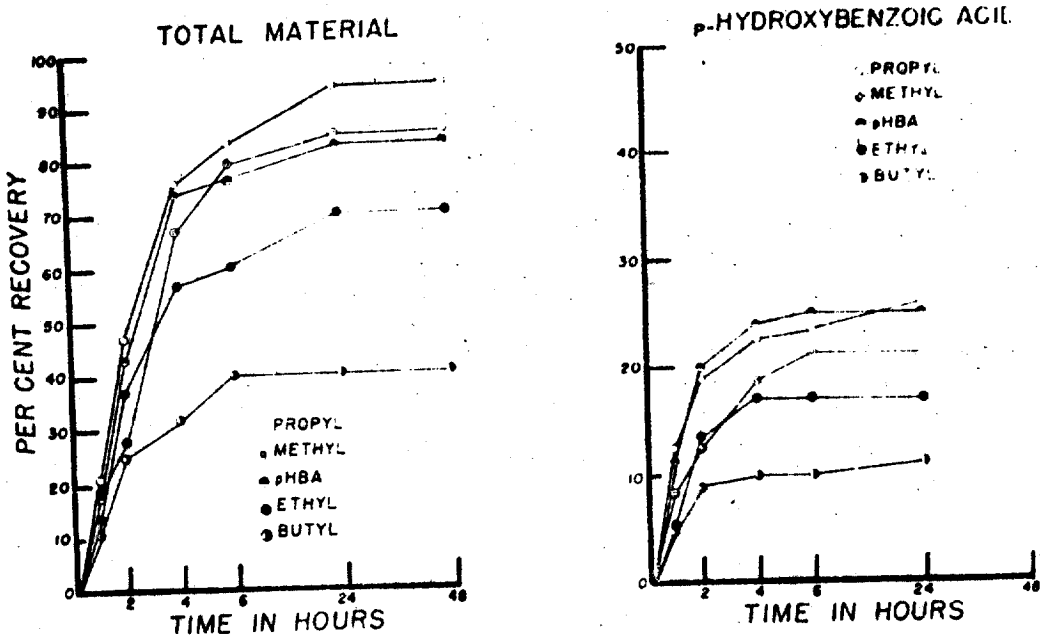

Fig. 1.—Recovery of products of metabolic alteration of *p*-hydroxybenzoic acid and its esters from the urine of dogs given 50 mg./Kg. of each drug intravenously. Each curve represents an average of at least 3 experiments.

following intravenous injections. Significant levels were found only in the plasma of some dogs a few hours after oral administration. The amount of free *p*-hydroxybenzoate excreted never exceeds 0.5% regardless of the method of dosage. The bulk of the "Total" material seems to be excreted in the conjugated form. This conjugation may take place before or after hydrolysis, and the biochemical methods employed cannot distinguish between the two metabolic routes. However, rapid hydrolysis of all but the butyl ester linkages is indicated since after intravenous doses of the compounds practically as much free PHBA is excreted as when the acid itself is injected.

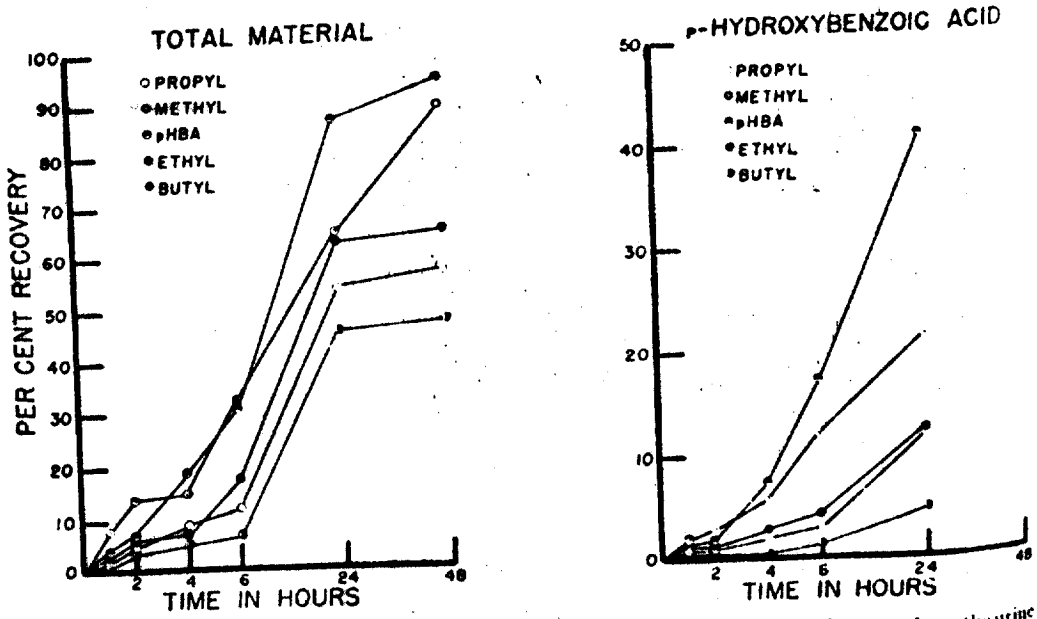

Fig. 2.—Recovery of products of metabolic alteration of *p*-hydroxybenzoic acid and its esters from the urine of dogs given 1.0 Gm./Kg. of each drug by mouth. Each curve represents an average of at least 3 experi-

Drug	Time	Average of Three Dogs Given 10 Gm. Kg. Orally of Each Compound: γ/cc. Plasma Levels: Parasept	Free PHBA	Total	Dose Excreted (Accumulative), %: Parasept	Free PHBA	Gluc. Acid Conjugate	Total	Average of Three Dogs Given 50 γ/Kg. i. v. of Each Compound: γ/cc. Plasma Levels: Parasept	Free PHBA	Total	Dose Excreted (Accumulative), %: Parasept	Free PHBA	Gluc. Acid Conjugate	Total
PHBA	5 min.	..	..	..	..	..	..	..	..	112	122	..		..	..
	15 min.	..	46	59	..		..	..	..	80	100	..	..	..	..
	30 min.	..	..	..	..	..	..	..	..	59	93	..	..	..	..
	1 hr.	..	249	260	..	1.7	.2	2.0	..	28	73	..	5.5	3.8	18.0
	2 hr.	..	399	420	..	2.0	1.1	6.0	..	1.3	56	..	20.0	18.2	43.1
	4 hr.	..	603	703	..	7.6	4.2	19.1	..	0	13	..	24.0	18.2	74.2
	6 hr.	..	660	660	..	17.8	8.2	30.3	..	0	6	..	25.0	18.2	80.3
	24 hr.	..	4	10	..	40.1	30.0	87.1	..	0	0	..	25.0	18.2	83.0
	48 hr.	..	0	4	..	40.1	30.0	94.0	..	0	0	..	25.0	18.2	83.0
Methyl *p*-hydroxybenzoate	5 min.	..	..	..	..	..	..	..	0	82	115				
	15 min.	0	15	17	..	..	..	..	0	68	120				
	30 min	..	..	..	..	..	..	..	0	48	115				
	1 hr.	0	110	273	0.003	0.7	0.5	8.1	0	19	113	0.03	8.4	4.3	14.1
	2 hr.	0	293	437	0.005	2.3	2.1	14.4	0	12	61	0.03	12.4	9.5	28.6
	4 hr.	0	553	827	0.005	6.2	4.9	15.5	0	4	27	0.08	18.1	28.5	67.3
	6 hr.	0	630	867	0.014	11.0	28.8	32.0	0	0	12	0.10	20.6	28.9	81.0
	24 hr.	0	19	77	0.014	21.2	33.1	66.1	0	0	0	0.58	20.6	32.0	85.0
	48 hr.	0	0	3	0.014	21.3	35.1	89.0	0	0	0	0.58	20.6	32.0	85.0
Ethyl *p*-hydroxybenzoate	5 min.	..	..						5	51	85				
	15 min.	2.3	4.5	22					5	62	77				
	30 min.								0	16	66				
	1 hr.	..	99	151	0.001	0.31	0.26	2.0	0	12	51	0.03	5.7	2.8	11.0
	2 hr	7.0	130	228	0.005	1.5	1.1	4.5	0	3	36	0.23	15.0	9.2	37.0
	4 hr.	0	375	480	0.005	3.1	2.4	7.1	0	0	30	0.35	17.0	14.0	56.0
	6 hr.	0	427	648	0.006	4.5	7.7	18.5	0	0	3	0.34	17.0	15.0	61.0
	24 hr.	0	0	17	0.034	12.5	35.5	64.0	0	0	0	0.31	17.0	15.0	70.0
	48 hr.	0	0	0	0.034	12.3	32.5	66.0	0	0	0	0.33	17.0	15.0	70
Propyl *p*-hydroxybenzoate	5 min.								3	85	93				
	15 min.	2	5.5	13					0	62	101				
	30 min.								0	47	83				
	1 hr.	0	32	79	0.003	0.09	0.11	0.37	0	17	49	0.03	13.5	7.4	20.3
	2 hr.	0	18	112	0.006	0.81	0.68	2.9	0	5	32	0.06	19.0	11.6	47.0
	4 hr.	0	95	332	0.001	2.3	1.8	9.0	0	0	13	0.06	25.0	17.5	75.5
	6 hr.	0	205	370	0.006	3.1	3.0	12.2	0	0	0.3	0.10	25.6	19.7	83.7
	24 hr.	0	13	43	0.012	12.1	12.0	53.2	0	0	0	0.10	26.0	22.6	91.0
	48 hr.	0	0	0	0.042	12.7	16.5	57.6	0	0	0	0.10	26.0	22.6	91.0
Butyl *p*-hydroxybenzoate	5 min.								0	47	64				
	15 min	5	0	0.7					0	39	54				
	30 min.								0	26	36				
	1 hr.	14	0	15	0.002	0.0	0.10	0.06	0	12	26	0.20	5.0	17.0	11.0
	2 hr.	3	1.3	34	0.018	0.09	0.40	0.87	0	5	11	0.30	9.0	17.0	26.3
	4 hr.	0	5.5	119	0.021	0.32	1.9	2.6	0	1	7	0.31	10.1	19.3	37.1
	6 hr.	0	15	141	0.075	1.05	4.3	6.9	0	0	1	0.31	10.1	20.1	40.1
	24 hr.	0	1.7	25	0.47	4.9	27.5	47.0	0	0	0	0.31	11.3	20.1	40.1
	48 hr.	0	0	0	0.54	4.9	27.5	48.2	0	0	0	0.31	11.3	20.1	40.1

After intravenous administration, the values for free PHBA and "Total" material in the plasma are similar, but the "Total" values remain high for a longer period of time. From this it would seem that the first detoxication mechanism called upon is one of hydrolysis. Peak "Total" concentrations range from 70-110 γ/cc. depending on the drug, and all fractions are gone from the plasma after six hours. After oral doses, levels are still detectable twenty-four hours after ingestion. Higher peak "Total" concentrations (400-600 γ/cc.) are obtained which appear six hours after feeding except in the case of butyl ester where the peak concentration at six hours is only 150 γ/cc. Extremely low free PHBA values are found in the plasma after oral administration of butyl *p*-hydroxybenzoate. This fact coupled with the smaller amounts of free PHBA excreted, again indicates a possible lowered hydrolysis rate of the butyl compound.

A study in which 70 mg./Kg. of methyl *p*-hydroxybenzoate was given orally to a fasted man revealed that the drug is handled similarly in human metabolism. No detectable ester was found in the plasma or urine. Fifty per cent of the administered dose was recovered from the urine after twelve hours as "Total" material of which 11% was accounted for as free PHBA.

No accumulation of products was noted in dogs which had been given 1.0 Gm./Kg. of methyl or propyl orally every day for a period of one year. The rate of urinary excretion in dogs had increased to such an extent that after twenty-four hours, 90% of the dose was recovered in the urine whereas in dogs given a single oral dose, it is not until forty-eight hours that the entire amount is accounted for. Plasma curves were almost identical in both these cases with the chronic dog's peak level appearing earlier.

When a paste of hydrophilic ointment to which 10% of methyl and propyl ester had been added, was applied to the skin on the back of white rabbits, no noticeable irritation was detected after forty-eight hours, and analysis of the kidney after sacrificing the animal revealed no ester or degradation product present.

DISTRIBUTION OF PHBA ACID AND ITS ESTERS

An experiment was designed to study the distribution of PHBA and its esters in animal tissues. Dogs were infused intravenously at the rate of 2 mg./Kg./min. until a total of 100 mg./Kg. of the compound under study was injected. When 95 mg./Kg. had been given, blood samples were taken. The dog was then killed with intravenous air and the tissues immediately isolated. The organs were cut up, mixed with an equal quantity by weight of saline in a Waring Blendor, and finely ground in a tissue grinder until the mash could be drawn through a syringe.

Analyses were made on the various tissues and the plasma to determine the levels of PHBA, ester and "Total" material present at the time of death. The results are shown in Table II. Appreciable ester is found only in the brain, spleen, and pancreas. High "Total" concentrations are found in the liver and kidney, and high concentrations of free PHBA are detected in the kidney. Other than these instances, levels in the various organs are below plasma levels. Apparently this rate of administration is slow enough to overcome most enzymatic [illegible] in the handling of these compounds, for even the higher ester concentrations, the butyl [illegible] does not appreciably differ from the other [illegible].

In vitro studies of normal tissue mashes [illegible]

TABLE I.—RATE OF HYDROLYSIS OF ESTERS OF PARA HYDROXYBENZOIC ACID BY DOG TISSUE MASHES. DATA CONSTRUCTED FROM [illegible] PLOTTED FROM [illegible] AND REACTION CAPACITY [illegible] ROOM TEMPERATURE.

Tissue	Concentration of Hydrolysis				
	Butyl Ester	Methyl Ester	Ethyl Ester	Propyl Ester	
Liver	[illegible]	[illegible]	[illegible]	[illegible]	64
	[illegible]	[illegible]	[illegible]	[illegible]	100
	[illegible]	[illegible]	[illegible]	[illegible]	100
Brain	[illegible]	[illegible]	[illegible]	[illegible]	0
	[illegible]	[illegible]	[illegible]	[illegible]	0
	[illegible]	[illegible]	[illegible]	[illegible]	0
Kidney	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	
	[illegible]	[illegible]	[illegible]	[illegible]	
Muscle	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	
Spleen	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	
	[illegible]	[illegible]	[illegible]	[illegible]	
Pancreas	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Plasma	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Whole blood	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

TABLE II.—DISTRIBUTION OF PARAHYDROXYBENZOIC ACID, ITS ESTERS, AND METABOLIC PRODUCTS IN TISSUES OF DOGS EACH COMPOUND INFUSED INTRAVENOUSLY AT A RATE OF 2 MG./KG./MIN. FOR FIFTY MINUTES

Tissues	Concentration in Tissues (γ per Gm. or ml.) — Concentration of PHBA					Concentration of Ester					Concentration of "Total"				
	Butyl	Propyl	Ethyl	Methyl	PHBA	Butyl	Propyl	Ethyl	Methyl	PHBA	Butyl	Propyl	Ethyl	Methyl	PHBA
Liver	28	19	28	74	56	0	0	0	0		210	215	280	520	540
Kidney	186	116	150	190	192	0	0	0	0		520	330	340	520	510
Brain	0	0	3	14	20	50	23	30	10		36	25	36	32	64
Spleen	14	35	20	36	64	32	0	0	6		76	53	80	[illegible]	108
Pancreas	2	10	16	..	30	70	0	16	..		80	50	132		124
Muscle	9	23	18	12	24	0	0	0	0		30	48	120	34	42
Plasma	72	72	47	155	225	25	5	0	0		193	275	177	263	300

with the various esters revealed that esterases in liver and kidney were extremely efficient in hydrolyzing the esters to free PHBA. The liver mashes were capable of 100% hydrolysis after three minutes of contact with all the esters except butyl in which case thirty to sixty minutes of contact was necessary. Muscle mash was capable of this hydrolysis to a small degree while the other tissues, plasma, and whole blood showed no *in vitro* ability to break the ester linkage. The liver, having low free PHBA levels in the intact animal, presumably conjugates or otherwise metabolizes most of its free PHBA to further degradation products.

DISCUSSION

From the studies reported above it is apparent that *p*-hydroxybenzoic acid and its esters are efficiently absorbed from the gastrointestinal tract and undergo rapid metabolic alteration. The esters in particular are rapidly hydrolyzed by esterases found in the liver and kidney. That this enzyme differs from that which ordinarily splits procaine is evident from the fact that procaine esterase is abundantly present in plasma, whereas the esters of *p*-hydroxybenzoic acid are not hydrolyzed by plasma or whole blood. Similarly it is unlikely that choline esterase is responsible for the hydrolysis since it was shown that the administration of physostigmine in highest tolerated doses had no effect on the rate of disappearance of the esters of *p*-hydroxybenzoic acid from the blood.

SUMMARY

1. Appropriate chemical methods have been devised for the quantitative analysis of *p*-hydroxybenzoic acid and its esters in tissues and biological fluids. With suitable modifications, this procedure may be employed to determine "Total" material, that is, ester plus free PHBA plus all metabolic products in which the phenolic group is not destroyed.

2. By means of these methods a comparative study has been made of the plasma levels and urinary excretion of dogs given 1.0 Gm./Kg. orally and 50 mg./Kg. intravenously of methyl, propyl, and butyl ester as well as PHBA itself. Per cent recoveries from the urine of these animals, regardless of the method of administration, ranged from 60-95 per cent except in the case of butyl ester where the recoveries were approximately 40 per cent. These results seem to indicate that PHBA and the esters are well absorbed after oral administration.

3. Only rarely do the plasma ester levels reach a significant value even immediately following intravenous injection. Highest ester levels are encountered after the oral administration of the butyl derivative. On the other hand, the high plasma levels and urinary output of free PHBA and conjugated products in dogs having received the various esters indicate that hydrolysis of the ester linkage and metabolic conjugation by the body constitute the chief paths of metabolic alteration. However, when the butyl derivative is administered rapidly there appear to be other pathways in addition which account for poor recovery.

4. Studies in which tissue mashes were allowed to incubate with the *p*-hydroxybenzoates revealed rapid *in vitro* enzymatic hydrolysis of the compounds by liver and kidney. The butyl ester was found to be the least easily hydrolyzed of the series.

5. Topical application of ointments which contained 10 per cent methyl and 10 per cent propyl *p*-hydroxybenzoate to the shaved backs of white rabbits produced no irritation after forty-eight hours of contact. Upon sacrificing the animals, no drug concentration was detected in the kidneys.

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(2) Williams, R. T., "Detoxication Mechanisms," John Wiley & Sons, Inc., New York, N. Y., 1947, p. 101.
(3) Maughan, G. B., Browne J. S. L., and Evelyn K., *Biol. Chem.*, **126**, 567 (1938).

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p-Hydroxybenzoic Acid Esters as Preservatives II.

Acute and Chronic Toxicity in Dogs, Rats, and Mice*

By CAROL MATTHEWS, JOAN DAVIDSON, ELIZABETH BAUER, JAMES L. MORRISON, and ARTHUR P. RICHARDSON

The results of a study of the acute and chronic toxicity of *p*-hydroxybenzoic acid and the methyl, ethyl, propyl, and butyl esters in mice, rats, and dogs are presented. All of these compounds possess a low order of acute toxicity. The toxicity of the sodium salts appears to be a function of the length of the alkyl substituent, with the butyl ester about three times as toxic as the methyl ester. The main toxic effect is an acute myocardial depression accompanied by hypotension, but this is transient in nature and not cumulative. No evidence of blood damage or histological changes in tissues of treated animals was noted. In concentrations below 5 per cent, none of the esters produces any evidence of primary irritation when applied to the intact skin of man. From the results obtained, it may be concluded that the methyl, ethyl, and propyl esters of *p*-hydroxybenzoic acid may be used safely as preservatives in effective concentrations for foods and drugs.

AALTO, FIRMAN, AND RIGLER (1) have recently described the antibacterial, antifungal, and chemical properties and uses of certain esters of *p*-hydroxybenzoic acid[1] as preservatives. They have drawn attention to the present wide usefulness of these compounds and to the possibility of an even wider use. Since one of the fields in which they may serve a useful purpose is in the preservation of certain food products it became important to obtain more detailed information on their pharmacology than is now available.

This is the first of two papers describing certain aspects of the pharmacology of these agents. It will deal chiefly with acute and chronic toxicity studies in mice, rats, and dogs. Since the methyl and propyl esters have been used most widely, greater attention will be paid to these two derivatives than to the others. The second paper in the series will deal with studies on the physiological disposition of these drugs.

EXPERIMENTAL

Acute Toxicity in Mice.—Although a number of studies have been carried out on the acute toxicity of esters of *p*-hydroxybenzoic acid[2] they have served only to show that under the limited conditions used this series of compounds possess a low order of acute toxicity when administered by mouth. Experiments designed to determine their inherent toxicity are not available. It seemed important therefore to carry out additional acute studies if for no other reason than for orientation of the contemplated chronic studies.

A uniform strain of albino mice from a single source was used throughout. The drugs to be administered were prepared as a suspension of the free ester in 3% starch, propylene glycol, or olive oil. Solutions of the sodium salts were prepared by adding slightly more than one mole of sodium hydroxide to one mole of free ester. The volume of each injection was adjusted so that all animals given parenteral injections received 0.1 ml. of solution or suspension per 10 Gm. bodyweight. In the case of oral tests the large doses made it necessary to increase the volume of some of the higher doses. All animals were fasted for twelve hours prior to being used, but immediately after being injected were returned to cages with food and water. The data were analyzed by the method of Miller and Tainter (2). Table I summarizes all the data.

All four of the esters produced the same type of symptoms. Typically there was a rapid onset of ataxia, paralysis, and deep depression resembling anesthesia. Only rarely were there evidences of increased motor activity. In nonfatal doses recovery was prompt, seldom lasting beyond thirty minutes. Generally speaking deaths also occurred within a short time, usually one hour. All animals, however, were observed for a period of one week and the results reported here represent effects obtained from the entire period of observation.

The results observed with *p*-hydroxybenzoic acid deserve special mention since the results recorded with this compound at first appear somewhat confusing. Whether one uses the free acid or the sodium salt makes a remarkable difference. As the salt this substance has a remarkably low toxicity when compared with the esters. As the free acid, however, it is approximately 15 times as toxic on intraperitoneal injection as is the salt. Autopsy of some of the animals which died showed a finding which might help explain this phenomenon. Animals injected with the free acid uniformly showed considerable amount of free fluid in the peritoneal cavity, probably caused by local irritation. Such an effect was not observed with the sodium salt. Although this result was somewhat mystifying, for

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[1] Although esters of *p*-hydroxybenzoic acid are easily described by their chemical names the methyl and propyl esters are described by U. S. P. XV as Methylparaben and Propylparaben. Available under the trademarked names of Methyl Parasept®, and Ethyl Parasept®. In this paper and in the succeeding one, all samples used were obtained from the Heyden Chemical Corporation.

[2] For a summary of earlier work see review by Neiling, C. P., and Burrell, H., *Drug and Cosmetic Ind.*, 54, 108(1944).

TABLE I.—ACUTE TOXICITY OF PARAHYDROXYBENZOIC ACID AND ITS ESTERS IN MICE

Compound	Oral						Intravenous		Intraperitoneal			
	Free Acid		Na Salt		Propylene Glycol		Olive Oil		Free Acid		Na Salt	
	PD_{50}[a]	LD_{50}[b]	PD_{50}	LD_{50}	PD_{50}	LD_{50}	PD_{50}	LD_{50}	PD_{50}	LD_{50}	PD_{50}	LD_{50}
p-Hydroxybenzoic acid	..	..	No paralysis	2200 ±89.4	..	..	..	..	1050 ±60.1	1200 ±58.9	No paralysis	210 ±18.8	No paralysis	3400 ±135.2
Methyl *p*-hydroxybenzoate	>8000	>8000	1300 ±123.4	2000 ±141.2	>8000	>8000	Approx. 8000	Approx. 8000	80 ±9.7	170 ±8.4	370 ±36.3	960 ±70.5	460 ±22.1	760 ±34.7
Ethyl *p*-hydroxybenzoate	..	..	..	Approx. 2500	..	..	..	..	..	..	..	..	290 ±50	520 ±60.0
Propyl *p*-hydroxybenzoate	No paralysis	>8000	No paralysis	3700 ±139.5	>8000	>8000	>8000	>8000	54 ±5.0	180 ±3.7	400 ±30.3	640 ±46.1	490 ±20.9	490 ±20.9
Butyl *p*-hydroxybenzoate	..	..	..	Approx. 950	..	..	..	..	..	..	..	..	200 ±65	230 ±24

[a] PD_{50} = Dose required to paralyze 50% of animals. [b] LD_{50} = Dose required to kill 50% of animals. All figures represent mg./Kg.

tunately no such differential effect was observed with any of the esters so that although it is difficult to compare the results obtained on the esters with *p*-hydroxybenzoic acid the data obtained do permit certain conclusions as to the relative acute toxicity of the four esters. In each instance where they were compared under the same conditions there was a consistent relationship between the length of the alkyl chain of the alcohol and order of toxicity. Thus it is quite apparent that in all instances the methyl ester was least and the butyl ester most toxic. This was illustrated in the intraperitoneal toxicity tests carried out with the sodium salts. In this particular series of experiments butyl *p*-hydroxybenzoate is approximately three times as toxic as the methyl ester. As will be pointed out later this difference in toxicity is correlated with the speed of hydrolysis of the various esters.

The oral tests do not contribute a great deal to the general understanding of the action of this group of compounds since the doses required to produce toxic effects are so large as to make it difficult to obtain an entirely satisfactory dosage-response curve. Since these compounds are more soluble in alcohols and fatty substances such as olive oil it seemed important to determine whether absorption from the gastrointestinal tract might not be speeded up by incorporating them in such a vehicle. The data in Table I clearly indicate a lack of effect of either olive oil or propylene glycol on absorption from the gut. On the other hand the use of sodium salts did appear to enhance absorption since the fatal doses for these preparations were significantly lower than when a suspension of the free esters was used. It is important to point out here that the great difference in response between oral and parenteral administration cannot be explained on a basis of incomplete absorption. Results to be reported in a succeeding paper (3) of this series showed excellent recoveries from the urine of animals after oral administration. The low oral toxicity, therefore, is to be explained by the fact that *p*-hydroxybenzoates have a rapid but brief action due to rapid metabolic alteration. Therefore rate of absorption is an important factor in explaining the wide variation of oral and parenteral toxicity.

Acute Toxicity in Dogs.—In order to more accurately determine the mechanism of acute death caused by this series of compounds, unanesthetized dogs and dogs anesthetized with 80 mg./Kg. of chloralose, or 25 mg./Kg. of pentobarbital were used for more detailed studies. Aside from some depression observed in unanesthetized animals the effects observed were the same regardless of preoperative preparation. One femoral artery was cannulated and connected to an Anderson glass capsule for recording of blood pressure. A femoral vein was cannulated and connected to a reservoir system to permit injection of drugs. In some experiments a glass cannula was inserted into a jugular vein to permit recording of venous pressure a few centimeters from the right auricle. A standard Sanborn Visocardiette was used for recording of electrocardiograms. Sodium salts of each of the compounds were prepared as previously described. Standard responses to intravenous doses of epinephrine, acetylcholine, and histamine were obtained as controls to test the function of the cardiovascular and autonomic nervous system. Following control

observations, gradually increasing doses of each of the compounds were injected and effect on the cardiovascular system, and autonomic nervous system determined.

The only important action observed was a precipitous but brief fall in blood pressure associated with a rise in jugular venous pressure as illustrated by Fig. 1. In these acute experiments, death was

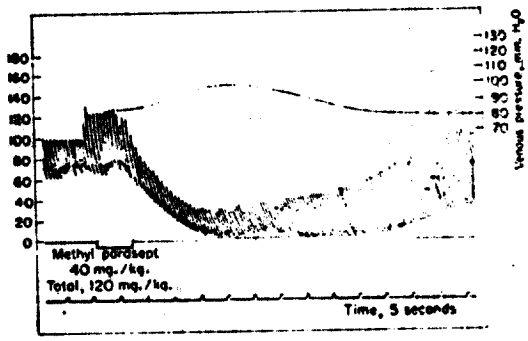

Fig. 1.—Effect of intravenous injection of 40 mg./Kg. of methyl parahydroxybenzoate. Dog anesthetized with chloralose. Upper line venous pressure at the level of the right auricle.

associated with the hypotensive action, and since cardiovascular effects were brief, it was not unexpected to find an important relationship between the action on the cardiovascular system and the rate of injection. For example in the case of the propyl ester the fatal dose is of the order of 85 mg. per Kg. when administered at the rate of 20 mg./Kg./minute, whereas when the same drug was administered at the rate of 5 mg./Kg./minute, the fatal dose is of the order of 600 mg./Kg. Table II summarizes the data obtained with the methyl and propyl ester on this point. Obviously then in order to compare the relative action of the compounds under study the rate of injection was carefully controlled. Arbitrarily, the effect on blood pressure of single doses injected within a ten-second period was determined. Figure 2 illustrates a typical record of the type of experiment carried out. The fall in systolic pressure was measured and dosage-response curves con-

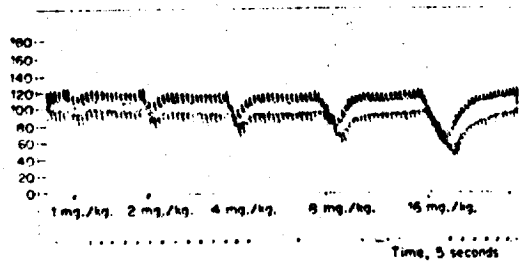

Fig. 2.—Effect of increasing intravenous injections of propyl parahydroxybenzoate on the blood pressure of dogs.

structed as illustrated in Fig. 3. To simplify presentation of the data the dose necessary to produce 35 mm. fall in pressure was determined. As in the case of acute toxicity studies in mice there is a correlation between the length of the alcohol alkyl chain and hypotensive effect. The "35-mm. hypotensive dose" being as follows: *p*-hydroxybenzoic acid 40 mg./Kg., methyl ester 16 mg./Kg., ethyl 16 mg./Kg., propyl ester 8 mg./Kg., and butyl ester 8 mg./Kg. Since the method of preparing the sodium salts utilized a slight excess of alkali and gave solutions of *p*H 9–10 it was important to rule out the possibility that these cardiovascular effects might have been due to alkali. This was controlled by a study of solutions prepared with sodium bicarbonate instead of sodium hydroxide. The *p*H of such solutions was 7.5–8.0. These nearly neutral solutions produced the same depressor effect as the more alkaline solutions. In addition the injection of sodium hydroxide solutions in doses several times those used in the above studies were without depressor effect.

The mechanism of the hypotensive action appears to be depression of the heart since it was accompanied by significant electrocardiographic changes. Figure 4 illustrates a typical set of tracings obtained in the course of blood pressure change shown in Fig. 1. At the nadir of the blood pressure response there was a widening of the QRS complex with a decrease in potential and inversion of the *T* wave. These changes are interpreted as being evidence

TABLE II.—EFFECT OF RATE OF INJECTION ON INTRAVENOUS FATAL DOSE OF ESTERS OF PARAHYDROXYBENZOIC ACID IN DOGS

Rate of Injection									
5 mg./Kg./min.		10 mg./Kg./min.		15 mg./Kg./min.		20 mg./Kg./min.		40 mg./Kg./min.	
Total dose mg./Kg.	Effect	Dose mg./Kg.	Effect	Dose mg./Kg.	Effect	Dose mg./Kg.	Effect	Dose mg./Kg.	Effect
						Methyl *p*-Hydroxybenzoate			
						360	Surv.	80	Died
						940	Died	320	Died
						1140	Surv.	600	Died
						1400	Died	920	Surv.
								1100	Surv.
Propyl *p*-Hydroxybenzoate									
470	Surv.	600	Died	210	Died	120	Died		
870	Surv.	720	Died	150	Died	80	Died		
400	Surv.	434	Surv.	90	Died	80	Died		
..	..	1080	Surv.	75	Died	80	Died		
..	..	..	..	100	Died	80	Died		
..	..	..	..	495	Surv.	100	Died		

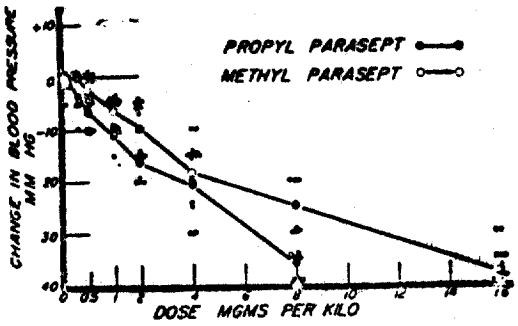

Fig. 3.—Summary of effects of single doses of methyl and propyl parahydroxybenzoates on the blood pressure of dogs. Small circles represent individual experiments. Large circles represent mean values.

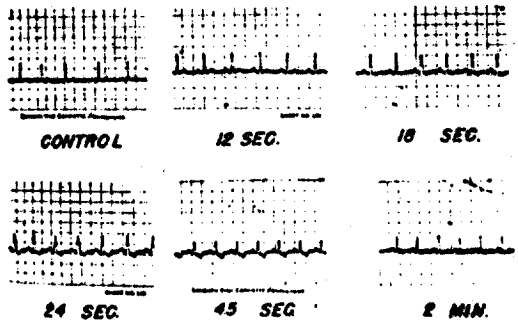

Fig. 4.—Effect of methyl parahydroxybenzoate on the electrocardiogram of the dog. Lead II taken in the course of experiment illustrated by Fig. 1.

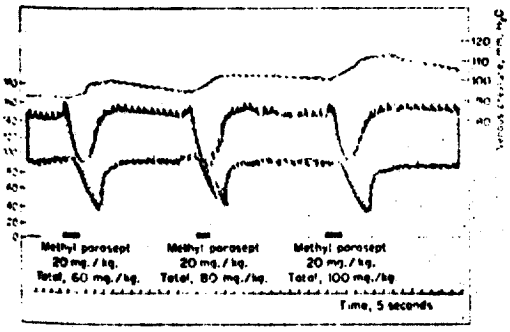

Fig. 5.—Effect of repeated doses of methyl parahydroxybenzoate on the blood pressure. Note lack of cumulative effect.

depression of the myocardium and are not unlike the effects produced by procaine and procaine amide. As in the case of the blood pressure fall, the electrocardiographic changes are quickly reversible and transient. As is shown in Fig. 5 there is little indication of cumulative effect. Associated with the hypotensive action there was uniformly a marked rise in right auricular pressure which is also indicative of cardiac depression.

Of some interest were the negative effects of the *p*-hydroxybenzoates on the autonomic nervous system. Even at near fatal doses there was no change in the response of the animals to epinephrine, acetylcholine, or histamine. Also full doses of atropine (1.0 mg./Kg.) or section of the vagi had no significant effect on the fall in blood pressure. Since metabolic studies indicated that all of the esters are rapidly hydrolyzed in the body it was of interest to find that doses of physostigmine sufficient to produce skeletal tremors (0.2 mg./Kg.) had no effect on the cardiovascular action; it seems fair to assume therefore that choline esterases are not responsible for the metabolic alteration of the compounds.

Chronic Toxicity in White Rats.—Since the indications at the outset of these studies were that the propyl and methyl esters were particularly apt to be used as food preservatives, extended chronic studies were carried out on these two compounds. Preliminary metabolic studies showed that absorption from the gastrointestinal tract was essentially complete (3) hence it seemed safe to study the chronic toxicity in animals in which the drug was incorporated in the diet. Purina Dog Chow was used as a diet throughout the entire experiment. Checkers were first ground roughly in a flour mill, and after the addition of an appropriate amount of drug the mixture was shaken and run through a flour mill four or five times to insure complete mixing. On the last run through the mill the food was ground as finely as the apparatus would permit. As a test to insure that the drug had been evenly and completely distributed in the diet, a test sample of dog checkers was ground with a known quantity of sulfanilamide and assayed chemically. Assay of a sulfanilamide diet before and after feeding showed that animals were unable to select food instead of drug and therefore it was assumed that this method of preparing diets insured continuous and even intake of drug.

Twenty-four weanling albino rats from a strain originally obtained from the Wistar Institute[3] were equally divided between males and females at each dosage level. Animals were selected at random from a large batch of weanling rats. At the start animals were placed in cages in groups of four, but as they matured they were separated so that there were two animals to a cage. Drug diet was placed in containers which prevented wasting by the rats; free access was permitted to both food and water. Food intake and weight of animals was determined every two weeks. Control animals were fed a similar diet of powdered dog checkers but without drug.

Three series of experiments as outlined above were carried out. In the first, a group of animals was given diets containing 2% of propyl and methyl esters of *p*-hydroxybenzoic acid, and compared with animals given no drug. After forty weeks on this diet, it was apparent that this concentration was having no effect on the growth curve and Series II was set up in similar fashion but containing 2% and 8% of each drug. In both Series I and II animals were treated for a period of ninety-six weeks. As these series of experiments were reaching completion it seemed advisable to also run a short term chronic toxicity on the ethyl and butyl esters.

[3] All rats used in these studies were obtained from Mr. Winfrey Winn, Department of Physiology of Emory University, who has maintained this colony unbroken for at least the past ten years.

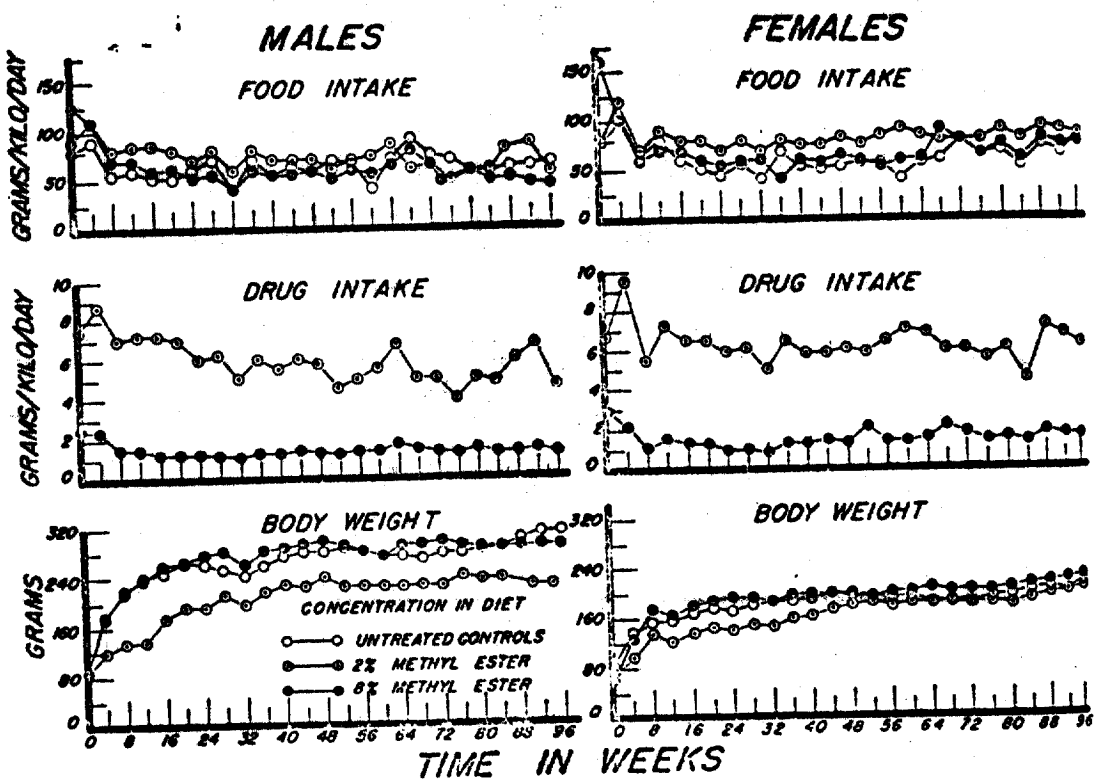

Fig. 6.—The effect of incorporation of methyl parahydroxybenzoate in the diet of weanling white rats. Each curve represents mean of 12 animals.

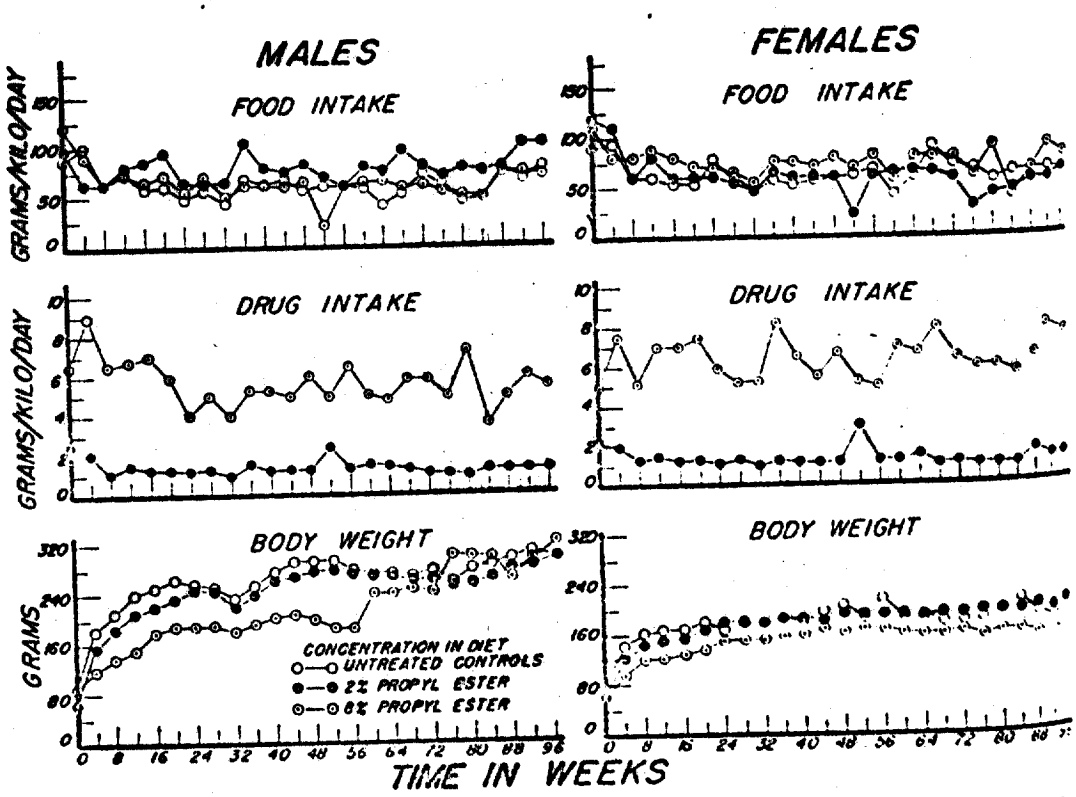

Fig. 7.—The effect of incorporation of propyl parahydroxybenzoate in the diet of weanling white rats. Each curve represents mean of 12 animals.

Therefore Series III was set up with rats on 2% and 8% of these two esters but in which the test period was allowed to run for only twelve weeks. At the conclusion of each experiment all animals, control and experimental, were sacrificed and appropriate tissues removed for pathological study. All animals dying in the course of the experiment were autopsied and tissues removed and fixed for later study by a pathologist.

Since the results obtained in Series II were similar but more extensive than Series I, only the results with the former are presented in detail here. Figures 6, 7, and Table III summarize the results obtained in Series II. Animals receiving diets 8% of both the propyl and methyl esters all showed a slower rate of gain in weight than did controls. This difference is much more striking in the early part of the experiment. In fact, as the end of the test was approached these differences tended to disappear. This effect was more striking in the case of the males than the females. On animals receiving 2% of these drugs, however, it was impossible to differentiate treated animals from untreated animals.

Series III was carried out in the same manner as the first two series except that younger rats were used and ethyl and butyl esters were incorporated in the diet. Essentially the same results were obtained with the ethyl ester as with the methyl and propyl esters, namely concentrations of 2% were without effect, but 8% showed not only a much slower rate of growth, but also signs of toxicity characterized by depression, decreased motor activity, and deaths within the first few weeks of beginning the experiment. In fact all the males on the 8% diet of butyl ester died before the end of the twelve-week period of observation. Although these symptoms were less striking with females on this same diet, this group also had many early deaths and depression.

Since it has already been pointed out that the most striking changes were observed within the first part of the experiment, Table III has been constructed so as to show a rough comparison of the effects produced by all four drugs at twelve weeks. The results clearly show the lack of effect of diets containing 2% of any of the four compounds whereas in each instance a definite decrease in weight was observed in all groups fed the 8% diets.

Food intake and hence drug intake remained fairly constant throughout the course of the various feeding experiments, nor were there significant differences between the various esters. Drug intakes for animals on the 2% diets averaged from 0.9 to 1.2 Gm./Kg. body weight per day. While the drug intake of rats on the 8% diets averaged from 5.5 to 5.9 Gm./Kg./day. If it is valid to transfer results from rat experiments to other species it would be safe to assume that on chronic studies the first signs of toxicity would not appear until at least a drug intake of 3 Gm./Kg./day had been reached.

Chronic Toxicity in Dogs.—A group of eleven young healthy mongrel pups were taken from their mothers after weaning and divided into five groups as shown in Table IV. Each dog was housed in an individual cage but permitted to run in an enclosed area from time to time. All animals were fed Purina Dog Chow *ad libitum* and in addition were given biweekly supplements of meat scraps. After they had become adjusted to the diet and seemed to be gaining weight, oral treatment with the methyl and propyl *p*-hydroxybenzoates was started. The drugs were put up in gelatin capsules of convenient size so that proper dosage could be readily administered. Treatment was given six days weekly (Sunday excluded). No toxic symptoms were observed in any of the animals. One control died 195 days after the beginning of the experiment apparently of pneumonia. The weights of animals before and after treatment are given in Table IV and for the most part showed respectable increases. Since these animals were mongrels of varying antecedents it was to be expected that final weights would vary greatly. Of most importance however was the fact that at the end of the experiment all animals appeared to be in excellent condition as far as general appearance was concerned. Of secondary interest it is worth noting that one of the females receiving 0.5 Gm./Kg./day of methyl ester was mated toward the end of the experiment and delivered a litter of healthy pups which were later raised in the laboratory.

Approximately one month before the end of the experiment all animals receiving 1.0 Gm./Kg. of either ester were studied for possible cumulation of compound in the blood. This was done in the following manner: Just prior to the regular daily dosing a control blood sample was taken and analyzed according to methods described elsewhere (3). After the usual dose had been administered blood samples were taken over the next twenty-four hours and analyzed for drug and metabolic end products. The results of this study are summarized in Table V. It is to be noted that at zero time

TABLE III.—A COMPARISON OF THE EFFECT OF ESTERS OF PARAHYDROXYBENZOIC ACID ON THE GROWTH CURVE OF WEANLING ALBINO RATS. EACH GROUP REPRESENTS THE MEAN OF 12 RATS. PERIOD OF TEST 12 WEEKS

Drug Diet	Sex	Mean Wt. of Rats Before Treatment, Gm.	Wt. After Treatment, Gm.	Gain in Wt., %
		Series II		
Control	M	90	240	144
2% Methyl ester	M	90	240	144
8% Methyl ester	M	93	141	52
2% Propyl ester	M	71	203	188
8% Propyl ester	M	87	144	63
Control	F	78	170	119
2% Methyl ester	F	69	164	136
8% Methyl ester	F	75	120	60
2% Propyl ester	F	65	160	146
8% Propyl ester	F	81	120	48
		Series III		
Control	M	38	263	610
2% Ethyl ester	M	39	264	601
8% Ethyl ester	M	30	161	430
2% Butyl ester	M	32	240	654
8% Butyl ester	M	31	Died	..
Control	F	31	198	538
2% Ethyl ester	F	33	180	440
8% Ethyl ester	F	33	151	350
2% Butyl ester	F	34	210	510
8% Butyl ester	F	34	115	235

(twenty-four hours following the previous treatment) a small amount of assayable material was still present in the blood, chiefly as conjugated end products. However the plasma concentration-time curve resembled closely that of dogs not previously treated. In addition urine was quantitatively collected over same period of time and it is highly significant that in the case of both compounds between 96% and 100% of the daily administered dose was accounted for in the urine, chiefly in the form of conjugated end products. Both the urine and plasma studies therefore furnish strong evidence of the lack of cumulation of either the methyl or propyl *p*-hydroxybenzoate when administered at the level of 1.0 Gm./Kg./day for one year.

Near the conclusion of the period of observation, complete blood counts and urine examinations were made on all dogs. Details of these experiments are omitted since the findings were entirely normal. At the end of the experiment all dogs were killed by the injection of air and turned over to the pathologist for careful autopsy and histological study of selected tissues.

Pathological Studies on Tissues.—As described above all animals dying in the course of chronic toxicity studies on rats and dogs were autopsied and selected tissues preserved for histological examination. At the conclusion of the chronic studies all animals were sacrificed and tissues selected for study. The various specimens were labeled with a code number and given to Dr. Abner Golden of the Pathology Department of Emory University School of Medicine. Particular attention was paid to kidney, liver, heart, lung, spleen, and pancreas. According to Dr. Golden's report, tissues from all rats surviving the chronic tests were entirely normal. The only significant findings were seen in the case of rats dying in the course of experiments; in all such animals there was extensive consolidation of the lung and pneumonia, and in this respect there was no difference between the control and treated animals.

Inasmuch as an important suggested use of esters of *p*-hydroxybenzoic acid is in cosmetics it is important to know whether these agents possess any local irritative property and whether they may produce sensitization. Studies on this point have been made by other investigators and a summary of their unpublished results is included below.

Allergic Reactions to Esters of *p*-Hydroxybenzoic Acid in Guinea Pigs.[4]—Guinea pigs were injected intracutaneously with a solution of the ester at regular intervals for three and one-half weeks, then allowed to rest for two weeks. During this period a sensitivity to the compound, if it existed, would have been built up. At the end of the period a challenging dose was given, and the reaction to it was read after twenty-four hours. A difference in the area of irritation between the first and challenging doses would have been a positive reaction.

The procedure used was the one suggested by H. Draize (4). This is a slight modification of the method outlined by Landsteiner and Jacobs.

Forty white male guinea pigs weighing 300 to 450 Gm., subsisting on a Purina rabbit pellet ration supplemented with greens (kale and lettuce), were identified and hair was removed from back and flanks by close clipping. The pigs were divided into four groups, ten for each ester.

Solutions of 0.1% in physiological saline were prepared, the methyl and ethyl esters used as such, and the propyl and butyl esters as the sodium salts made by dissolving the esters in the calculated amount of 5% sodium hydroxide solution, then diluting. The solutions were injected intracutaneously using a 27-gauge hypodermic needle. The animals were injected three times weekly at random in an area of the back and upper flanks four centimeters square. A total of ten injections was given. The first injection consisted of 0.05 ml.; the remaining ones were 0.1 ml. No reaction was noted twenty-four hours after the first injection.

Two weeks after the tenth injection, a retest injection was made using 0.05 ml. of a freshly prepared solution. The retest injection was made in an area just below the sites of the sensitizing injection. Twenty-four hours later the guinea pigs were closely observed for a reaction; there was none.

Primary Irritation and Sensitization in Man[5]

Primary Irritative Effect.—The methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid were dissolved in propylene glycol in increasing concentrations, and applied to the skin of 50 human subjects. It was found that the following con-

TABLE IV.—SUMMARY OF CHRONIC ORAL TOXICITY IN DOGS

Methyl Parasept[6] Orally						Propyl Parasept[6] Orally					
1 Gm./Kg. Daily			0.5 Gm./Kg. Daily			1 Gm. Kg. Daily			0.5 Gm. Kg. Daily		
No. of Days	Initial Wt., Kg.	Final Wt., Kg.	No. of Days	Initial Wt., Kg.	Final Wt., Kg.	No. of Days	Initial Wt., Kg.	Final Wt., Kg.	No. of Days	Initial Wt., Kg.	Final Wt., Kg.
378	3.6	9	318	3.5	8.8	394	4.5	6	394	4.1	5
422	3.6	12	331	8.6	9.4	313	4.7	4.5	...	...	...
394	4.5	5.5	...	...	...	384	6.4	12	...	...	...

Control

No. of Days	Initial Wt. Kg.	Final Wt., Kg.
422	2.3	12.3
195	4.6	7.5

[4] By J. Marrus and W. M. Ziegler, Heyden Chemical Corporation, Princeton, N. J.

[5] By Herman Shelanski, Industrial Toxicology Laboratories, Philadelphia, Pa.

TABLE V.—PLASMA CONCENTRATION—TIME CURVES OF DOGS RECEIVING 1.0 GM./KG. OF METHYL AND PROPYL PARAHYDROXYBENZOATE DAILY FOR APPROXIMATELY 350 DAYS. TESTS STARTED 24 HOURS AFTER LAST DOSE

	Plasma Concentration mcg./ml.		
Time	Free Parasept®	Para-hydroxy-benzoic Acid	Total Material
0	0.5	8.0[a]	54.0
15 Min.	0	23.0[a]	93.0
1 Hr.	4.0	56.0[a]	261.0
2 Hr.	14.0	197.0[a]	313.0
4 Hr.	0	492.0[a]	655.0
6 Hr.	0	450.0[a]	591.0
24 Hr.	0	27.0[a]	43.0
0	0	10.0[b]	47.0
15 Min.	0.3	33.0[b]	55.0
1 Hr.	0	39.0[b]	122.0
2 Hr.	0	100.0[b]	315.0
4 Hr.	8	91.0[b]	226.0
6 Hr.	1	87.0[b]	265.0
24 Hr.	0	0.0[b]	17.0

[a] Methyl Parasept® (mean of 2 dogs).
[b] Propyl Parasept® (mean of 3 dogs).

trations produced no irritation when applied during a period from four to eight hours every other day and a total of ten applications; methyl *p*-hydroxybenzoate 5%, ethyl *p*-hydroxybenzoate 7%, propyl *p*-hydroxybenzoate 12%, and butyl *p*-hydroxybenzoate 5%. Higher concentrations than this produced some evidence of irritation.

Repeated Insult Method.—In 50 unselected subjects (25 males, 25 females) each of the esters was applied in the maximum tolerated concentration as described above. At the end of this period three weeks was allowed to elapse during which time there was no contact with the drugs. At this point each drug was reapplied for a period of from 24 to 48 hours in the same concentration which was used on original contact. In no instance was there evidence of development of sensitization.

SUMMARY

A study has been made of the acute and chronic toxicity of *p*-hydroxybenzoic acid and its methyl, ethyl, propyl, and butyl esters in mice, rats, and dogs.

1. Each of these compounds has a low order of acute toxicity, which in the form of the sodium salt increases with the length of the alkyl ester, the butyl ester being about three times as toxic as toxic as the methyl ester.

2. In the case of the esters the chief toxic effect is an acute myocardial depression with hypotension. This effect is transient in nature and is not cumulative.

3. Rats fed 0.9 to 1.2 Gm./Kg./day of the four esters showed no effect even when treated as long as ninety-six weeks. Rats given 5.5 to 5.9 Gm./Kg./day showed a definite depression of rate of growth particularly during the first twelve weeks of feeding. At this higher dosage the butyl ester was definitely more toxic and killed all animals within the first twelve weeks.

4. Dogs fed 1.0 Gm./Kg./day for one year showed no evidence of toxicity.

5. There was no evidence of blood damage or histological changes in kidney, liver, heart, lung, spleen, and pancreas in any of the treated animals.

6. In concentrations up to 5 per cent, none of the esters produce evidence of primary irritation when applied to the intact skin of man. Repeated applications do not produce sensitization.

7. It would appear that the methyl, ethyl, and propyl esters of *p*-hydroxybenzoic acid may be used safely as food and drug preservatives in concentrations which have been recommended.

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(3) Jones, P. S., Thuppen, D., Morrison, J. L., and Richardson, A. P., THIS JOURNAL, 45, 268(1956).
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CHROM. 4219

CHROMATOGRAPHY OF FOOD PRESERVATIVES ON POLYAMIDE LAYERS AND COLUMNS

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SUMMARY

The separation of eight kinds of food preservative by one- and two-dimensional chromatography on polyamide layers was investigated. A comparison was made between the detection limits on polyamide and silica gel layers, and the superiority of the polyamide thin layers was confirmed.

The separation of the food preservatives on a polyamide column with two types of elution system was established. The recovery of these compounds was satisfactory.

INTRODUCTION

After establishing the efficacy of polyamide in chromatography[1-7], it has been applied extensively to column and thin-layer chromatography where it was found that the separation was very much influenced by the hydrogen bond formed between the HO-group in phenolic compounds and the -CONH-group in polyamide[1,2,8].

This paper deals with the separation of food preservatives (aromatic acids with and without a phenolic HO-group and its alkyl esters, and some other conventional preservatives) by column and thin-layer chromatography using polyamide as the chromatographic medium.

EXPERIMENTAL

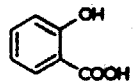
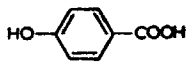
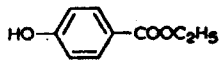
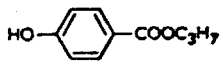
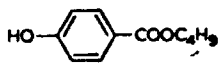
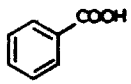
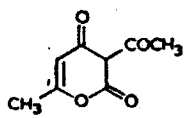
Materials

Polyamide powder from two sources, Polyamide Woelm TLC and Wakō Polyamide B-10* were used for thin-layer chromatography while for the column chromatography only Polyamide Woelm was used.

* "Wakō Polyamide B-10" contains 10% anhydrous calcium sulfate as a binder (Wakō Pure Chemical Industries, Ltd., Nihonbashi-honcho, Chuō-ku, Tokyo, Japan).

TABLE I

WAVE LENGTHS AND MOLAR ABSORBANCY INDICES OF FOOD PRESERVATIVES AT THEIR UV ABSORPTION MAXIMA

Sample		*λmax (mμ) and ε × 10⁻³*					
		In water		*In 1% (w/w) NH_3*		*In H_2O–AcOH–MeOH (5.5:0.5:4, v/v)*	
Salicylic acid		232 298	6.66 3.37			305[a]	3.60[a]
p-Hydroxybenzoic acid		251	11.8	280	17.5	256	15.0
Ethyl *p*-hydroxybenzoate		256	15.0	297	23.4	256	15.9
n-Propyl *p*-hydroxybenzoate		256	15.0	297	24.1	256	15.9
n-Butyl *p*-hydroxybenzoate		256	15.5	297	24.2	256	15.9
Benzoic acid		227.5	9.18			—[b]	—[b]
Sorbic acid	$CH_3—CH=CH·CH=CH·COOH$	260	23.6			262	29.3
Dehydroacetic acid		227.5 306	14.5 12.1			308	14.1

[a] λ max and its molar absorbancy of salicylic acid were measured in H_2O–AcOH–MeOH (3:1:6).
[b] λ max and its molar absorbancy of benzoic acid could not be measured in this medium.

The food preservatives used in this experiment are of a specially refined grade. The chemical and optical characteristics of the preservatives are as listed in Table I.

Preparation of the polyamide chromatoplates

(a) One gram of Polyamide Woelm TLC was mixed with 12 ml of ethanol to give a homogeneous suspension, which was spread evenly with a suitable applicator on a glass plate (20 × 20 cm) to a thickness of 0.25 mm. The plate was dried in air at room temperature and stored in a desiccator.

(b) Fourteen grams of Wakō Polyamide B-10 were made into a homogeneous suspension by mixing it with 56 ml of distilled water. The chromatoplate was prepared in the same way as (a).

Solvent systems for thin-layer chromatography

The solvent systems (A–F) used in the experiments are listed in Table II.

Application of samples and development of chromatoplates

For one-dimensional chromatography, 0.5 μl of an 0.2 % (w/v) ethanolic solution of each preservative was applied except for dehydroacetic acid and benzoic acid where 1.5 μl of a sample of the same concentration was used. Sample application was made on a line 3 cm from the lower edge, and the sample was developed by the ascending method.

In order to ensure the equilibration of the vapor in the chamber, the inside was lined with filter paper soaked in the solvent.

The development was continued until the solvent reached 12 cm from the point of sample application.

For two-dimensional chromatography, 1 μl of an 0.2 % (w/v) ethanolic solution of the samples was applied; as above increased amounts (2 μl) of dehydroacetic acid and benzoic acid were applied. Samples were spotted at a point 3 cm from both edges.

Detection of the spots on the chromatoplates

The spots can be seen under UV light as dark areas against a bright background, but for confirmation's sake *p*-hydroxybenzoic acid, its esters, and salicylic acid were detected as reddish violet spots by spraying with diazotized sulfanilic acid reagent; and dehydroacetic acid, sorbic acid and benzoic acid gave reddish spots against a yellow background with Methyl Red reagent.

The composition of the reagents used are as follows:

Diazotized sulfanilic acid. One gram of sulfanilic acid, dissolved in 8 ml of conc. HCl and 100 ml of distilled water, is added to the same volume of 0.09 % $NaNO_2$ solution.

Methyl Red. A mixture of 10 ml of 0.1 % ethanolic solution of Methyl Red and 20 ml of 0.1 *M* phosphate buffer (pH 7.0).

Preparation of the polyamide column and application of samples

Forty-five grams of Polyamide Woelm were washed with distilled water, methanol and chloroform, successively. After drying in air, the washed polyamide powder was suspended in the same solvent as that used for elution, and the powder was packed into a glass column (1.7 × 50 cm) to form a column bed of 45 cm height. The samples

TABLE II

SOLVENT SYSTEMS FOR CHROMATOGRAPHY OF FOOD PRESERVATIVES ON POLYAMIDE LAYERS

Symbol	*Components*	*Ratio (v/v)*
A	MeOH–H_2O	6:4
B	Acetone–H_2O	5:5
C	MeOH–AcOH–H_2O	5:1:4
D	MeOH–conc. NH_3–H_2O	2:1:7
E	AcOH–H_2O	3:7
F	*n*-Hexane–AcOH	8:2

TABLE III

R_F VALUES OF FOOD PRESERVATIVES ON POLYAMIDE LAYERS

(*a*) *R_F values on polyamide (Woelm)*

Sample	*Solvent system*					
	A	*B*	*C*	*D*	*E*	*F*
Salicylic acid	0.05	0.05	0.18	0.56	0.14	0.25
p-Hydroxybenzoic acid	0.13	0.18	0.30	0.96	0.16	0.04
Ethyl *p*-hydroxybenzoate	0.32	0.41	0.38	0.64	0.16	0.11
n-Propyl *p*-hydroxybenzoate	0.27	0.37	0.32	0.55	0.10	0.15
n-Butyl *p*-hydroxybenzoate	0.22	0.32	0.29	0.44	0.07	0.19
Benzoic acid	0.14	0.21	0.46	0.85	0.23	0.56
Sorbic acid	0.20	0.29	0.56	0.92	0.32	0.64
Dehydroacetic acid	0.18	0.25	0.73	0.79	0.54	0.45

(*b*) *R_F values on polyamide (Wakō B-10)*

Sample	*Solvent system*					
	A	*B*	*C*	*D*	*E*	*F*
Salicylic acid	0.12	0.16	0.25	0.75	0.29	0.40
p-Hydroxybenzoic acid	0.31	0.34	0.53	0.95	0.33	0.06
Ethyl *p*-hydroxybenzoate	0.53	0.63	0.61	0.76	0.34	0.15
n-Propyl *p*-hydroxybenzoate	0.49	0.58	0.56	0.68	0.25	0.21
n-Butyl *p*-hydroxybenzoate	0.44	0.54	0.52	0.58	0.18	0.28
Benzoic acid	0.34	0.33	0.66	0.91	0.43	0.62
Sorbic acid	0.43	0.44	0.72	0.93	0.55	0.68
Dehydroacetic acid	0.45	0.40	0.83	0.86	0.73	0.50

(each 10 μmoles) were dissolved in 5 ml of the same solvent and applied on the top of the column. The elution was performed at a flow rate of 0.22–0.25 ml per min, and each 3.5 ml fraction was collected. The concentration of the food preservatives in the fractions was determined by measuring the absorbancy at its λ max* in the medium used for elution.

* In the case of benzoic acid, the absorbancy was measured at 240 mμ, as benzoic acid has no absorption maximum in H_2O–AcOH–MeOH (5.5:0.5:4).

RESULTS AND DISCUSSION

Thin-layer chromatography

The solvent systems suitable for the separation of the food preservatives on polyamide layers are summarized in Table II. They are classified into two types, solvents A–E and solvent F, according to the polarity of the constituent solvents. Tables III a and b show the R_F values of the food preservatives in the above solvent systems.

It may generally be concluded from these tables that the Woelm Polyamide possesses stronger adsorption properties than the Wakō Polyamide B-10.

Hydrogen bonding effects can be observed from the R_F values of salicylic acid and *p*-hydroxybenzoic acid which are known to form an intra-molecular hydrogen bond and inter-molecular hydrogen bond, respectively.

Comparison of the differences in the R_F values of these compounds in any two types of solvent A–E or one of these and solvent F shows that a complete separation could be obtained by two-dimensional development in either of the following combinations:

Solvent B–Solvent F
Solvent B–Solvent E

Of all the possible combinations of the solvent systems listed in Table II, these two combinations gave the best distribution of spots on the chromatograms (Fig. 1).

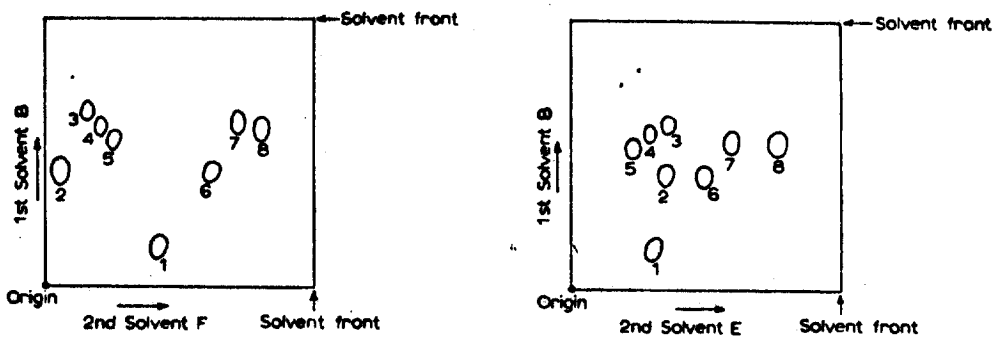


Fig. 1. Two-dimensional chromatograms of food preservatives on polyamide layers. 1 = salicylic acid; 2 = *p*-hydroxybenzoic acid; 3 = ethyl *p*-hydroxybenzoate; 4 = *n*-propyl *p*-hydroxybenzoate; 5 = *n*-butyl *p*-hydroxybenzoate; 6 = benzoic acid; 7 = sorbic acid; 8 = dehydroacetic acid.

One of the characteristics of the polyamide thin layers is to give a bright background in transmitted UV light, and this phenomenon provides a highly sensitive method of detection of UV-absorbing substances on the chromatoplate[8,10]. As shown in Table I, all the food preservatives tested absorb the UV light, thus they are all detectable as dark spots under transmitted UV light. In Table IV, a comparison is made between the detection limit of the spots on polyamide layers and on silica gel layers. It can be seen from Table IV that the detection of these substances is more sensitive on polyamide layers than on silica gel layers. With regard to other methods of detection of the spots, the Methyl Red reagent and the diazotized sulfanilic acid reagent can be used on the polyamide layers as well as on silica gel layers (see EXPERIMENTAL).

TABLE IV

DETECTION LIMITS OF FOOD PRESERVATIVES BY UV ABSORPTION ON POLYAMIDE LAYERS AND SILICA GEL LAYERS

Sample	*Polyamide in µg*	*Silica gel*[a] *in µg*
Salicylic acid	0.1	0.5
p-Hydroxybenzoic acid	0.25	5–10
Ethyl *p*-hydroxybenzoate	0.25	5–10
n-Propyl *p*-hydroxybenzoate	0.25	5–10
n-Butyl *p*-hydroxybenzoate	0.25	5–10
Benzoic acid	3[b]	50[b]
Sorbic acid	0.25	5–10
Dehydroacetic acid	2[b]	25–50[b]

[a] Fluka Silica gel (Fluka AG, Chemische Fabrik, Switzerland) was used.

[b] The low sensitivities of both these compounds are due to the large discrepancy between their absorption maxima and the wave length (2537 Å) of the UV light (see Table I).

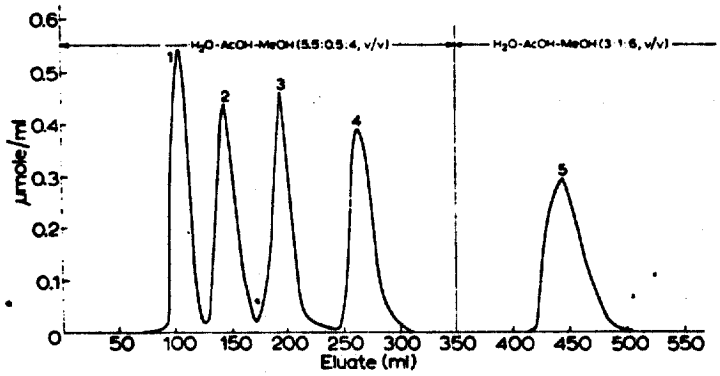


Fig. 2. Separation of food preservatives by polyamide column chromatography. Samples: 1 = dehydroacetic acid; 2 = sorbic acid; 3 = benzoic acid; 4 = *p*-hydroxybenzoic acid; 5 = salicylic acid; each 10 µmoles applied. Column: Polyamide Woelm for column use; 1.7 × 45 cm.

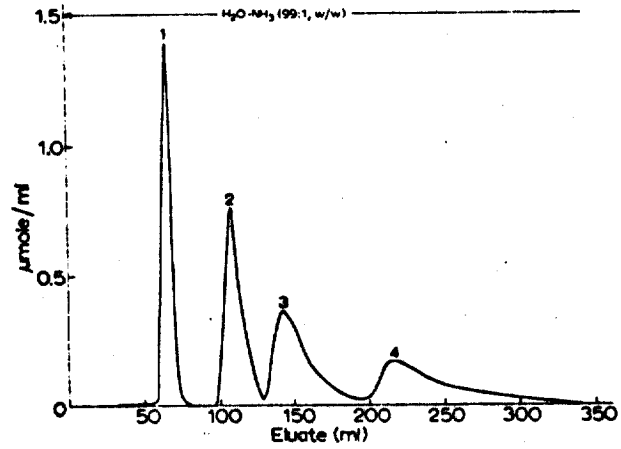


Fig. 3. Separation of food preservatives by polyamide column chromatography. Samples: 1 = *p*-hydroxybenzoic acid; 2 = ethyl *p*-hydroxybenzoate; 3 = *n*-propyl *p*-hydroxybenzoate; 4 = *n*-butyl *p*-hydroxybenzoate; each 10 µmoles applied. Column: Polyamide Woelm for column use; 1.7 × 45 cm.

Column chromatography

Suitable conditions for the separation of the food preservatives on column chromatography have been established as shown in Figs. 2 and 3. A mixture of salicylic acid, *p*-hydroxybenzoic acid, benzoic acid, sorbic acid and dehydroacetic acid was separated on a polyamide column by stepwise elution using two elution systems: (1) H_2O–AcOH–MeOH (5.5:0.5:4) and (2) H_2O–AcOH–MeOH (3:1:6) successively (Fig. 2). The recovery of the samples in this separation system was satisfactory, as is shown: dehydroacetic acid 95.5 %, sorbic acid 96.5 %, benzoic acid 101.2 %, *p*-hydroxybenzoic acid 100.1 %, salicylic acid 103.2 %. An elution procedure using water containing NH_3 (1 %, w/w) was selected for the separation of a mixture of *p*-hydroxybenzoic acid and its alkyl esters. Separation was attained as shown in Fig. 3 and recovery was also good: *p*-hydroxybenzoic acid 92.0 %, ethyl *p*-hydroxybenzoate 96.0 %, *n*-propyl *p*-hydroxybenzoate 98.3 %, *n*-butyl *p*-hydroxybenzoate 91.0 %.

Because neither of the elution media above used has any absorption in the UV, the concentration of each preservative in the fractions was directly determined by measuring the absorbance at the λ max (see Table I).

CONCLUSION

Eight kinds of food preservative, which include five phenolic hydroxyl compounds, one enolic hydroxyl compound and two carboxylic compounds, were separated on polyamide layers. From the correlation of the mobility of the samples and the solvents used, it was noticed that some hydrogen bonding forces have a definite effect on the interaction between samples having phenolic hydroxyl groups and the polyamide molecule, and that the polarity of the solvents exerts a considerable influence on the hydrogen bonding. The detection of the spots on the polyamide layers by the UV absorption method is 5–20 times more sensitive than for those on silica gel layers. This confirms that polyamide layer chromatography is suitable for the micro-analysis of food preservatives.

The conditions for the separation of these substances on a polyamide column were established. The simultaneous separation of all the samples was not possible, but by combining two types of elution system, *viz.*, a H_2O–AcOH–MeOH system and a H_2O–NH_3 system, good separations in two groups of samples were attained. The recovery of the samples separated on the polyamide column was also satisfactory.

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Action of Methyl Hydroxybenzoate on Nervous Conduction

METHYL hydroxybenzoate and its sodium derivative are recommended by the B.P.C. and by Martindale[1] as preservatives for creams, emulsions, lotions and ophthalmic solutions, and they are used to preserve local anaesthetics. During an investigation of the action of phenol on nerve fibres[2,3], we examined the action of methyl hydroxybenzoate and its sodium derivative on conduction in fibres of the spinal roots of the cat, and also on the vagus and sympathetic nerves.

These substances, dissolved in Krebs's solution to form 0·1 per cent and 0·2 per cent solution, when applied directly to the spinal roots or to the vagus and sympathetic bundle in the neck, block conduction in nerve fibres of all sizes, myelinated and unmyelinated. The effect was typical of a local anaesthetic in that small fibres were blocked before large and that the conduction block was reversible. The blocking effect of a 0·1 per cent solution of methyl hydroxybenzoate was found to be similar to that of a 0·05 per cent solution of procaine.

The substances are recommended as preservatives as, according to Martindale, they are "inert and non-toxic". The results mentioned above were obtained with the weakest solutions recommended by the B.P.C., the concentrations advocated being 0·1 and 0·2 per cent methyl hydroxybenzoate and 0·1–0·3 per cent of the sodium derivative.

The fact that in acute experiments conduction returned in fibres which had been blocked, following removal of the substance and washing the root or nerve bundle, cannot be taken to mean that no subsequent degeneration of the affected nerve fibres takes place. Exactly this situation occurs with phenol in non-aqueous solution. Although electrophysiological techniques have shown recovery of conduction in fibres which have been blocked by phenol, there is histological evidence of subsequent massive degeneration among these fibres[4]. If methyl hydroxybenzoate causes similar effects, it is possible that the degeneration of a proportion of nerve fibres following the injection of local anaesthetics in painful states may account for the prolonged or permanent relief of the pain that sometimes occurs. Whether this is the explanation of the prolonged therapeutic effect of local anaesthetics or not, it remains important to note that such recommended preservatives are not inert and that their presence should be taken into account both in experimental and in therapeutic situations.

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Queen Square, London.

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Studies on Protein Binding I

Interaction of *para*-Hydroxybenzoic Acid Esters with Bovine Serum Albumin

By NAGIN K. PATEL, PAI-CHANG SHEEN, and KATHLEEN E. TAYLOR*

The binding of methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid (parabens) by bovine serum albumin (BSA) was investigated by an equilibrium dialysis procedure. All the compounds were found to interact with BSA. The association constants were ascertained from the dialysis data, and free energy change, ΔF°, enthalpy change, ΔH°, and entropy change, ΔS°, of the interaction were computed from the data at two temperatures. The possible mechanism of the interaction is discussed. The spectrophotometric study of the interaction revealed hypochromic effects in the absorption spectra of all the parabens in the presence of BSA. The *in vitro* antifungal activity of methyl- and propylparaben was found to be related to the concentration of free or unbound drug.

INTERACTION OF NUMEROUS small molecules with serum proteins has been extensively reviewed by Goldstein and most of the drugs studied have been found to bind primarily to the albumin fraction of plasma proteins (1). Although a majority of the earlier reports were of a qualitative nature, attempts have been made to quantify the data with an ultimate aim of elucidating the nature of equilibria and forces involved in the drug-macromolecular interaction (2, 3). With the advancement of the theoretical knowledge of these interactions, considerable research has been conducted on the binding of salicylates (4, 5), sulfonamides (6-8), penicillins (9), xanthines (10), steroids (11), and other therapeutic agents with serum albumins.

In their extensive reviews Goldstein (1) and Brodie and Hogben (12) have discussed the importance of drug protein association in the transport, distribution, and elimination of medicinal agents. In addition, it is generally recognized that the specificity of pharmacologic agents lies in the structure of complexes which they form with macromolecular systems. The investigation of the factors involved in such interactions may shed some light on the understanding of drug action.

Interactions of some benzoic acid derivatives (5) with serum albumins have been investigated. The degree of ionization and the relative size of the anion of the interacting molecule influenced the protein binding, and Boyer *et al.* (13) concluded that an anion with a fairly large nonpolar group imparted an increase in its binding capacity for BSA. Both textbook and reference works (14-16) indicate a significant loss of biological activity of some antifungal agents in the presence of protein materials and Alto *et al.* observed that the antimicrobial activity of *p*-hydroxybenzoates was diminished in the presence of protein (17). No reports could be found of experimentation on the direct correlation of binding data with the *in vitro* antimicrobial activity of *p*-hydroxybenzoates. The purpose of this work was to evaluate quantitatively the binding of a series of *p*-hydroxybenzoic acid esters by bovine serum albumin (BSA) and to show that the *in vitro* antifungal activity of methyl- and propylparaben was primarily a function of free or unbound drug.

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* Recipient of the E. L. Woods Memorial Competition Award.

EXPERIMENTAL

Materials.—Bovine serum albumin, fraction V (BSA) purchased from the Armour Pharmaceutical Company (lots B24011 and D25001), was used throughout this study. The albumin[1] content of lots B24011 and D25001 were 97 and 95%, respectively. Both lots of albumin exhibited similar binding tendencies for methyl *p*-hydroxybenzoate. The moisture content of protein was determined and appropriate corrections were made in weights; a molecular weight of 69,000 was assumed. Reagent grade monobasic potassium phosphate and dibasic potassium phosphate were employed to prepare the buffer solutions. Reagent grade potassium chloride was used to obtain an equilibrium ionic strength of 0.15 where necessary. The *p*-hydroxy-

[1] The analytical results were kindly supplied by Dr. Douglas of the Armour Pharmaceutical Company, Kankakee, Ill.

benzoic acid esters (parabens) and the synthetic culture medium were the same as described in a previous paper (18). All paraben and protein solutions were prepared immediately prior to use.

Dialysis Studies The general approach, technique, and treatment of data of the study have been described previously (19, 20). Dialysis cells were the same as those employed by Patel and Foss (20) with the exception that seamless cellulose membranes (Fisher Scientific Company) were employed in the present work. The membranes were placed in distilled water and heated to 90° for 1 hr., and they were washed with distilled water several times in order to remove any contaminating substances in the cellophane (7). The membranes were permeable to hydroxybenzoates and impermeable to BSA. Results of an extensively dialyzed sample of the BSA yielded results similar to those from an undialyzed sample of commercial material. Thus undialyzed BSA was used in the entire investigation. Binding of parabens by the membranes and the dialysis cell was found to be insignificant. Equilibrium was established at the end of 16 hr. by agitation on a rotational shaker in an Aminco constant-temperature laboratory bath (American Instrument Co. Inc.).

The dialysis studies were performed on 0.1 m*M* BSA, with the exception that in the case of propylparaben BSA interaction, an additional 0.25 m*M* BSA was included. All parabens were studied at pH 7.4 and their initial concentrations ranged from 5.15×10^{-4} *M* to 1.32×10^{-3} *M*. Binding data were also collected for the methyl- and propylparaben BSA systems at pH 6.6 for the purpose of approximating the pH of the synthetic culture medium (18).

Spectrophotometric Studies Spectrophotometric examination of paraben solutions in the absence and presence of BSA was made at room temperature on a Hilger Ultrascan recording spectrophotometer, model H999. The concentration of paraben used was 6.4×10^{-5} *M*. Any interference due to the protein was eliminated by using the appropriate concentration in the reference cell.

Analysis After Equilibration At the end of equilibrium time 1- or 2-ml. aliquots were removed from both sides of the membrane and were diluted with 0.1 *N* HCl to suppress the dissociation of parabens. The concentrations of parabens were determined using a Beckman DU spectrophotometer at the wavelength of their maximum absorbance, 256 mμ. Any interference due to the protein was eliminated by using the appropriate concentration in the reference cell and it was found that at very high dilutions employed for the analysis of parabens, there was no effect on the absorbance of parabens in the presence of BSA. Any absorption contribution by the dialyzable components in BSA was assumed to be negligible in view of the high dilutions employed in the analysis. The pH values of the solutions were recorded with a Beckman aerometric pH meter, model 1 76, after each dialysis experiment, with no appreciable change noted.

Microbiological Studies Observations were made on the influence of BSA on the antifungal activity of methyl- and propylparaben for *Aspergillus niger*.[2] The culture medium and methods as described previously (18, 21) were employed with the following exception in sterilization procedure. In order to avoid denaturation of protein at the high autoclave temperature, the solutions were sterilized by Millipore filtration using a hypodermic syringe, Swinney adapter, and Millipore filter.[3] Inhibitory concentrations for methyl- and propylparaben, in the presence and absence of BSA, were obtained through visual observation of samples each day for a period of 2 weeks.

RESULTS

Treatment of Data The mathematical analysis of binding data from equilibrium dialysis studies has been described and discussed by Klotz (2, 22) and Scatchard (23). If the intrinsic association constants for all polymer sites are identical, the data can be plotted according to the equation of Scatchard (23),

$$\frac{r}{(A)} = nk - rk$$

where r = moles of bound drug/mole of protein, (A) = free drug concentration, n = number of binding sites available on each protein molecule, and k = association constant. Plotting $r/(A)$ *versus* r will give a straight line with slope = $-k$, and ordinate and abscissa intercepts of nk and n, respectively. By the method of least squares the quantities nk and k were calculated using an IBM 360/67 computer. From the value of k, the thermodynamic functions were calculated according to the following equations (2, 24):

$$\Delta F^0 = -RT \ln k$$

where ΔF^0 = the free energy change, R = gas constant, and T = absolute temperature.

$$\ln \frac{k_2}{k_1} = \frac{\Delta H^0}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

where ΔH^0 = the enthalpy change and k_1 and k_2 are binding constants at temperatures T_1 and T_2.

$$\Delta F^0 = \Delta H^0 - T\Delta S^0$$

where ΔS^0 = the entropy change.

Dialytic Data of the Paraben-Protein Interaction—The data for the interaction of methyl-, ethyl-, propyl- and butylparaben at pH 7.4 and at two temperatures are plotted in Figs. 1-4. Within the concentration ranges of the drugs and the protein, a linear relationship of $r/(A)$ *versus* r, as described by Scatchard's equation, was noted. The k and n values were obtained by linear extrapolation to the ordinate and abscissa axes.

Absorption Spectra in the Presence and Absence of Protein The absorption spectra of certain molecules are altered in the presence of proteins and this spectral change is indicative of an interaction between two compounds. This change usually consists of a decrease in the absorption coefficient. The pronounced hypochromic effects of methyl-, ethyl-, propyl-, and butylparaben in the presence of increasing concentrations of BSA are depicted in Figs. 5-8. The method has been employed by var-

[2] AMH No. 1156, kindly supplied by Miss Marjorie Dinsen of the Provincial Laboratory of Public Health, Edmonton Canada.

[3] Mixed esters of cellulose, marketed as MF-Millipore, Type HA. Millipore Ltd., Montreal, Canada.

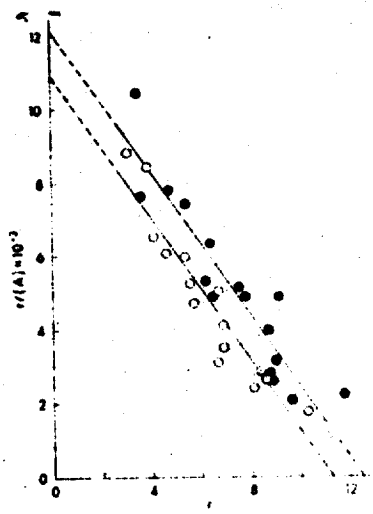

Fig. 1—Binding of methylparaben by BSA, pH 7.4. Key: ○, 30°; ●, 20°.

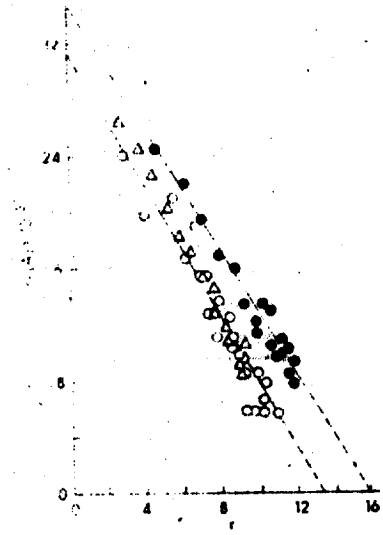

Fig. 3—Binding of propylparaben by BSA, pH 7.4. Key: ○, 30°; ●, 20°; Δ, data collected employing 0.25 mM BSA, pH 7.4, at 30°; all other data of Figs. 1–4 were obtained using 0.1 mM BSA.

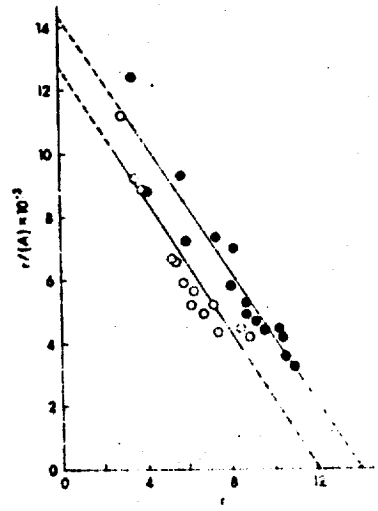

Fig. 2—Binding of ethylparaben by BSA, pH 7.4. Key: ○, 30°; ●, 20°.

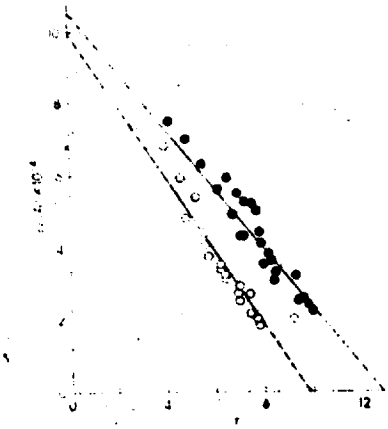

Fig. 4—Binding of butylparaben by BSA, pH 7.4. Key: ○, 30°; ●, 20°.

ious investigators (25–27) to study the binding of some drugs by proteins.

Inactivation by BSA Related to Binding. It was shown in earlier studies that the ratio, R, of total to free preservative for a given concentration of polysorbate 80 and cetomacrogol 1,000 was fairly constant (18–21). If the antifungal activity of parabens can be assumed to be due to the free or unbound form, the R value may be used to predict the minimum inhibitory concentration of antifungal agent in the presence of a definite quantity of BSA by a procedure described previously (18, 21). It is recognized that in the case of BSA the R value would not be constant over a wide range of concentrations of the parabens. However, within a narrow range of concentrations, especially around the minimum inhibitory concentration of the drug, it can be assumed to be reasonably constant as outlined below. The values as obtained employing 0.1 m*M* BSA tion, at pH 6.6 and 30°, were 1.19 (range 1.16 with standard deviation of 0.026) and 1.75 (range 1.60–1.95 with standard deviation of 0.12) for methyl- and propylparaben, respectively. The total paraben concentrations on the protein side at the end of the dialysis experiment were 0.062–0.110% and 0.030–0.05%, in the cases of methyl- and propylparaben, respectively. The results of the *in vitro* correlation of binding data with the antifungal activity of methyl- and propylparaben for *Aspergillus niger* in the presence of 0.1 m*M* BSA are summarized in Table I. It is evident from these data that there is a good correlation between the predicted minimum inhibitory concentration and the experimental antifungal concentration for both the parabens.

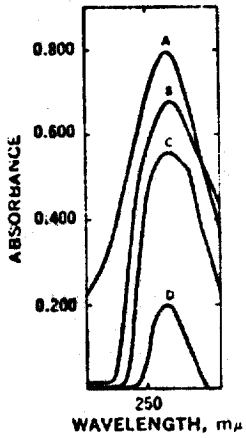

Fig. 5—Absorption spectra of methylparaben at pH 7.4. Key: A, buffer; B, 0.025 mM BSA; C, 0.050 mM BSA; D, 0.10 mM BSA.

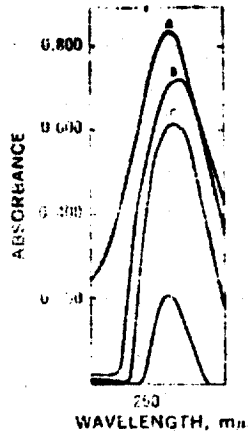

Fig. 7—Absorption spectra of propylparaben at pH 7.4. Key: A, buffer; B, 0.025 mM BSA; C, 0.050 mM BSA; D, 0.10 mM BSA.

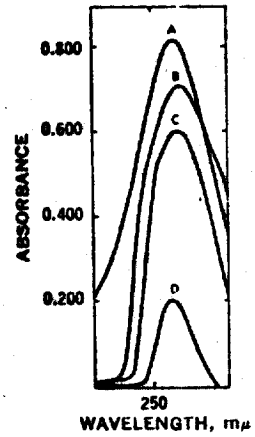

Fig. 6—Absorption spectra of ethylparaben at pH 7.4. Key: A, buffer; B, 0.025 mM BSA; C, 0.050 mM BSA; D, 0.10 mM BSA.

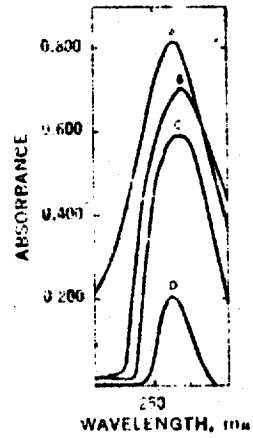

Fig. 8—Absorption spectra of butylparaben at pH 7.4. Key: A, buffer; B, 0.025 mM BSA; C, 0.050 mM BSA; D, 0.10 mM BSA.

DISCUSSION

The ability of *p*-hydroxybenzoic acid esters to interact with several macromolecules has been well documented (18, 19, 28, 29). Based upon this observation, it was expected that BSA might provide binding sites for the parabens. From Figs. 1–4, it is evident that all the parabens interacted with BSA. The values for the association constants, *k*, and number of binding sites, *n*, together with the thermodynamic parameters of the interaction, are summarized in Table II. From this table it is noted that the values of *n* are not integers. This might be either due to the experimental error in dialytic studies as explained by Klotz (30) or due to the view that protein might not be molecularly homogeneous with respect to binding properties as theorized by Karush (31).

The exact mechanism of binding of *p*-hydroxybenzoates by BSA cannot be elucidated from this study, since the results were obtained primarily from the binding of methyl-, ethyl-, propyl-, and butylparaben. However, certain tentative conclusions may be drawn. A comparison of association constants for various parabens at a definite temperature (Table II) reveals that the binding increases from methyl- to ethyl- to propyl- to butylparaben. Due to the limited solubility of hexyl- and heptylparaben it was not possible to evaluate the association constants for these compounds by dialysis studies. An increase in the solubility of these compounds in aqueous BSA solution at pH 7.4 over their water solubility at the same pH indicated that hexyl- and heptylparaben would be bound to a greater extent than butylparaben (32). About 10% of the total amino acids in the protein are nonpolar residues (33) and it is quite possible that the association between these residues and the nonpolar moieties of the paraben molecule (aromatic ring and hydrophobic chain of ester) may contribute to the overall interaction. An examination of the data of Table II shows that the magnitude of binding increased with an increase in temperature, demonstrating that the binding process was endothermic (unfavorable positive enthalpy). More significantly, the binding was accompanied by an increase in entropy term which is typically a thermodynamic behavior in common

TABLE I—CORRELATION OF PROTEIN BINDING DATA WITH *In Vitro* ANTIFUNGAL ACTIVITY OF PARABENS, OBSERVED FOR TWO WEEKS

Concn., BSA	Minimum Inhibitory Concn. Methylparaben Pred.[a]	Expt.[b]	Propylparaben, g./100 ml Pred.	Expt.
0		0.060		0.020
0.1 m*M*	0.071	0.074	0.035	0.035

[a] Predicted from the binding data. [b] Experimental values.

TABLE II—ASSOCIATION CONSTANTS AND THERMODYNAMIC PARAMETERS OF PARABEN-PROTEIN INTERACTION IN AQUEOUS SOLUTION AT pH 7.4

Paraben	Temp.	Binding Constant (k), L./mole × 10^{-3}	n × 10^{-2}	No. of Sites, n	Free Energy Change (ΔF°), kcal./mole	Enthalpy Change (ΔH°), kcal./mole	Entropy Change, ΔS
Methyl-	30°	0.987	10.9	11.2	−4.15	0.0845	14
	10°	0.968	12.1	12.5	−3.8[illegible]		14
Ethyl-	30°	1.05	12.8	12.2	−4.19	0.529	15
	20°	1.02	11.1	11.1	−4.04		15
Propyl-	30°	2.33	31.3	13.4	−4.67	0.876	18
	20°	2.22	34.9	15.7	−4.49		18
Butyl-	30°	10.1	99.4	9.9	−5.36	4.00	31
	20°	8.11	105	12.9	−5.24		31

with that described by Schachman (34) for hydrophobic bonding. The positive entropy change for this type of interaction has been ascribed by Kauzman (35) and others (36, 37) as resulting from the energy change accompanying the disruption of the "iceberg" structure of water molecules around hydrocarbon groups in aqueous solution (hydrophobic bonding). The large difference in the values of ΔH_0 and ΔS_0 of propyl- and butylparaben was probably due in part to the experimental error which resulted from a significant scatter in the experimental points in the case of butylparaben (Fig. 4), especially at 30°.

In Figs. 5–8, the degree of hypochromism increases with an increase in concentration of protein. The decrease in the absorption has been interpreted by Klotz (25) as an effect due to the formation of drug-protein complex, if the complexation involves a chromophor system of drug. Although spectrophotometric study can often yield information as to the magnitude of the interaction as well as to the nature of forces responsible for the interaction, and such study is in progress, this part of the work was limited to the qualitative nature of the interaction.

The results of correlation of binding data with the inhibitory concentration of methyl- and propylparaben for *Aspergillus niger* in the presence of 0.03% BSA are in good agreement. This part of the study shows that the protein-bound paraben is devoid of *in vitro* antifungal activity.

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Keyphrases

Protein binding

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Dialysis—bovine serum albumin-parabens

Parabens, antimicrobial activity—binding effect

IR spectrophotometry—analysis

J. Chromatog., 50 (1970) 436–441

CHROM. 4777

IDENTIFICATION AND QUANTITATIVE ANALYSIS OF *p*-HYDROXYBENZOIC ACID AND ITS ESTERS USING REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

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(Received March 31st, 1970)

SUMMARY

The *p*-hydroxybenzoic acid and its methyl, ethyl, propyl, butyl and benzyl esters are separated on thin layers of silanized silica gel using borate buffer (pH 11) as mobile phase, adding a given volume of organic solvent as required. The separation of the various compounds is complete and an accurate quantitative determination of the *p*-hydroxybenzoic acid and its esters is possible using a simple technique of elution and spectrophotometric determination.

INTRODUCTION

The increasing use of *p*-hydroxybenzoic acid esters as food and drug preservatives has led to the development of many analytical techniques for identifying and assaying these compounds. In recent years, thin-layer chromatography (TLC) in particular has often been used with good results[1–20].

In this report we shall briefly describe a reversed-phase TLC method based on the use of silanized silica gel as stationary phase. Coupled with a simple elution and spectrophotometric determination technique, it has proved highly effective in separating and determining *p*-hydroxybenzoic acid and its principal esters.

EXPERIMENTAL

Preparation of the chromatographic plates

About 30 g of HF_{254} (Merck) silanized silica gel were suspended in 60 ml of a (2 : 1) water–methanol mixture by shaking vigorously for a few minutes in order to obtain a homogeneous suspension. The latter was then distributed on the plates using a Desaga spreader so as to obtain a uniform layer of 0.25 mm thickness. The plates were dried in air for about 3 h and stored over silica gel in a desiccator. They should not be used for at least 24 h after preparation.

Preparation of the chromatography solutions

Methanol solutions of pure commercial products (Merck, BDH, Carlo Erba)

were used as reference solutions for *p*-hydroxybenzoic acid and its esters. The methods described in a previous report [21] were used to extract the preservatives from pharmaceutical specialities and to prepare the sample solutions.

Application of the solutions to the plates

To identify the substances, the sample and reference solutions were applied as spots in parallel on the same plate using a micropipette and taking care to pipette no more than 5 μl each time, and to deposit from 2 to 10 μg of each preservative on the plate. For the quantitative determination, the solutions to be analyzed were applied in bands on 5 × 20 cm plates, pipetting from 0.025 to 0.1 ml of solution so that from 20 to 100 μg of each substance are applied to each plate. The solutions were applied 2 cm from the starting edge leaving a margin of approx. 0.5 cm on both sides of the plate.

Chromatographic development

Chromatographic development was carried out using borate buffer, pH 11 (502 ml of 0.2 *M* H_3BO_3 solution diluted to 1000 ml with 0.2 *M* NaOH solution) as mobile phase, adding a given volume of organic solvent as required. The following buffer–solvent combinations have proved most suitable for separation of the various preservatives: buffer–methyl alcohol (90 : 10), buffer–ethylcellosolve (90 : 10), buffer–ethyl acetate (saturated solution at 25°), buffer–ethyl ether (saturated solution at 15°), buffer–tetrahydrofuran (95 : 5), buffer–dioxane (90 : 10).

The plates are developed in glass chromatography chambers lined with filter paper. Before putting the plates in them, the chromatographic chambers were saturated with the solvent vapours for about 1 h. The plates are allowed to develop until the solvent front has risen about 14 cm from the point where the substance was deposited (90 to 120 min). When double development is desired, the plate is dried at room temperature for about 1 h and then returned to the chromatographic chamber. Chromatographic development is performed at about 25° except when the mixture contains ethyl ether in which case the temperature of the chromatographic chamber should be 15°.

Detection of the compounds

After the plates have been developed they are dried at room temperature. The spots or bands of the various compounds were visualized under a 250-mμ UV light source.

Quantitative analysis

After detection, the band of each substance is isolated by cutting away the sides of the layer of adsorbent with a spatula, and then performing the quantitative determination by removing the silica gel and extracting it with methyl alcohol. A silica gel collection method using a vacuum described by several authors[22–25] has proved useful for this purpose. We have adopted the simple device shown in Fig. 1 which permits the easy collection of the silica gel and quick elution of the substances under vacuum. The device consists basically of a small glass funnel inserted, with a special plastic (polyvinylchloride) fitting, into a Pyrex glass volumetric flask (Fig. 1A). Near the lower end of the funnel stem is a perforated sintered glass filter plate with

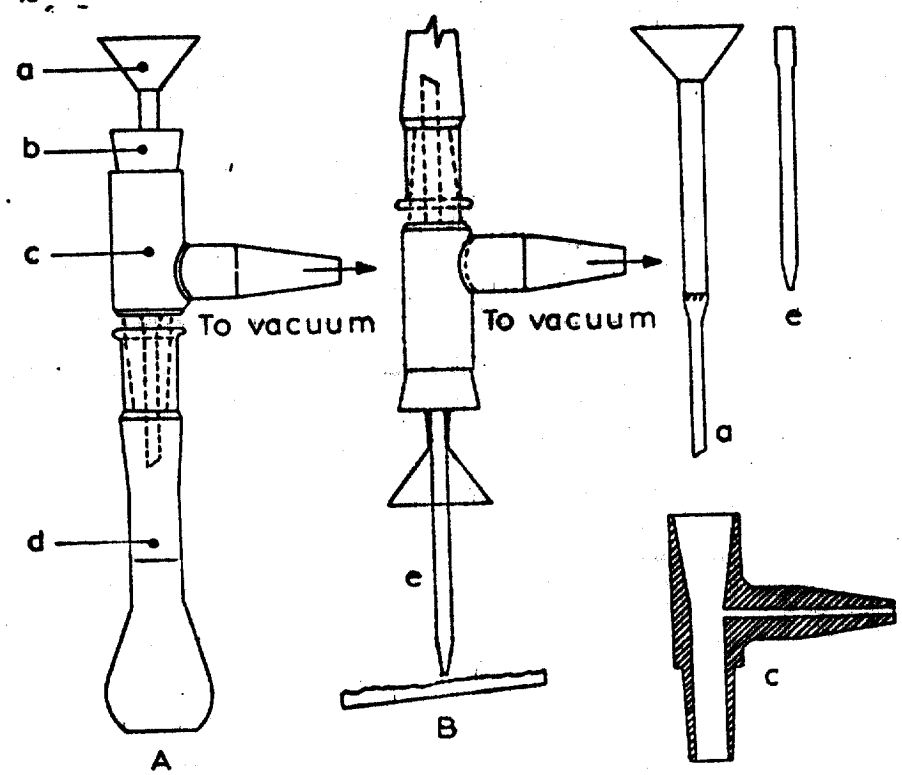

Fig. 1. (1:2 scale). A = system for elution of substances; B = system for suction of silica gel. a = Pyrex glass funnel with perforated sintered glass filter plate, b = rubber stopper, c = polyvinylchloride fitting with side inlet for vacuum, d = volumetric flask, e = glass tube with tygon ring.

gauze or fiber glass pressed onto it; the system can be connected to the vacuum pump by means of the side attachment on the plastic fitting. The silica gel is collected by inserting a small glass tube with a pointed end in the funnel using a tygon ring. The device is turned upside down, and the silica gel is sucked in by applying a low vacuum (Fig. 1B). When the silica gel has been collected, a few tenths of ml of methyl alcohol are sucked through to wash the inlet tube (taking care that no solvent emerges from the bottom end of the funnel stem), and the device is quickly uprighted. The vacuum is broken, the inlet tube is removed, 1 or 2 ml of methyl alcohol are pipetted into the funnel, the vacuum is again applied, and the solvent is collected in the volumetric flask. Methyl alcohol is added a couple more times until elution of the substance to be determined is complete (generally after 4–5 ml of methyl alcohol have passed through the funnel, elution is complete).

The eluent in the volumetric flask is diluted to volume with methyl alcohol and divided into two portions. One was acidified and the other was made alkaline by adding 2% of 1 *N* HCl and 1 *N* NaOH, respectively. Light absorption is measured at 297 mμ using the alkaline solution as test solution and the acidified solution as blank following the differential spectrophotometric method described in our previous report[21].

TABLE I

VALUES OBTAINED ON HF_{254} SILANIZED SILICA GEL FOR THE VARIOUS COMPOUNDS STUDIED

Compound	R_F values[a]					
	Mixture A pH 11 buffer–methyl alcohol (90:10)	*Mixture B pH 11 buffer–ethyl cellosolve (90:10)*	*Mixture C pH 11 buffer saturated with ethyl acetate at 25°*	*Mixture D pH 11 buffer saturated with ethyl ether at 15°*	*Mixture E pH 11 buffer–tetrahydrofuran (95:5)*	*Mixture F pH 11 buffer–dioxane (90:10)*
Benzyl-*p*-hydroxybenzoate	0.04	0.08	0.03	0.02	0.03	0.08
Butyl-*p*-hydroxybenzoate	0.06	0.11	0.06	0.05	0.08	0.13
Propyl-*p*-hydroxybenzoate	0.15	0.22	0.14	0.15	0.14	0.20
Ethyl-*p*-hydroxybenzoate	0.23	0.32	0.25	0.25	0.24	0.28
Methyl-*p*-hydroxybenzoate	0.30	0.38	0.34	0.35	0.33	0.35
p-Hydroxybenzoic acid	0.60	0.70	0.69	0.75	0.57	0.59

[a] Each R_F is the mean value of six separate chromatographic runs.

RESULTS AND DISCUSSION

Table I shows the R_F values obtained on developing the chromatograms with the various buffer–solvent mixtures.

Of the mobile phases studied, mixtures C, D, E, and F gave the best separation of the various compounds, especially of benzyl- and butyl-*p*-hydroxybenzoates. However, mixtures C and D are rather difficult to use because the solvents are volatile and the solutions are saturated; good chromatographic development and reproducibility of data are closely controlled by temperature stability in the chromatographic chamber. Mixtures E and F are more practical, and we normally use them for the analysis of preservatives in pharmaceutical preparations.

In quantitative zone chromatography, the separation may not be good enough

TABLE II

R_F' VALUES CALCULATED AFTER SECOND DEVELOPMENT ON HF_{254} SILANIZED SILICA GEL

Compound	R_F' value[a]	
	Mixture E pH 11 buffer–tetrahydrofuran (95:5)	*Mixture F pH 11 buffer dioxane (90:10)*
Benzyl-*p*-hydroxybenzoate	0.06	0.17
Butyl-*p*-hydroxybenzoate	0.14	0.25
Propyl-*p*-hydroxybenzoate	0.24	0.38
Ethyl-*p*-hydroxybenzoate	0.36	0.49
Methyl-*p*-hydroxybenzoate	0.47	0.58
p-Hydroxybenzoic acid	0.68	0.75

[a] Each R_F' is the mean value of six separate chromatograms.

for elution and quantitative determination of the components of the mixture when the amounts of some of the compounds to be separated are much larger than others. In this case, the mobility of the substances and the chromatographic separation can be greatly enhanced by performing a second development with the same solvent mixture, especially when low R_F paraseptics are involved.

Table II shows the R_F' values obtained with mixtures E and F after a second development. In this case the R_F' values represent the proportion between the distance of the spots of substance from the point of application after the second development and the distance of the solvent front from the point of application (the solvent run was the same in the first and second development).

The quantitative analysis of the eluents by differential spectrophotometry eliminates background absorption due to the extraction of the silica gel with methanol. The amount of the test compounds may be calculated from the spectrophotometric results using a calibration straight line plotted from results of chromatographic analyses performed on solutions containing known amounts of preservatives.

Greater precision in quantitative analyses may be obtained by using the internal standard method. In this case a known amount of a compound with a different R_F from those of the test compounds is added to the test solution before chromatography (since the six preservatives discussed here are not generally used together in the same pharmaceutical speciality, one which is known to be absent in the test sample may be used as an internal standard). After chromatographic development and spectrophotometric determination of all the compounds, it is

TABLE III

QUANTITATIVE CHROMATOGRAPHIC ANALYSIS OF METHANOL SOLUTIONS CONTAINING DIFFERENT CONCENTRATIONS OF METHYL AND PROPYL-*p*-HYDROXYBENZOATE

Chromatography was performed on 5 × 20 cm H_{254} silanized silica gel plates using a pH 11 buffer–dioxane mixture (90:10) as mobile phase; 0.05 ml of sample solution was pipetted as a band on the plates. Method A = determinations performed using the calibration straight line. Method B = determinations performed by adding butyl-*p*-hydroxybenzoate as internal standard.

Solution No.	*Theoretical (mg/ml)*	*Found*[a]			
		Method A		*Method B*	
		mg/ml	*% difference*	*mg/ml*	*% difference*
(a) Methyl-p-hydroxybenzoate					
1	0.424	0.426	+0.5	0.424	0.0
2	0.824	0.807	−2.1	0.836	+1.5
3	1.227	1.196	−2.5	1.260	+2.7
4	1.595	1.552	−2.7	1.585	−0.6
5	2.000	1.902	−0.7	2.046	+2.0
(b) Propyl-p-hydroxybenzoate					
1	2.008	2.045	+1.8	2.053	+2.2
2	1.621	1.580	−2.5	1.613	−0.5
3	1.206	1.164	−3.5	1.211	+0.4
4	0.804	0.781	−2.9	0.807	+0.4
5	0.419	0.420	+0.2	0.416	−0.7

[a] Mean of three chromatographic determinations performed under the same conditions.

easy to calculate the amount of each single compound in the test solution using the formula:

$$\frac{P \cdot Ec \cdot F}{Est} = \text{mg of compound contained in the sample solution}$$

where P is the amount of internal standard added, expressed in mg; Ec and Est are the differential absorption of the eluents of the test compound and of the compound used as internal standard respectively; F is a correction factor calculated from the ratio between the differential absorptions of equal amounts of the internal standard and test compounds (experimentally; the F factor is determined by repeated quantitative chromatographic analyses of mixtures containing known amounts of the two compounds).

Tables III (a) and (b) show the results of a quantitative chromatographic determination performed on solutions with different concentrations of methyl-*p*-hydroxybenzoate and propyl-*p*-hydroxybenzoate using the calibration straight line method and the internal standard method.

The data given in Table III show that the quantitative analysis, especially using the internal standard method, is quite accurate; the internal standard method is also more precise than the calibration straight line method because the quantitative determination is not affected by pipetting errors which may occur when the solutions are applied to the chromatographic plates. A repeatibility test consisting of 15 chromatographic determinations on a methyl- and propyl-*p*-hydroxybenzoate mixture showed that the coefficient of variation for both paraseptics is about 3% for the calibrated straight line method and about 1.5% for the internal standard method.

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ON THE BEHAVIOR OF P-OXYBENZOIC ACID ESTERS IN THE HUMAN BODY

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Baumann & Herter found that after per os ingestion of p-oxybenzoic acid in dogs and rabbits, there was considerable increase of esterified sulfuric acid, but that this was not the case for man. The latter eliminated p-oxybenzoic acid in the urine partly as such partly as p-oxy"benzur" acid, and a very small fraction of the acid was split to the accompaniment of phenol generation. C. Schotten followed quantitatively the changes of the acid in the human body, and found that for ingestion of 26 gm of acid as sodium salt, that after 28 hours about 33% of the acid ingested could be recovered as such and 16% as p-oxybenzur acid, or somewhat more than 50%; Quick, for ingestions of 3.4 gm of acid as sodium salt in the following 4 hours recovered 39% of the ingested acid as such and 45% of it as p-oxybenzur acid, somewha less than 85%. In contrast to his findings in man, this author found that in dogs, p-oxybenzoic acid in the urine mainly combines with glucuronic acid and etherified glucoside-like as well as esterified, so that 1 mole of acid reacted with 2 moles of glucuronic acid.

After one of us (S) with various collaborators some 30 years ago determined the strong anti-microbic effects of the p-oxybenzoic acid alkyl esters and since thereby their use as preservatives in food and medicinal products was thus suggested, there followed immediately a deeper study of the effects of p-oxybenzoic acid esters on the animal bodies and their conversions in same. Sabalitschka & Dietrich in preliminary tests observed no adverse effects from daily intakes of 2 gm p-oxybenzoic acid esters (methyl) for one month in man. Bijlsma etal. then researched more thoroughly the effects of those esters in higher organisms and found that in dogs, cats and rabbits, there was saponification of the methyl, ethyl and propyl esters of p-oxybenzoic acid and elimination in salt form of the acid thus generated through the urine and with slight combining of same with sulfuric acid and glycocoll. Part of the acid seemed to be decomposed by the body. "hen feeding the same esters, depending on kind of ester and kind of animal, 7-49% of the ingested acid was recovered in the urine. Dropping contained neither ester nor free acid. For dogs and rabbits, toxic and lethal doses were resp. 2gm/kg and 3gm/kg for methyl ester, 4 gm/kg and 5gm/kg for ethyl ester, and 3-4 gm/kg and 6 gm/kg for propyl ester. These results were corroborated by work at the Reichsgesundheitsamt on the part of Rost. Freund administered the esters as an oil mash by means of a stomach probe in rabbits, only 10 gm/kg led to death while 9 gm/kg produced no disorders whatever. Using sc. injections of aqueous solutions of the esters' sodium compounds, Basilisco found that the lethal minimum dose was 3.5 gm/kg for methyl ester and 2.0 gm/kg for both ethyl and propyl esters, in mice. Kohn found no changes in the blood sugar level of rabbits after an iv. injection of 2-10 cc of aqueous solutions of 4 different p-oxybenzoic acid esters with a maximum possible concentrations of 0.01-0.2%. The esters also proved wholly inert with respect to circulation and breathing.

Approximately at the same these authors performed their experiments, we researched the ester behavior for ingestion over several days and per os in man. Presently we wish to report on those erstwhile researches and results in view of the recently published successes of such esters in fighting moniliases and the likes

as are increasingly noticed with respect to interference in antibiotic therapy. McVay & Sprunt first mentioned such successes and this caused the use of these esters for prophylactic purposes in antibiotic therapy in order to prevent monoliases and the likes. Siegel too proposed reacting neomycin ointment with propyl-p-oxybenzoate. Rossier & Wegmann observed massive occurrences of monilia in the sputum and stool for lung mycosis and extended mouth stomatomycosis and where antibiotics failed, then an impulse-like improvement of the clinical picture, namely critical drop in temperature, total reversal of mouth stomatomycosis, disappearance of monilia in sputum and stool within 3 days for daily administering of 0.8 gm of p-oxybenzoic acid methyl ester. Peterson states that peroral administration of p-oxybenzoic acid propyl ester will lead to healing even in tenacious cases of interstitial infant pneumonia in which use of vitamin K_1 fails if alone if in combination with this vitamin. Recently we too observed in vitro a strongly lethal effect of the esters with respect to isolated irritants in small children sick with moniliasis and the like, and more will be reported on this subject later. The evaluation of the anti- microbic effects of these esters when fighting infections in the body however is not entirely novel, even if not as well known as the fighting of skin infections. Already long ago Oppenheim reported on success with iv. injections of 2.5% solution of the sodium compound of the p-oxybenzoic acid propyl ester (Nipasol-sodium) made to react with 1% coffeine-sodiumbenzoate when treating non specific urine path infections where other means had failed.

When for 5 days daily doses of 2 gm of propyl or benzyl ester were ingested (of p-oxybenzoic acid), where the 2gm amount was divided into several doses, there was no propyl ester in the urine; there was only 13.7% of p-oxybenzoic acid and 3.7% of p-oxybenzur acid from the acid introduced as ester, i.e., a total of 17.4%; however for benzyl ester we found still 6% of ester as such and 13.6% of p-oxybenzoic acid and 3.7% as p-oxybenzur acid from the acid introduced with the ester, i.e., a total of 6% of the ester and 17.3% of the acid introduced with the ester. One must take into account the losses affecting these yields, namely the required purifications, and in fact the amounts of the substances eliminated in the urine should be higher

Because of the minor distribution coefficient lipoid/water for p-oxybenzoic acid, - Boesecken & Waterman only found 0.6 for oil/water acid pairing with glycocoll in the body may occasionally be doubted, and was doubted, we used methylisation and comparison of the substance thus obtained, with synthetically made anisuric acid, the substance that was considered p-oxybenzuric acid, further it was compared with synthetically made p-oxybenzuric acid. The substances proved identical.

To the extent the esters are saponified, the present knowledge of the facts mentioned earlier apply to the behavior of p-oxybenzoic acid in the body and as regards elimination. Because of the above indicated distribution coefficient, free p-oxybenzoic acid will be easily and readily eliminated with the urine, and it need not be transferred to an easily water-soluble compound. Smith also observed the rapid elimination and he showed that after ingestion of 0.4 gm of sodium-p-oxybenzoate per kg of body weight in rats, 100 cc of blood already after 30 minutes will hold 29 mg, and after one hour 8.1 mg, and after 2 hours zero mg of p-oxybenzoic acid.

To the extent the esters will not be saponified, their elimination in the urine will be furthered by pairing with sulfuric acid, as observed already by Kase & Seki. They ingested p-oxybenzoic acid and

methyl esters into the stomachs of rabbits and found no changes in sulfuric acid in the urine for the former, but for the latter there was increase in the esterified urine sulfuric acid. W also found a clear increase in esterified sulfuric acid in human urine following ingestion of propyl and benzyl-p-oxybenzoate. A/B according to Baumann amounted to 10.6 for propyl ester, on the average and before and after, 12.1 before and after on the average for benzyl ester, during ingestion and on the average 5.1 for propyl ester and 4.5 for benzyl ester-- thus it sank always to half or less. Without acid addition A/B for the same person was 9.6 at the ingestion of p-oxybenzoic acid and 6.9 during, - on the average -,and therefore the drop fell within normal fluctuations, hence one may not conclude there was increase in esterified sulfuric acid due to the added p-oxybenzoic acid, at least not with assurance. This agrees with the initially mentioned test results by Baumann & Herter for man.

From the average increase in esterified sulfuric acid during ingestion of p-oxybenzoic acid esters one might compute pairing of 55% of added propyl ester and of 87% of the added benzyl ester. Because of the normal daily fluctuations of eliminated paired sulfuric acid, because of the urine amounts only being estimated and because of the non-daily obtention of paired sulfuric acid, the figures so computed are not wholly valid. Probably they are too high because for the tests carried out by the other authors and by us with p-oxybenzoic acid, pairing of the free acid with sulfuric acid could neither be ascertained nor wholly excluded, and further, a very small part of the p-oxybenzoic acid also appears as phenol in the urine. But at least the major part of the increased esterified sulfuric acid might be due to pairing of sulfuric acid with the esters, so that the above figure still do reveal the elimination of a considerable part through the urine of of the added p-oxybenzoic acid esters paired with sulfuric acid. As expected and corroborated by us, the p-oxybenzoic acid esters released during saponification of sulfuric acid esters by means of hydrochloric acid, were saponified in part or intoto, examination of the dissociation products arising fr this saponification does not reveal which amounts of ester are elimina paired with sulfuric acid.

The computed higher sulfuric acid pairing of benzyl ester with respect to propyl ester cannot be explained by a lesser alkaline saponificaion of the former in the intestine. We, as did Efron, found that the rate constants for alkaline saponification are 3 times higher for benzyl ester as for propyl ester. Perhaps the stronger pairing of benzyl ester with sulfuric acid is caused by a slower enzyme or enzyme-like saponification of the ester passing from the intestine into the body or by its better suitability for pairing. That benzyl ester is not as easily saponified in the body as propyl ester is shown by the elimination of unchanged 6% benzyl ester in the urine.

We did not find at all any unchanged propyl ester that had bee orally administered, as had already been the case for Schuebel & Manger with their animal experiments. They had not fed benzyl ester. Furthermore, for both esters we found about 17.5% of the acid added as esters in the form of p-oxybenzoic acid or paired with glycocoll.

An appreciable part of the ester leaves the human body paired with sulfuric acid. For propyl ester, part of the added amount of ester or of the amount of acid from its saponification can no longer be found. Schuebel & Manger made the same observation in their tests on animals. They concluded there was decomposition of p-oxybenzoic acid in the organism that might lead to breaking the benzol ring. The feasibility discovered by Boeseken & Waterman, namely to grow penicillu glaucum with p-oxybenzoic acid as the sole nutrient, shows that organisms are capable of such decomposition.

We isolated 1.3 gm of hippuric acid for 6 days of propyl ester intake and 2.1 gm for benzyl ester intake. It is not known whether the higher hippuric acid content for benzyl ester is due to accident or determined by the benzyl residue in the ester. According to Snapper etal, increase in hippuric acid may be due the benzyl alcohol due to ester saponification and its oxidation to benzoic acid. However we paid no attention in our isolating process to fully isolating the hippuric acid, for lack of interest in the matter. In any event the values we found lie in the range of normal amounts of daily urine as listed, from 0.1 to 1 gm (according to Landois and Rosemann).

Other communications that have appeared since must be mentioned as regards the pharmacology of p-oxybenzoic acid esters. Mancini administered sodium compounds of methyl, ethyl and propyl esters intravenously to rabbits and for 0.43 g/kg, 0.48 gm;kg and 0.51 gm/kg of methyl, ethy and propyl esters resp.&found no toxic effects. Ghirardi administered 0.7 gm/kg of p-oxybenzoic acid ester daily for 90 days, i.e., a total of about 60 gm/kg without therefor observing any disorders at all in the behavior of the animals or egg white and sugar in the urine. The animals were killed at the end of the test; section showed completely macroscopic normalcy of the inner organs. Zink found that mice may tolerate 1.7 gm/kg and guinea pigs 1.2 gm/kg, iv. and im. respec., of propyl ester. When using esters for preserving medecines, Haznagg found no effect regarding oral or parenteral administration on blood sugar for 0.2 gm methyl-p-oxybenzoate , and Reissmann found no noxious effect from an addition of 0.1% methyl and propyl p-oxybenzoate in blood reserves nor,when there was transfusion, on the blood corpuscles of the recipient, and Pilich found no disorders from an addition o.4% sodium-propyl-p-oxybenzoate in digitalis infusions where he tested the effectivenss according to Focke. Guinea pigs, rats and pigons weakened by scurvy and avitaminoses according to Cremer showed n damages after lengthy feeding with the same diet that had been made to react with a mixture of methyl and propyl esters. The continuous administration of this diet also left growth and weight of normal rabbits unaffected, and similarly for rats and guinea pigs; as opposed to the sulfuric acid also used as preservative, this ester mixture did not lessen the blood's bactericidal capacity and therefore the animals' general resistance to infection, nor did it alter the blood picture, nor did it adversely affect the oxidation of the intermediate metabolism or the animals' fertility. Without any damages, Rendel was able to feed the fruit fly drosophila melanogaster with a diet of food solely preserved by means of methyl p-oxybenzoate.

Recently Sokol dealt exhaustively with the effects of alkyl-p-oxybenzoates on higher organisms and with their conversions following oral, intraperitoneal and intravenous administration. There were no effects in mice for those doses that could be given them orally at all,

for maxima of 8 gm/kg of methyl, ethyl and propyl esters and for 5 gm/kg of butyl ester. These esters were even less damaging to health when administered intraperitoneally than the free p-oxybenzoic acid which is held to be relatively innocuous. For instance the LD_{50} of methyl ester was 0.96 gm/kg and that of its acid was 0.21 gm/kg. For iv. administration, the dangerous dosis depended strongly on the rate of intake. If the latter was 20 mg/kg-minute, then it was already reached for a total intake of 85 mg/kg, but if it only was 5 mg/kg-min, then it was reached only for a total intake of 600 mg/kg. This indicates rapid detoxification in the body as regards esters. Paralysis caused by excessive ester doses either led to death or disappears without leaving detectable permanent damages. Kopsch made use of the ester-induced paralysis in his experiments with amphibious larvae and adult frogs to anesthetize them. A non-damaging, non-interfering anesthetic was sodium-propyl-p-oxybenzoate.

For daily administration of 0.15 g/kg of ethyl or propyl ester to rats for 18 months, no damage was observed by Sokol, rather stimulation of their growth. Ten-fold higher doses too caused no pathological changes in the animals, though there was delay in growth. If the rats' diet contained 2% methyl or propyl ester for 18 months, corresponding to a daily intake of 1 gm/kg, no damages were observed. Similar results are obtained for similar tests with dogs for one year. Dogs being orally administered 1 gm/kg evidenced 89% of methyl ester, 66% of ethyl ester, 58% of propyl ester, 48% of butyl ester of the ester amounts added in the urine, and when fed iv. 0.05 gm/kg, 85% of methyl ester, 70% of ethyl ester, 94% of propyl ester and 40% of butyl ester; these substances were almost completely changed but had kept the phenol structure. The fairly clear agreement between the amounts found in the urine following both administrations leads to the conclusion of a complete transition of the added esters or of their conversion products from the intestine into the body, and this corresponds to the lack of same as found by Schuebel and Manger in the droppings. The major part of the esters from methyl to propyl appears in this fashion already in the first 6 hours following iv. injection. After these administrations of the smaller amounts of esters, these before long could not be found anymore in the plasma. However the dogs' plasma following oral administration did retain detectable ester quantities. There were only traces left in the urine of the esters from methyl to propyl, and butyl ester could be detected in the amounts of 0.5% of the added ester quantities in unchanged form. Following ingestion of the four esters, the urine contained somewhat more unchanged ester, namely 0.1 to 0.6% of the amounts ingested. The main part of the esters found in the urine was combined. The autho who performed the work referred to in this paragraph, leaves the question unanswered, whether there is present a compound of the ester proper or a compound of the p-oxybenzoic acid caused by saponification because his procedure cannot distinguish between these two possibiliti

After Sokol administered iv. 2 mg/kg-minute to dogs up to a total of 100 mg/kg and instant death, he found all four esters as suc in the brain, the maximum amounts being for butyl ester, and also certain amounts in lesser concentrations in the spleen and pancreas. There was no free p-oxybenzoic acid in the brain or very little, but there was more of it in the liver and the maximum amounts were in the kidneys. The total amount of ester reaction products also was much higher in the liver and the kidneys than in the other organs, but th

contained no free esters. The esters also saponify rapidly liver and kidney tissues in vitro, other organs are not saponified at all; muscle saponification is very slow.

When administering 70 mg/kg per os to humans, Sokol found a behavior similar to the animals with respect to esters. There was no ester proper in the urine, and there was about 50% in combined form or changed in other ways, of which 11% free oxybenzoic acid (p). The occurrence of the latter corresponds to our own results, whereas his amount of combined ester or of conversion products which Sokol reports as his main part approaches that determined by us for paired sulfuric and p-oxybenzoic acids.

Sokol found, as had already Schuebel & Manger, that for chronic feeding, there was no accumulation in the body but only accelerated elimination.

According to Sokol, larger amounts of p-oxybenzoic acids are detectable in the plasma for oral feeding. At least part of the ingested amounts of ester therefore will be saponified only after transition from the intestine into the body in the liver and and the kidneys or will be paired with sulfuric acid in the liver. Prior to this change the esters therefore spread in the body as such and may unfold their anti-microbic effects. If the orally administered part of the esters were already saponified to a large extent or in toto, in the intestine, then the antimicrobic effect following per os administration in therapy outside the intestine could not be explaine since free p-oxybenzoic acid is only slightly antimicrobic.

Recently Davis found that p-oxybenzoic acid is the required nutrition or growth substance for escherichis coli and therefore considered it to be a bacteria-vitamin similar to vitamin H' of p-amino benzoic acid. The antagonism discovered for the p-aminobenzoi acid growth substance and such similar substances like sulfonamides is expected by Davis also to occur for the growth substance p-oxybenzo acid and structurally similar substances, which just like the sulfonamides are enriching chemotherapy by their selective toxic effects on pathogenous microbes. Such antagonists might be the p-oxybenzoic acid esters. Already some time ago MacDonald assumed a mechanism similar to the sulfonamides' for the anti-microbic action of p-oxybenzoic acid esters. In earlier work performed by one of us (TS) with Marx and not yet published, making use of the Warburg apparatus, the esters showed strong inhibiting properties with respec to beer yeast respiration. In contrast to the salicylic and benzoic acids that were simultaneously tested, it was practically independent of the pH's used.

TESTS.

In order to isolate with ether the ingested substance and their reaction products as created in the body from fairly large amounts of urine, we used a specially adapted device which allowed continuous extraction from 1½ liters of urine. It consisted of a high glass cylinder with a ball-form condenser at the top. Ether introduce at the bottom continuously streamed through the urine in the cylinder the ether being made to pass flat glass bells close one to another an each pierced with fine holes, before collecting above the urine. Ther the ether spills into a round-bottom flask that is heated by an elect water bath and the ether evaporates in same, the vapor passes into th ball-form condenser above the cylinder and the condensed ether is t introduced at the bottom into the urine. This continuous extraction allows full recovery of the p-oxybenzoic acid, even though the

distribution coefficient ether/water of this acid is unfavorable.
As a trial we reacted 1.5 l of normal urine with 3 gm of oxybenzoic acid, and after these urine amounts are extracted following acidification with diluted sulfuric acid, for 8 hours in the apparatus with ether, and drying the remaining part of ether in the round-bottom flask with sodium sulfate and evaporating, we were able to fully recover from the dry residue the added p-oxybenzoic acid propyl ester, as described below.

One of us (HC) for 5 days ingested each day 2 gm of ester or 1.53 gm of p-oxybenzoic acid distributed over several doses, i.e. a total of 10 gm, and the urine was continuously collected from beginnin to 2 days after termination and 1½ liters of same each time were ethered-out after addition of excess sodium bicarbonate for a period c 6 hours. In the ether extract so obtained, we found (?) no propyl este

After that we acidified the urine with diluted sulfuric acid and in the same manner ethered it out for 8 hours, evaporated in the round-bottom flask the remaining ether after the last extraction following sodium sulfate drying, dried that residue further in the dessicator and after several hours drew it out with absolute ether in (illeg.)* where the p-oxybenzoic acid passed into the ether. WE shook out the acid from the ether solution with aqueous soda solution, and shook out the latter again with ether after acidification with sulfuric acid till the ether no longer absorbed, then dried the (illeg.) ether shakings with sodium sulfate, evaporated them and purified the p-oxybenzoic acid by repeated recrystallization from wat

The non-dissolved residue in the absolute ether was treated with warm ethyl acetate for removal of the hippuric acid and the stil remaining residue was treated with ether containing alcohol and by treating the ether with soda solution, we isolated as before (?) had been the case for p-oxybenzoic acid in this instance the p-oxy"benzur" acid.

We obtained (?) 1.05 gm of p-oxybenzoic acid (=13.7% of the acid introduced as ester and 0.4 gm of p-oxybenzur acid (? 3.7% of t acid ingested as ester).

p-oxybenzoic acid: m.p. = 210.5°C. Titration with Poitriers Blue 0.1233 gm=12.45 cc (?) n/10 lye (?) 19.43 cc. 0.1284 gm of substance; 0.285 gm CO_2; 0.? gm H_2O

found: C= 60.9%; H=4.7%; computed: C= 60.9%; H=4.38%

p-oxybenzur acid: m.p.=236-258°C. Titration with Poitriers Blue, 0.1233 gm = 12.45 cc n/10 lye (?) over 19.43 cc. Substance =0.1259 gm; CO_2= 0.2397 gm; H_2O=0.0537

found: C=55.0%; H=4.7%; computed: C= 55.4%; H=4.65%.

For further identification a p-oxybenzur acid of the second substance obtained in the urine, we methylated on one hand the substance and compared the methylation product with the anisuria acid obtained fro the (?)anisoyl chloride and glycocoll, on the other hand we prepared synthetically p-oxybenzur acid via the p(carbomethoxy-oxy)benzoyl-glycine-ethyl ester and compared with the substance isolated from th urine.

Anisuria acid - 4-methoxy-hippuric acid $CH_3.O.C_6H_?.CO.NH.CH_2CC$

1. From anisoyl chloride and glycocoll.

We obtained the required anisoyl chloride by heating of 1½

* original becomes partly illegible hereafter; transl.

mole of thionyl chloride with 1 mole of anisic acid for 1½ hours in the water bath. After termination of reaction, the excess thionyl chloride to the largest part was distilled in an oil bath at (?) 110°C and the residue was subjected to vacuum distillation. The distillate congealed in ice. m.p. =?

We dissolved one mole of glycocoll in 2 moles of 2 N soda lye and reacted the solution heated to 60°C under constant shaking gradually with 1 mole of anisoyl chloride, exhausted the precipitated anisuria acid and by means of extraction removed , by means of absolute ether, small amounts of anisic acid.

M.p.= 171°C. Titration 0.1220 gm acid = 5.8 (?) cc, n/10 lye (computed: 5.83 cc)

2. Methylating p-oxyhippuric acid from urine.

The p-oxybenzur acid from the urine was reacted with in coole alcoholic solution with excess diazomethane solution and the reaction product after alcohol evaporation was recrystallized from water.

M.p.=170.5°C. M.p. after mixing acids 1 and 2: 170.5°C.

p-oxybenzur acid and p-oxybenzoic acid

$HO.C_?H_4CO\ NH\ CH_?CO_?H$

We obtained the acid according to Fischer after reacting glycocoll ethyl ester with 4-(carbomethoxy-oxy)-benzoyl chloride, which we had prepared from 4-(cabomethoxy-oxy)-benzoic acid and thion chloride. The glycocoll ethyl ester so substituted was treated with lye, with transient strong red coloring, was followed by hydrochloric acid neutralisation with 30 minutes' standing.

M.p.=238°C with early decomposition. M.p. for mixture with p-oxybenzic acid isolated from urine: 237°C. (?)

We also obtained the amount of hippuric acid that had passed to the warm ethyl acetate by evaporating the ethyl acetate and absorbing the residue with alcohol-containing ether, then we shook ou with aqueous soda solution and ethered-out the latter after it had been acidified with sulfuric acid, and we recrystallized from water the residue obtained when evaporating the ether. We obtained 1.3 gm of hippuric acid with a m.p. of 187°C.

Prior, during and after ingestion, the A/B quotient according to Baumann was obtained for 50 cc of the daily urine, where A is unpaired and B is paired, and we found

prior to ingestion	9.9;	A=0.0920 gm; B= 0.0093 gm.
during		
1st measurement	4.9;	A=0.0958 gm; B=0.0197 gm.
2nd	6.8;	A=0.1762 gm; B= 0.0258 gm (?)
3rd	3.7;	A=0.1162 gm; B= 0.0318 gm;
2 days after ingestion	11.3;	A=0.1130 gm; B= 0.0100 gm.

Prior and after ester ingestion, there was on the average 0.009 gm of paired sulfuric acid, whereas on the average the ester ingestic was 0.0258 (?) gm, or 0.0161 gm more, for 50 cc of daily urine, and this corresponds to 0.0296 gm of propyl ester. If one assumes a urine elimination of 9 liters for the five-day ingestion period and the next one, then the amount of paired sulfuric acid contained therein amounts to 53% of ingested esters.

p-oxybenzoic acid benzyl ester.

As a preliminary test, we fed 2 gms of ester to each of several guinea pigs each day; they weighed about 700 gm. No adverse effects could be observed. Then we ingested 2 gms of ester each day for 5 days (corresponding to 1.21 gm of p-oxybenzoic acid) which were spread into several doses and we tested the urine as for propyl ester ingestion.

We were able to isolate the urine reacted with sodium bicarbo by ethering-out and obtained 6 gm of ether (=6% of the amount ingest

M.p.= 114°C. Titration with Poirriers Blue 0.100 gm = 4.5 cc n/10 lye (computed: 4.38 cc).

After acidification we found 0.82 gm of p-oxybenzoic acid (=13.6% of the acid ingested as esters) and 0.32 gm of p-oxybenzur acid (3.7% of the acid ingested as esters) in the ether extract.

p-oxybenzoic acid: m.p.=210.5°C; Titration with Poirriers Blue 0.1306 (?) gm = 22 cc n/10 lye (computed: 21.8 cc). Substance= 0.1256 gm; CO_2=0.2801 gm; H_2O=0.0493 gm.

found: C=60.9%; H=4.80%; computed: C= 55.4%(?); H=4.65%

p-oxybenzur acid . M.p.= 238°C(?). Titration with Poirriers Blue 0.1334 gm = 13.9 cc n/10 lye (computed: 13.88cc). Mixed m.p. with synthetic p-oxybenzur acid: 237°C (?). Substance= 0.1314 gm(?); CO_2=0.2633(?); H_2O=0.0364 (?).

found: C=55.1%; H= 4.80%; computed: C=55.4%. H= 4.65%.

We also isolated 2.1 gm of hippuric acid.

Thus according to Baumann we found:

	A/B	
prior to ingestion	11.5;	A= 0.1172 gm; B= 0.0162 gm?.
during ingestion		
1st measurement	2.6;	A= 0.1326 gm; B= 0.0505 gm.
2nd	7.4;	A= 0.0920 gm; B= 0.0124 gm.
3rd	3.6;	A = 0.1030 gm; B= 0.0289? gm
2 days after ingestion	7.8;	A= 0.1303 gm; B= 0.0095 gm.

Prior and after ingestion, 50 cc of urine (daily) on the aver contained 0.0098 gm of paired sulfuric acid while the ester ingestio on the average amounted to 0.0306 gm, that is 0.0208 gm more, corresponding to 0.0484 gm of benzyl ester. If urine elimination of 9 liters is assumed for the 5 days of ingestion plus one, then the amount of paired sulfuric acid contained therein would amount to 87% of the ingested esters.

In a similar preliminary test the same person daily took a 2 gm of p-oxybenzoic acid, the following A/B quotients were obtainc

prior to ingestion,	1st measurement:	9.7;	A,B=0.0915, 0.0094
	2nd	11.3	0.094, 0.0505
during ingestion	1st measurement:	6.1	0.0971, 0.0158
	2nd	7.6	0.0954, 0.0126
2 days after ingestion		7.8	0.1372, 0.0177

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Allergic Contact Dermatitis to Methyl and Propyl Paraben

Ira Leo Schamberg, MD, Philadelphia

A patient with chronic recurrent vesiculosquamous dermatitis of the palms and soles was found sensitive to methyl and propyl paraben in high dilution. The parabens are present in many therapeutic agents which come in contact with the skin, and sensitivity to them should be considered in patients with chronic dermatoses.

SCHORR AND MOHAJERIN[1] recently made the first case report of paraben sensitivity in the United States. The report of our patient, diagnosed in 1959, is the second in this country to appear in the literature.

Report of a Case

A white girl, 6 years of age, was brought to my office in June 1953 because of a pruritic vesiculosquamous dermatitis of the right sole, with erosions between the toes and secondary bacterial infection intermittently present since the age of 2 years. Bacterial culture of fluid from a pustule revealed *Pseudomonas aeruginosa* and β-hemolytic *Staphylococcus albus*, coagulase negative. The bacterial infection cleared on systemic antibiotic therapy and bland wet dressings, but within a week bullae containing clear glairy fluid appeared on the palms and both soles. Scaling appeared after the bullae and vesicles began to resolve. This bullous and vesiculosquamous dermatitis on the hands and feet has continued to wax and wane for over 17 years until the present time (January 1967). Topical therapy included wet dressings, 40% sulfur paste, liquor carbonis detergens, a preparation containing halquinols and tar (Tarquinor Cream), iodochlorhydroxyquin, (Vioform) ointment and cream, and various corticosteroid and antibiotic creams and ointments. In July 1953, as treatment for many large bullae, a hexachlorophene and menthol preparation (Appliderm No. 2) was prescribed for application to the left palm and a corticosteroid ointment to the right palm. Two days later the left palm was swollen and studded with more and larger bullae; the right palm showed much less involvement. In December 1958 chlorquinaldol (Sterosan) ointment was prescribed for application to the central left palm where a number of bullae presented. The ointment was used only twice because its application was immediately followed by severe itching and the appearance of larger confluent bullae in an annular distribution at the border of the treated area. A diagnosis of contact dermatitis to chlorquinaldol ointment was made (and in retrospect, of contact dermatitis to Appliderm No. 2).

The manufacturers of the suspect preparations (White Laboratories Inc., and Geigy Pharmaceuticals) supplied not only their formulae but also forwarded all the ingredients for patch testing. Tables 1 and 2 indicate that patch tests to methyl and propyl paraben and to chlorquinaldol ointment were positive. All other tests were negative. The results of quantitative patch tests to methyl and propyl paraben are shown in Table 2. Appliderm No. 2 contains both chemicals in concentration of 0.15% by weight, and chlorquinaldol ointment in concentration of 0.12%. A paraben-free mixture of hydrocortisone, tar, and diiodohydroxy-

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Re[illegible] gen[illegible]

Table 1.—*Thirty-Hour Reading of Closed Patch Tests to Ingredients of Chlorquinaldol (Sterosan) Ointment and Appliderm No. 2* *

Chlorquinaldol Ointment	
Chlorquinaldol	0
White wax, USP	0
White petrolatum, USP	0
Lanolin, anhydrous, USP	0
Methyl paraben, USP	3
Propyl paraben, USP	3
Talcum, USP	0
Chlorquinaldol ointment	[illegible]
Appliderm No. 2	
Hexachlorophene	0
Menthol	0
Propylene glycol monostearate	0
Magnesium aluminum silicate	0
Polysorbate	0
Methyl paraben	3
Propyl paraben	3
Water	0

*Tests carried out in January 1959.

Table 2.—*Forty-Eight Hour Reading of Closed Quantitative Patch Tests* *

Dilution	Methyl Paraben	Propyl Paraben
1 to 1,000	3	2
1 to 10,000	2	0
1 to 100,000	2	0
1 to 1,000,000	1	0
1 to 10,000,000	0	0

*Tests carried out in January 1959.

quin (Cor-Tar-Quin Cream) 0.5% was prepared (Mr. Irving Wershaw, Dome Chemicals, Inc.) for this patient's use in August 1961 and in October 1962. Tubes of the cream prepared in October 1962 and kept for over four years at room temperature showed no signs of bacterial or fungal growth in January 1967.

Recurrent pruritic vesiculosquamous episodes have been controlled by avoidance of contact with parabens, by conservative and short-term systemic corticosteriod, antibiotic, and sulfonamide therapy when indicated, and by wet dressings and topical application of the specially prepared paraben-free Cor-Tar-Quin Cream and a hydrocortisone and iodochlorhydroxyquin preparation (Vioform-Hydrocortisone Cream).

Comment

The parabens (parahydroxybenzoates, parasepts), bacteriostatic, fungistatic, and antioxidant in their action, are widely employed as preservatives. The methyl and propyl esters are used most frequently. Table 3 lists various medicinal and other products containing these chemicals. This is not an exhaustive compendium but simply includes substances which we have discovered to contain these agents over the nine years since our interest in them was aroused by the subject of this case report. Chlortetracycline (Aureomycin) and tetracycline (Achromycin) ointments contain 2.4% methyl and 0.6% propyl paraben, far higher concentrations than are present in any other therapeutic agents made for use in this country. (They are usually added to topical preparations in 0.1 to 0.3% concentration.) The high level in these ointments was recommended by the Food and Drug Administration to prevent monilial overgrowth, according to a written communication from Stephen A. Szumski, PhD, Medical Advisory Department, Lederle Laboratories (Dec 16, 1966). In Europe, especially in Denmark, parabens are present in 5% concentration in widely used antifungal topical agents. It is believed that topical application of preparations containing this high concentration is responsible for the relatively high incidence of paraben sensitivity reported in Denmark.[2]

In the United States, it is required by the Food and Drug Administration that the label indicate the presence of parabens in foods (permitted up to a concentration of 0.1%) and in all prescription drugs, except oral preparations. Nonprescription drugs, cosmetics, and dentifrices need not reveal on the label the presence of parabens.[3] As a result, the patient with paraben sensitivity may find it impossible to avoid contact with this agent.

Not only are patients with chronic dermatitis especially prone to become sensitized to additional contactants,[1] but also, as revealed by Table 3, they are, if under treatment, probably subjected to contact with the parabens over long periods of time. Therefore, clinically significant sensitization to the parabens is most likely to occur in patients with chronic localized eczematous dermatitis which has been subjected to a good deal of topical therapy. If the initial cause of this chronic dermatitis is allergic eczematous contact dermatitis to other agents, and positive patch tests are elicited to these other

Table 3.—Substances Containing Parabens

[illegible]
Steroid
Ointments
[illegible]
Creams
[illegible]
Neo-Cortef
Neo-Dermacortef
Neo-Oxylone
Lotions
Cortisporin
Flexinefrin
Heb-Cort V
Neo-Cortisone
Neo-Hydrocortisone
Vioform-Hydrocortisone
Creams
Celestone with Neomycin
Heb-Cort N
Heb-Cort V
Mycolog
Neo-Synalar
Steroid with Antibiotic
Antibiotic
Ointments
Achromycin
Aureomycin
Lotions
Neo-Cortef Antibiotic
Vioform
Creams
Achromycin
Chlorquinaldol
Tetracycline
Other (specify)
Ointments
Hydrophilic Ointment, USP
Lotions
Antiva
Rupe-Cream
Creams
Lotrimin
Mycostatin
Tarcortin
Solutions
Hydrogen peroxide
Other (general)
Cosmetics
Dentifrices
Deodorants
Face creams
Hand creams
Suppositories
Oral Preparations
Food, cake (one bakery only)
Achromycin Pediatric Drops
Aristocort Syrup
Mysoline Suspension
Tofranil Suspension
Parenteral Preparations
Lidocaine hydrochloride (xylocaine injection, USP
Penicillin, and streptomycin solutions for treatment of bovine mastitis
Sterile dried
Methylparaben sodium parenteral
[illegible]
Gauze
Pads
Sterile Dressings

agents, the physician may be led away from the true current diagnosis of sensitivity to parabens and the disease may therefore be prolonged. Also, as emphasized by Rudner and Cronin,[4] patch tests may be negative to steroid creams containing parabens in paraben-sensitive patients because of the anti-inflammatory effect of the corticosteroid. The low concentration of parabens, in these creams may also contribute to the failure to obtain positive tests. Schorr and Mohajerin[1] advise patch testing with 5% paraben in petrolatum.

Generic and Trade Names of Drugs

Iodochlorhydroxyquin—*Vioform*.
Chlorquinaldol—*Sterosan*.
Tetracycline—*Achromycin*, *Panmycin*, *Polycycline*, *Steclin*, *Tetracyn*.
Chlortetracycline—*Aureomycin*.

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Paraben Allergy

A Cause of Intractable Dermatitis

William F. Schorr, MD

Paraben esters are incorporated in most dermatologic and cosmetic creams and lotions as preservatives. A two-year clinical study on the allergenic potential of these bacteriostatic and fungistatic chemicals showed that of 273 consecutive patients with chronic dermatitis who were routinely patch tested to 5% paraben in petrolatum had paraben allergy, an 0.8% incidence. Strongly positive patch test reactions in four patients with marked paraben allergy correlated well with the clinical course. Patients with contact dermatitis caused by paraben allergy present with long histories of intractable dermatitis which continues to flare regardless of the corticosteroid cream prescribed. Once the proper diagnosis is recognized and a paraben free medication is applied, these patients make a rapid and complete recovery. Paraben allergy must be suspected in any patient who gives a long history of recalcitrant dermatitis which has not responded to customary dermatologic diagnostic and therapeutic technique.

The paraben esters—methyl, ethyl, propyl, or butyl *p*-hydroxybenzoate—are incorporated as preservatives in the vast majority of dermatologic creams, lotions, and ointments prescribed by physicians. They are also commonly incorporated in cosmetic preparations, foods, drugs, dentifrices, and suppositories. This wide usage attests to their excellence as bacteriostatic and fungistatic agents.

Because of this wide usage, however, a great percentage of our population has had repeated exposure, both topically and systemically, to the parabens.

In June 1966 we published the first report of paraben-induced contact dermatitis to be identified in the United States.[1] Schamberg reported a second case in 1967[2] and Fisher in the same year reported two more cases in the United States.[3]

Since 1965 paraben allergy has been diagnos and treated in four patients at the Marshfield Cli ic. Two patients with paraben allergy were fou in a group of 273 consecutive patients with histori of long-standing dermatitis who were patch test to the parabens. This is an incidence of 0.8% ir group of patients with chronic dermatitis. The r maining two cases were selected patients not i cluded in the series of 273 consecutive patients.

The great importance of parabens as a cause long standing, progressive, and severe iatroge contact dermatitis rests not in their incidence disease production, but rather in their insidio pattern and the great ease with which this diagr sis can be missed.

Patch tests to the paraben-containing corticos roid or antibiotic creams or lotions will frequent give false negative reactions. It is necessary to t patients to a high (5% in white petrolatum) pa ben concentration for 48 hours with a closed pat test to establish the diagnosis. The usual conc tration of less than 0.5% paraben in corticoster and antibiotic creams, while able to produce a lentless progression and extension of disease wh applied to dermatitic skin, is insufficient to produ a true positive allergic patch test reaction wh applied to normal skin on the same patient. T phenomenon is well known to the dermatolog and has been explained in detail.[1] Closed pat tests with 5% methylparaben, ethylparaben, p pylparaben, or butylparaben do not irritate norr skin when applied for 48 hours. No false posit reactions have been found on testing more th 260 control patients without paraben allergy.

All patients in this series showed marked cro sensitivity to all four paraben esters and a pat test to any one of the four esters would have est lished the diagnosis.

The suffering, disability, and financial burden patients allergic to paraben preservatives when t

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diagnosis is missed can be best exemplified by our first patient (case 1) who was treated with numerous medications by many physicians over a period of seven years during which time his dermatitis was in a state of constant flare. Within 24 hours of the application of a paraben free cream, his skin began to heal for the first time in seven years.

The following case reports demonstrate the problem.

Report of Cases

CASE 1.—A 48-year-old white man, a punch press worker, was seen in November 1965. His dermatitis had begun on the perianal skin after a hemorrhoidectomy seven years previously. Over the years, there was a gradual extension of the dermatitis. Most recently, it had spread to the eyelids, face, neck, and external ears. This extension occurred shortly after treatment with eardrops containing neomycin and dexamethasone. Examination showed a relatively severe, pruritic, recurrent contact dermatitis involving the genitocrural area, axillae, face, ears, eyelids, lateral aspects of the neck, parts of his arms, and lower back. No fungi were found in the scales from his groin. Therapy during the past seven years consisted of a large number of topically applied and systemic medications prescribed by many physicians. These included a variety of topically applied corticosteroids as well as iodochlorhydroxyquin, neomycin sulfate, and other dermatologic agents. Only the neomycin had caused an obvious acute flare. The remainder of his medications resulted in a gradual but relentless progression of the dermatitis.

The patient was admitted to the hospital for study. All items of contact were removed from his room except for bed linen and eating utensils. Therapy was begun with betamethasone cream (0.2%) and triamcinolone cream (0.1%) applied to separate areas by nursing personnel. The dermatitis continued to flare during the first four days of hospitalization. All therapy was discontinued on the fifth hospital day and his skin improved spontaneously. Reactions to the parabens as well as to other medications were tested by applying closed patch tests for 48 hours. The patient had positive reactions to all parabens. He also reacted to neomycin and iodochlorhydroxyquin. Patch test reactions to triamcinolone, flurandrenolone, and fluocinolone acetonide creams, which have low paraben concentrations, were either negative or weakly positive.

Additional patch tests were made to cadmium, nickel, cobalt, potassium dichromate, elastic, mercaptobenzothiazole, thiram (tetramethylthiuram disulfide), formaldehyde solution, *p*-phenylenediamine, lanolin, lanolin with 5% salicylic acid, petrolatum with 5% salicylic acid, spermaceti, polyoxyethylene stearate, polysorbate 80 (Tween 80), squalene, propylene glycol, sorbitan monooleate (Span 80), sorbitan sesquioleate, stearyl alcohol, sodium lauryl sulfate, sorbic acid, turpentine, benzocaine, dibucaine (Nupercaine) hydrochloride cyclomethycaine sulfate (Surfacaine), peruvian balsam, cinnamon oil, acacia, and flurandrenolone powder. No positive reactions were found at 48, 72, or 96 hours.

Therapeutic challenge with flurandrenolone cream which contained ethylparaben produced a second flare. This exacerbation cleared rapidly with hydrocortisone, 1%, in a paraben free base (Hydrotex with water, 1:2).

The patient returned to work in November 1965, and his skin has remained clear. His only treatment is the strict avoidance of preparations containing paraben, neomycin, and iodochlorhydroxyquin and the topical application of the paraben-free steroid cream one or two times a week.

On Jan 21, 1966, all patch tests were repeated, with similar results.

CASE 2.—A 43-year-old business man with a ten-year history of recurrent stasis dermatitis was seen in June 1967 with a 10 × 7 mm stasis ulceration on the lower portion of the leg superimposed on an area of stasis derma titis. The ulcer was treated with a liniment containing 6,9 diamino-2-ethoxyacridine, ichthammol (Tumenol), an zinc oxide in corn oil (Rivanol Zinc Oil), three times dail after a warm water compress. Flurandrenolone crea (0.025%) was prescribed for the surrounding dermatit skin. The patient returned six days later with a seve bright red dermatitis of both legs, volar aspect of bot arms and forearms, and the front and sides of his nec His eyelids were red and swollen. He was hospitalized an placed on a regimen of 40 mg of prednisone a day. Th dermatitic flare persisted despite continued oral admi istration of prednisone and discontinuance of the Rivan Zinc Oil therapy. Topically applied therapy was chang to betamethasone cream and then to triamcinolone crea both of which contain paraben preservatives. All items contact were removed from the patient's room except f his eating utensils, magazines, and hospital bedclothe Despite these measures the dermatitis could not be brougl under control (Fig 1).

On the 12th hospital day topically applied therapy wa changed to hydrocortisone incorporated in a vanishi cream base in which sorbic acid was used in place paraben. Within 24 hours the dermatitis was marked improved (Fig 2) and oral administration of prednisor was discontinued. The dermatitis continued to heal con pletely over the next five days (Fig 3), even though th prednisone therapy had been stopped.

After the dermatitis healed, patch tests to ethylparabe 5%, and methylparaben, 5%, in white petrolatum were a plied. The patch tests were removed at 48 hours and bo showed a 3+ reaction with erythema, edema, and fi vesiculation (Fig 4). Patch tests to the vanishing crea with its low paraben content produced a very weak rea tion which could have been missed. Patch tests to th corticosteroid creams and the ingredients in Rivanol Zi Oil were negative.

The patient was sensitized to paraben when flura drenolone cream with its 0.1% ethylparaben content wa applied. The dermatitis was repeatedly flared during the apy with betamethasone cream containing methylparab and butylparaben and by triamcinolone cream containi methylparaben and propylparaben preservatives. The p tient promptly recovered when a paraben-free hydrocort sone cream was applied.

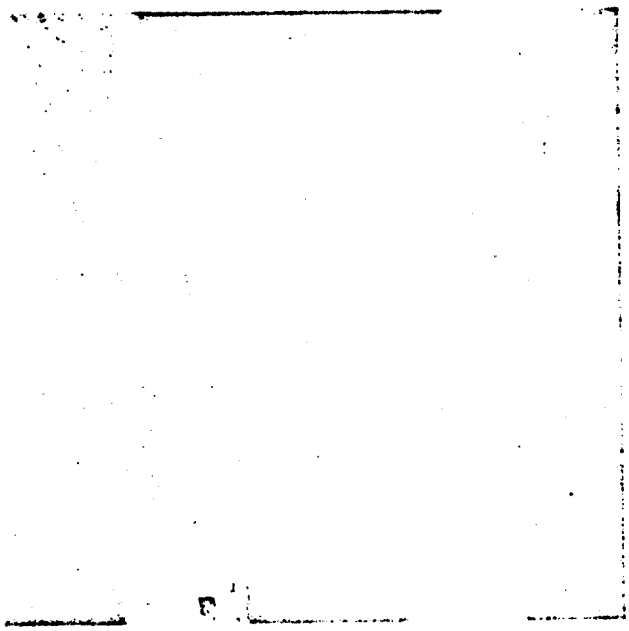
All patch tests were repeated two months after th patient was discharged from the hospital and identical r sults were obtained. The dermatitis had been healed i two months, thereby ruling out the possibility of fal positive tests. Patch testing patients with hyperreacti skin when the dermatitis is acute is a known source false positive reactions.

CASE 3.—This 34-year-old farmer had a seven-year hi tory of pruritus ani which was flared by the application flurandrenolone lotion containing methylparaben and pr pylparaben. This patient showed positive patch tests to four paraben esters as well as to fluocinolone cream co taining methylparaben and propylparaben preservativ and to betamethasone cream containing methylparab and butylparaben. Although the patch test reactions to c standard testing concentration of 5% paraben in whi petrolatum showed a stronger reaction, the diagnosis cou have been made by testing to the steroid containing crea with its much lower paraben concentration in this case

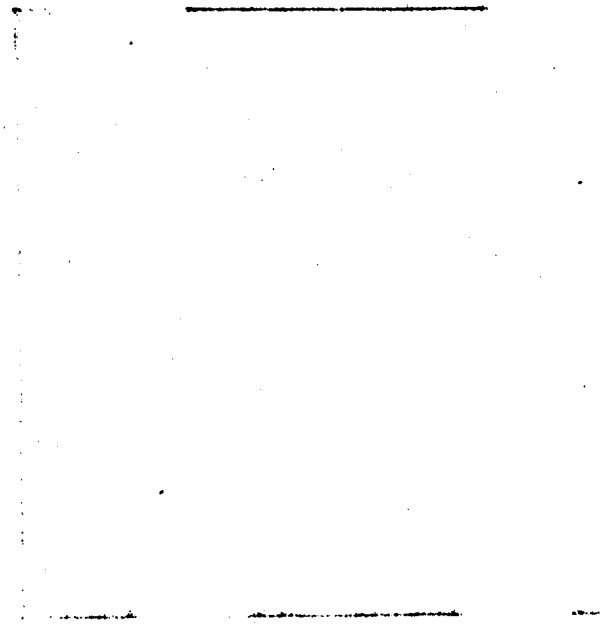
CASE 4.—This 58-year-old building inspector had derm titis of the hands and a long history of dermatologic tre ment. His clinical history, diagnosis, and treatment we very similar to the previous three patients and will not farther elaborated upon.

Comment

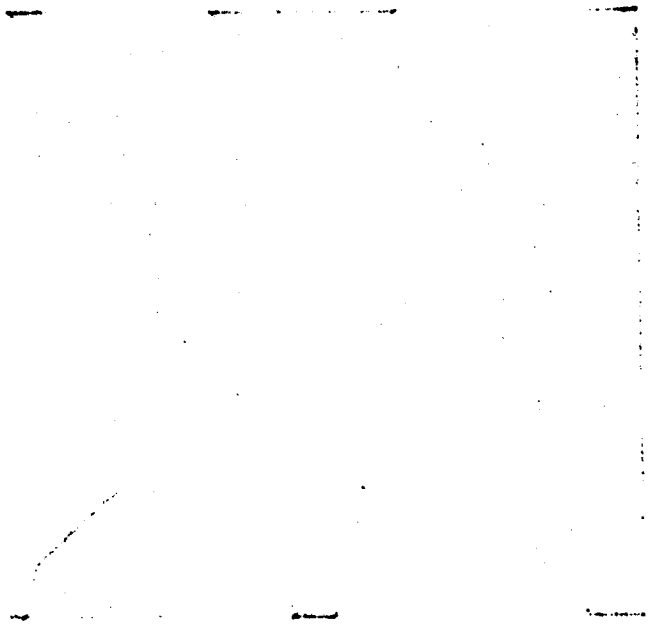
The skin of patients with chronic dermatitis h a greater chance of becoming sensitized to topical applied therapeutic agents than does the skin



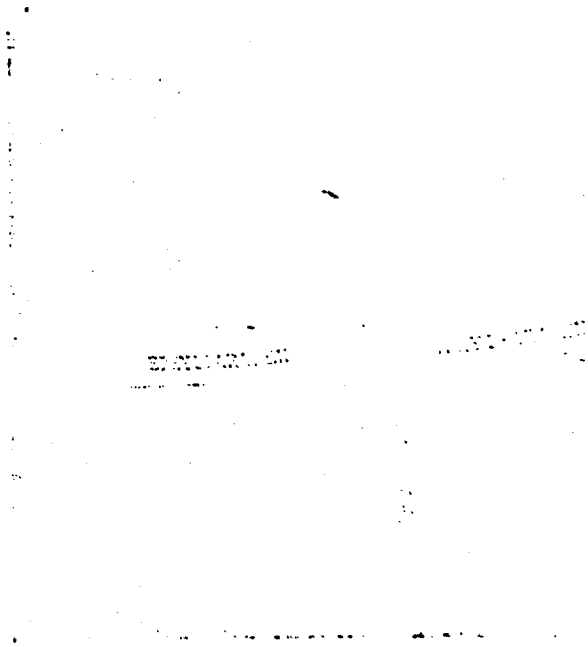
1. Photograph taken on the 12th hospital day (case 2). The dermatitis had continued to flare despite continued oral administration of prednisone, isolation from all outside contactants, and the topical applications of three potent corticosteroid creams.



2. Photograph taken on the 13th hospital day, 24 h after therapy was changed to a paraben free hy cortisone cream (case 2). Therapy with orally adm tered prednisone was discontinued.



3. The dermatitis had completely healed five days after the paraben free corticosteroid cream was substituted (case 2).



4. Closed patch tests to 5% ethylparaben and m paraben in petrolatum after 48 hours demonstrate strongly positive reactions characteristic of all pa in our series who had paraben allergy

other patients. Patients with chronic dermatitis are often treated over the years with numerous medications thereby increasing the risk of iatrogenic disease. The risk is greatest in stasis dermatitis and the principle sensitizers are neomycin, lanolin, and the *-caine* group of drugs (local anesthetics). Hjorth and Trolle-Lassen[4] have shown that nearly half of all positive patch tests performed on 1,664 consecutive patients with eczema at the Finsen Institute were due to therapeutic agents. Paraben preservatives should be recognized as another source of doctor-induced relentless progressive dermatitis.

We reviewed the world literature on this subject in our original publication.[1] Paraben allergy has been well recognized in Europe.[4-8] Parabens are used in concentrations below 0.5% in the United States; in contrast, in Europe concentrations up to 5% are used. In the past it has been generally assumed that these higher paraben concentrations used in Europe accounted for the predominance of paraben allergy there. However, this study, as well as the reports by Schamberg and Fisher, have demonstrated that even the lower concentrations can produce a therapeutic contact dermatitis. The assumption that contact dermatitis to parabens is a uniquely European disease which does not occur in the United States is a fallacy. It is perhaps true that the higher concentrations used in Europe have a greater sensitizing capacity; however, standard American dermatologic and cosmetic preparations are not 100% safe because of their lesser paraben content and physicians must be aware of this potential problem.

Once the diagnosis is made, treatment can be instituted by incorporating 1% hydrocortisone in a paraben free base. Hydrotex is free of paraben. Its greasy consistency, however, makes it less suitable for the treatment of acute dermatitis or of dermatitis involving intertriginous folds. This difficulty can be overcome by adding a sufficient amount of water to make a creamy emulsion.

A new awareness of this problem on the part of our pharmaceutical industry is apparent from a recent campaign advertising a paraben free hydrocortisone cream. In this preparation the paraben preservatives have been replaced with sorbic acid. Particular attention must be paid to the fact that contact dermatitis to sorbic acid[9] has been reported. There is no substitute for proper specific diagnosis by established dermatologic testing techniques.

Erythromycin (Ilotycin) ointment is paraben free according to Fisher[3] and can be used as substitute for other topically applied antibiotics. Like Fisher, we have not seen a case of contact dermatitis caused by this preparation.

Schamberg[2] noted that the Food and Drug Administration requires the declaration of paraben on the label of prescription drugs. Unfortunately, however, such a demand is not placed upon nonprescription drugs, cosmetics, and dentifrices and as a result the patient with paraben sensitivity may find it impossible to avoid contact with this chemical.

Cosmetic houses especially, with their patent rights, have made it very difficult, and at times impossible, to adequately diagnose and cure contact dermatitis produced by these agents. It is virtually impossible to determine the proper testing concentrations, and to diagnose and remove all sources of a commonly used offending chemical from the patient with dermatitis because the physician is often not able to obtain the necessary information from cosmetic manufacturers.

Since submission of this manuscript for publication, an additional report on paraben allergy in the United States has appeared in the literature.[10]

The hydrocortisone incorporated in a vanishing cream base in which sorbic acid was used in place of paraben was supplied through Robert E. Lyons, MD, of Texas Pharmacal Company, Houston.

Cases 3 and 4 were patients of Richard Rowe, MD.

Generic and Trade Names of Drugs

Betamethasone—*Celestone.*

Triamcinolone acetonide—*Aristocort Topical Cream, Aristoderm Foam, Kenalog.*

Iodochlorhydroxyquin—*Domoform, Quinoform, Quin-O-Crem, Vioform, Entero-Vioform.*

Flurandrenolone—*Cordran.*

Fluocinolone acetonide—*Synalar.*

Prednisone—*Deltasone, Deltra, Meticorten, Paracort.*

Cyclomethycaine sulfate—*Surfacaine.*

Polysorbate 80—*Monitan, Sorlate, Tween 80.*

Sorbitan sesquioleate—*Arlacel C.*

Dibucaine hydrochloride—*Nupercaine Hydrochloride.*

Neomycin sulfate—*Mycifradin Sulfate, Myciguent, Neobiotic.*

Sorbitan monooleate—*Span 80.*

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ON THE TOXICOLOGY OF SEVERAL RECENT PRESERVATIVES: p-CHLOROBENZOIC ACID AND p-OXYBENZOIC ACID ESTERS.

Prof. Dr. K. Schuebel

The benzoic and salicyl acids and their sodium salts for years have been used as antiseptics and preservatives. Recently significance has been achieved on the part of the p-chlorobenzoic acid and its sodium salt, of microbine, of methyl -, ethyl-, and propyl esters of the p-oxybenzoic acid in the food industry and especially as regards fish preservation of same. Therefore it was of interest not only from the theretical, but also from the practical viewpoint, to test the compounds mentioned for toxic effects in different warm-blooded animals and where possible to ascertain the effects in organisms *.

M. NEISSER & TILLMANN (1), also C. VON HEIDE & R. FOELLEN (2) have investigated the development-inhibiting and killing effect of microbine with respect to diverse mushrooms and bacteria. Microbin is reported to be coli-inhibiting for a concentration of 1:150, and sodium benzoate at 1:100 as regards bacteria. For coli fermentation, 0.3 percent microbine are supposed to be as effective as 0.5 percent sodium benzoicum. On the basis of these observations, salicyl acid and sodium fluoride should be eliminated as preservatives because of the appreciably higher toxicity. NEISSER & TILLMANN are of the opinion that there is no toxicity associated with microbine, nor benzoate, since quantities of microbine 100 times larger than are being used for humans. cause no effects in animals. Microbine supposedly is an excellent preservative when used in a concentration of 0.5 - 1% for fruits products such as jam, juices and red-currant juice. Even though yeast fermentation already is being inhibited for a concentration of 0.05/1,000 V.D. HEIDE & FOELLEN (3) reject microbine as a preservative in any event on account of statutory facts, as regards wine and fruit wine. Para-chlorobenzoic acid is hardly soluble in water. 97 mg of pure acid dissolve in one liter of water at 15°C, whereas 277.2 gm of its sodium salt will dissolve in the same amount. However it easily dissolves in linoids. Thus its properties lead us to expect the same effects as from bactericidal chemicals. The alkali salts of the benzoic, salicyl and p-chlorobenzoic acids are readily soluble in water, but not in linoids. They will therefore behave entirely differently with respect to mushroom and bacteria. Adsorption at cell surface, diffusion rates, penetration and reaction with the cell's components, that is, physical-chemical changes must have a significant influence on the development-inhibiting and bactericidal effects of chemical substances on one-cellular organism

The sodium salts of said acids may only affect bacteria in nutritional media if simultaneously there is enough pure or free acid present. However the freed acids partly are tied to eggwhite because of precipitation and flocculation and are made harmless.

The esters of organic acids behave entirely differently towards microbes than do the alkali salts. Even if according to H. KOLBE (4) the salicyl acid becomes ineffective with respect to yeast fermentation because of transformation into methyl ester, this decrease in effectiveness cannot however be extrapolated to all acids from ester formation. The free p-oxybenzoic acid affects micro-organisms more than does phenol Introducing a carboxyl group into the phenol, and in the para position, therefore reinforces the bactericidal effect while the toxic effect on the animal organism is being eliminated.

Ester are less soluble in water than the corresponding acids. Often the bactericidal effect is determined from the division coefficient, that is, the ratio of lipoid solubility to water solubility. The lesser the water solubility, the more favorable the outlook for strong, bactericidal effect -- within certain limits. This effect further depends on the magnitude of the alkyl that was introduced. When esterifying parabenzoic acid, there is a gradual increase in effectiveness from methyl to benzyl ester. Methyl ester is 2.6 as destructive of staphylococcus, and benzyl ester is 83 times as destructive as free carbolic acid. Methyl ester is 1.6 times more development-inhibiting, and benzyl ester 57 times, with respect to staphylococci as free para-oxybenzoic acid, and with respect to destruction, the former is 0.7 as effective, the latter is 23 times more effective than the associated acid. By means of alkyl sealing of the free oxy group, there is reinforcement further of the bactericidal effect.

SABALITSCHKA (5) has made the significant observation, that by usin a mixture of esters of the p-oxybenzoic acid, greater solubility and therefore stronger bactericidal effects may be achieved. Preserving foods, pharmaceutical and technical preparations naturally requires a certain ester solubility. While methyl ester dissolves only to 0.2% in water, a solubility of 0.6% may be achieved by melting this compound (1:9) with sugar. The esters of p-oxybenzoic acid are neutral, almost odorless and tasteless compounds and cause no pure coloring in substrates.

If p-chlorobenzoic acid and the ester of p-oxybenzoic acid are used as preservatives in foods, the important question arises, how much of said compounds may be tolerated without damage by animal and espcially human organism; further, does metabolism transform, detoxicat or eliminate such compounds?

Upon introducing p-chlorobenzoic acid or its sodium salt (microbine solbrol) into the stomach, loss of appetite and vomiting occurs in dogs and cats even for small doses in view of local irritation at "emetico-sensitive" nerve endings. p-chlorobenzoic acid and its sodium salt have a slight narcotic effect and slow breathing in rabbits and slightly decrease breathing volume. While no pathological components are eliminated in the urine upon dosing of ½-½g/kg, upon dosing with 1-2g/kg the urine always shows sugar and egg white. The toxic effects from p-chlorobenzoic acid show more or less as fattenings of liver and kidneys. 10-70% of p-chlorobenzoic acid could be found again in the urine. It is mainly eliminated as salt. If the urine is made to react with concentrated hydrochloric acid, p-chlorobenzoic acid is precipitated. Through reprecipitation and repeated recrystallisation from alcohol, the acid is obtained again in pure chemical condition.

The methyl, ethyl and propyl esters of p-oxybenzoic acid probably are saponified to a large extent in the small intestines of dogs and cats. This causes formation of p-oxybenzoic acid which will be eliminated as such through the kidneys and according to ester by 4-49% of theoretical. A small part of this acid seems to combine with sulfuric acid and glycocoll. Detoxication occurs because of saponification, since a bactericidal acid is being formed which however for man and animal is fairly lacking in toxicity. Presumably ferments activity decomposes part of the p-oxybenzoic acid to phenol and carbon dioxide, possibly though decomposition goes further. Of the three esters investigated, methyl ester is the most toxic, and propyl ester is the compound most effective against microbes. Their toxicity apparently depends on the division coefficents. The ester are easily fat-soluble, but not the p-oxybenzoic acid which is so created. It is dubious that part of the esters should be reabsorbed unchanged. The alcohols formed during saponification are of some interest in toxicology. The saponification rate here holds considerable interest. For relatively slow hydrolysis of methyl ester, a small amount of methyl alcohol is being continuously generated. Parallel combustion and elimination therefore prevent reaching the blood toxic threshold at the sensitive cells. Rapid saponification on the other hand may lead to sudden flooding and poisoning of the organism. This viewpoint is of utmost significance when using methyl ester. 1 gm of methyl ester yields 0.2 gm, or one fifth of the ester weight, in methyl alcohol. Ingesting 5 and 10 gm of methyl ester theoretically would cause formation of 1 and 2 gm of methyl alcohol. These amounts may not be dangerous as regards adults and probably never would apply when eating from cans. When administered in larger doses, this methyl ester easily may cause damage to kidney and li

The formic acid due to oxidation of methyl alcohol could never be demonstrated in the urine of the test animals. Upon administration of larger quantities of the three esters, egg white, reducing substances and left rotation occur in the urine to a more or less pronounced degree For rabbits, the normally alkaline urine may become neutral because of the elimination of the free p-oxybenzoic acid, or even acid. Cumulative effects for chronic feeding of the three esteres could not be ascertaine

2 g/kg and 3g/kg of the p-oxybenzoic acid methyl ester resp. act toxically and fatally for dogs and rabbits; the amounts are resp. 4 and 5 g/kg for ethyl ester and 3-4 g/kg and 6 g/kg resp. for propyl ester. The esters from the p-oxybenzoic acid are appreciably less toxic than the salicyl acid methyl ester, and also less toxic than benzoic acid and its sodium salt, also less toxic than p-chlorobenzoic acid, which latter becomes toxic for 3/4-1 g/kg and fatal for 2-2.5 g/kg for rabbits an guinea pigs. The degree of toxicity of p-chlorobenzoic acid might be nearly that of benzoic acid.

From observations regarding different animal species, conclusions may not be jumped at concerning toxic effects in man. Above all it is a question of the amounts ingested per meal from preserved foods. Caution is required about the methyl ester of the p-oxybenzoic acid on purely theoretical grounds, because of the formation of methyl alcoho The same applies to p-chlorobenzoic acid. If o.25 gm of those acids are among 250 gm of Bismarck herrings, presumably healthy individuals will not suffer damages. But it cannot be stated how a liver sufferer would fare so long as empirical research in man has not been carried out, and it behooves to be cautious. Apparently ethyl and propyl esters may be considered harmless for chronic use for the amounts in which they occur in preserves.

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RECENT CONTRIBUTION TO THE TOXICITY AND THE POSSIBLE THERAPEUTIC APPLICATIONS OF THE METHYLIC ESTER OF PARAHYDROXYBENZOIC ACID

By Doctors M. Simonelli and R. Marri

From the many investigations which have been carried out on the methylic ester of parahydroxybenzoic acid it has been determined that this substance has a rather low toxicity. We were also able to confirm this property based on our experimental research,

The experiments which we carried out in this regard were conducted on rabbits. The substance in question was injected into these rabbits endovenously. At the same time we recorded the changes in pressure and respiration.

To a rabbit weighing 1.850 kilo we administered, by means of 15 endovenous injections given at an interval of 3 minutes from each other, 0.535 g of methylic ester of p-hydroxybenzoic acid (equal to 0.289 g per kg of body weight). We observed only very small drops in arterial pressure during the injection, followed by a rapid return to normal.

Owing to the poor solubility of the substance in water, and since we were thus not able to inject doses capable of killing the animal, in subsequent experiments we used a solution of 15% made in propylene glycol.

To a rabbit weighing 1.850 k we were able to administer, by means of 15 endovenous injections given at an interval of 10 minutes from each other, 8.50 cc of that solution, equal to 1.27 g of the substance (i.e. 0.69 g per kilo) , without encountering noticeable changes in pressure and respiration, with the exception of a hypotension and a reduction in the amplitude of the respiration, both changes being of a transitory nature.The animal survived the experiment, being completely normal in the next few days.

A third rabbit, weighing 1.780 kg died only after 11 endovenous injections of 1 cc of the solution at 15% made in glycol, applied at an interval of 10 minutes one from the other, thus receiving 0.92 g per kilo of the substance under examination.

After thus having confirmed the low toxicity of the methylic

ester of the p-hydroxybenzoic acid we carried out some research for the purpose of determining the antiseptic and disinfecting power, in particular as could concern the applications in eye therapy.

It seems evident from table I that this substance has a protective action against the emergence and the development of moulds if added even in weak concentrations to the major collyria (eye salves).

In other investigations carried out to determine the disinfecting power in comparison with coco palms we were able to confirm a bactericidal action even for low concentrations.

Given this useful practical application of methylic ester of p-hydroxybenzoic acid in the preservation of collyria, we endeavored to study the action of contact of its solutions on the conjunctiva.

In the rabbit the aqueous solutions in concentration less than 0.20% did not produce any reaction. Those at 0.20% only caused a slight conjunctival hyperemia of brief duration.

In the human conjunctiva we instilled acqueous solutions varying from 0.10 to 0.30% (the latter solution was in the supersaturated state). We noticed only a moderate hyperemia accompanied by slight lacrimation and the sensation of slight burning. All of these symptoms vanished within the space of approximately 1 minute.

We were able to confirm these results by repeating these instillations several times a day in more than 100 patients and no inconvenience was complained about.

In conclusion, from all of our investigations the following are confirmed:

1. the low toxicity of the methylic ester of para-hydroxybenzoic acid,
2. its antiseptic power,
3. its disinfecting power,
4. the low contact action on the conjunctiva and the usefulness of using it in preserving collyria.

(from the Institute of Pharmacology of the Royal University of Florence , Section of Florence, seating of May 14 1939-XVII).

Soluzioni sperimentate	%	Concentrazioni dell'estere metilico dell'ac. p. ossibenzoico aggiunto alle soluz. %	Sviluppo di muffe dopo giorni		
			8	15	90
Cocaina	2	0	+	++	+++
»	»	0.1	—	—	—
»	»	0.2	—	—	—
Atropina	1	0	—	+	++
»	»	0.1	—	—	—
»	»	0.2	—	—	—
Scopolamina	2	0	—	—	—
»	»	0.1	—	—	—
»	»	0.2	—	—	—
Sodio cloruro	10	0	—	+	+++
» »	»	0.1	—	—	—
» »	»	0,2	—	—	—
Glucosio	25	0	++	+++	+++
»	»	0.2	+	++	++
»	»	0.5	—	+	+
»	»	0.1	—	—	—
»	»	0,2	—	—	—

[illegible] and possible clin[illegible] [illegible] of abiol

Table 1

a = experimental solutions

b= concentrations of methylic ester of the p-hydroxybenzoic acid added to the solutions %

c = growth of molds after days

d = cocaine

e = atropine

f = scopolamine

g = sodium chloride

h = glucose

Arch. Exp. Pathol. Pharmakol. 146:208-222, 1929.

CONTRIBUTIONS TO THE PHARMACOLOGY OF A FEW PARAOXYBENZOIC ACID ESTERS: THE ORGANISM'S FATE AND THE TOXICITY.

K. Schuebel & J. Manger

Physical-chemical changes such as cell-surface adsorption, diffusion rates, penetration into and reaction with cell components must be of decisive significance as concerns the growth-inhibiting and bactericidal action of chemical substances on one-cellular life. Acids such as benzoic acid and salicyl acids and their salts have long been used as preservatives. However precise research has shown that sodium salts of those acids no longer affect bacteria growth. Such salts may only then affect bacteria in nutritional media when enough free acid is present in order to release the salicylic or benzoic acids from their salts. The effects from free acids on bacteria will be stron influenced by the substratum to that extent, that such acids may be partially rendered innocuous through precipitations and flocculations, that is when combining with egg white.

H. Kolbe (1) was able to show that salicylic acid will become ineffective with respect to yeast fermentation if methyl ester is prepa from it. This lowering however may not be generalized as regards ester fied acids, since paraoxybenzoic acid esters do behave quite differentl Free paraoxybenzoic acid, which has been described in the literature as being innocuous to man and antiseptic (H. Kolbe, (1). C. Wehmer (2)). affects micro-organisms more strongly than phenol. Introducing a carbox group into phenol and in para-position reinforces the bactericidal effect but the toxicity with respect to animal organism is neutralized. The esters are less soluble in water than are the corresponding acids. Frequently the bactericidal efficacy of an ester is influenced by the ratio of lipoid solubility to water solubility, that is, by the so-called distribution coefficient. Within certain limits, the outlook for strong, bactericidal effects is the better, the lesser the water solubility. The effectiveness further depends on the magnitude of the alkyl introduced into the acid. There is a gradual increase in effectiveness in paraoxybenzoic acid for esterification from methyl alcohol to benzyl alcohol. Methyl ester is already 2.6 times as lethal for staphylococcus as free carbolic acid: benzyl ester is 83 times as lethal as phenol. With respect to growth inhibition for staphylococcus, methyl ester is 1.6 as strong, benzyl ester 57 times as strong as free paraoxybenzoic acid; with respect to lethal effects on staphylococcus, the former is 0.7 times as strong, the latter 23 times as strong as the associated acid. Even when sealing the free oxy group with alkyl, this effect is not neutralized, on the contrary, there is further reinforcement (3).

For practical applications as concerns preservatives in foods, pharmaceutical and technical preparations, the esters must of course be endowed with a certain solubility required for effectiveness. Now Sabalitschka (3) has made the significant observation that a mixture o esters provides not only higher solubility, but also a reinforced bacte ricidal effect. Further, the resistance (specific) of certain microbes may thus be easier to deal with. Further yet, it has been shown feasible to achieve a solubility in water up to 0.6% by fusing 1 part

ester with 9 parts sugar, whereas the methyl ester from paraoxybenzoic acid alone is soluble in water only up to about 0.2%, (cold water).

Esters further provide the advantage of being neutral, odorless and tasteless compounds that do not dye the substrate to be sterilized.

It having been shown with certainty that the esters of paraoxybenzoic acid show a strong bactericidal effect, and since they are alre being used on a large scale as preservatives, the important question now arises, how much of it the animal organism can take without being damaged and how these compounds are converted, detoxicated or eliminate The answer to these questions is of the greatest significance for their application in food products as regards their preservation. In this respect, we tested methyl ester, ethyl ester and propyl ester of paraoxybenzoic acid.

Test animals were cats, rabbits and dogs.

I. EXPERIMENTS WITH PARAOXYBENZOIC ACID METHYL ESTER.

a) Tests with cats

Cats were rather sensitive to methyl ester. For various animals, there was reliably vomiting immediately after introduc the ester into the stomach. Others first evidenced saliva flow and naus Already a one-time dose of 1 gm of methyl ester per kg led to loss of appetite. For oral administration, the lethal dose cannot be ascertain A test on a cat is shown below:

Cat, white, female, 2.7 gk weight
Methyl ester feeding

date (1928)	g/kg	total amount (gm)	urine volume (cc)	albumin	saccharum	polarisation	reaction	remar
29 Oct	½	1.35	-	0	0	0	acid	1
30 "	3/4	2.03	-	+	0	0	"	2
31 "	½	1.35	40	+	0	0	"	3
1 Nov	½	1.35	55	+	0	0	"	4
3 "	½	1.35	73	+	Nylander	0	"	5
4 "	½	1.35	50	+	-	-	"	6
5 "	-	-	90	++	0	1=0.5	-	7
6 "	-	-	none	0	0	0	-	8

Remarks:

1. Slight tremor after 30 minutes: no appetite.
2. Vomiting after 20 minutes: does not eat
3. Seems sick
4. Vomiting after 15 minutes; does not eat
5. Very sick. Averse to eating
6. -
7. Weight: 2.12 kg
8. Does not eat. Eliminated.

(A total of 308 cc of urine were collected, of which 143 were evaporate under vacuum at 25°C. 7-8 November: exitus. Weight at death: 1.91 kg. Section: bilateral pleuritis exsudativa fibrinosa purulenta. No other macroscopic finding; kidney examined under microscope).

The animal received a total of 8.775 gm of paraoxybenzoic acid methyl ester. 0.7gm + 0.8gm, i.e., 0.15 gm of paraoxybenzoic acid were recovered from 143 cc of urine. Thus about 3gm of free acid from 308 gm This amounts to about 40% of theoretical.

For another cat, the dose administered was once 1g/kg per os, i.e., for a body weight of 2.88 kg, about 2.88 gm. Urine recovered free paraoxybenzoic acid was about 1 gm. This corresponds to 40% of theoretical.

b) Tests with rabbits

Methyl ester was introduced per os in gradually increasing doses. Only a comparison will be rendered below:

Rabbit, wild-grey, female, weight: 2.65 kg
(methyl ester)

date (1928)	g/kg	total amount in cc	urine volume in cc	albumin	saccharum	polarisation	reaction	remarks
8 Nov.	¼	1.325	180	-	-	l=0.6	alkal.	none
9 "	¼	1.325	120	-	-	-	-	behaves
10 "	¼	1.325	200	-	-	-	-	"
11 "	¼	1.325	135	cloudy	-	-	alkal.	none
12 "	¼	1.325	240	"	-	-	acid	"
13 "	¼	1.325	74	"	-	-	basic	"
14	1	2.65	272	-	-	-	alkal.	"
15	2	5.3	166	+	+	left	acid	behav.(
16	3	7.95	174	cloudy	+	-	"	doesn't e
17	-	-	44	"	+	-	"	another
18	-	-	32	+	-	-	"	-
19	3	7.95	none	-	-	-	-	(1)

(1): somnolent, slow breathing after 60 minutes; ataxic motions; anomalous position; exitus letalis after 84 hours.

Specific weights on 16, 17 and 18 November: 1028, 1045 and 1045 resp.

A total of 31.8 gm of methyl ester were administered per os; 2531 cc of urine were vacuum evaporated. Egg white and sugar were found in the urine of the dead animal.

By shaking with ether, 2.6 gm of paraoxybenzoic acid were obtained from the urine and after heating with sulfuric acid another 1.7 gm were obtained after repeated shaking. Thus a total of 4.3 gm were obtained. This corresponds to 15% of the theoretically possible amount.

c) Tests with dogs

In general dogs are more resistant to paraoxybenzoic acid methyl ester. But these animals too show nausea and vomiting after larger doses. The preparation was administered per os in gradually increasing doses, - in one case, in other cases, we began at once with more massive doses of 1-1½ gm / kg and we rapidly increased those to 3gm. Toxic symptoms were evident after these doses. Several test recor are rendered below:

Dog, longhaired, male: weight: 6.2 kg

date (1928)	g/kg	total amount in gm	urine volume in cc	albumin	sacha-rum	polari-zation	reaction	remarl
30 Oct	1	6.2	-	-	-	-	-	none
31 "	½	3.1	250	cloudy	-	-	acid	"
1 Nov.	½	3.1	520	0	0	1=0.5	"	"
2 "	½	3.1	405	0	0	-	"	little vomitir
3 "	½	3.1	260	cloudy	pvl.+	-	"	none
4 "	½	3.1	550	"	0	-	"	no dia
5 "	½	3.1	305	"	0	1=0.3	"	good di eats

A total of 2530 cc of urine were evaporated in vacuum. This corresponds 21.7 gm of incorporated methyl ester.

3.7 gm of paraoxybenzoic acid were obtained from the urine by shaking with ether. This corresponds to about 17% of the theoretically possible amount. After a pause of 10 days, the dog was tested again.

date (1928)	g/kg	total amount in gm	urine volume in cc	albumin	sacha-rum	polari-zation	spec. weight	reac-tion	
17 Nov.	1.5	9.3	305	-	-	-	1033	acid	1
28 "	2.0	12.4	122	0	+	-	1011	-	2
29 "	2.0	12.4	505	cloudy	+	-	1020	acid	3
10 Nov*	2.0	12.4	375	"	+	-	1037	neutr.	
11 "	3.0	18.6	91	+	+	-	1040	acid	4
22 "	-	-	300	+	+	-	1019	"	5

Notes: 1. active; 2. vomiting after 6 hours; 3. no diagnosis;
4. immediate vomiting of 2/3 of incorporation; does not eat; lies anathetically; twitching after 10 hours and fearfulne
5. does not eat; increased reflex irritability; deep breathing; lies on side; occasional tetanus-like twitching.

1584 cc of urine were evaporated under vaccum; a total of 65.1 gm of paraoxybenzoic acid methyl ester had been administered per c No recovery from poisoning. Exitus letalis on 23 NOvember.

9.1 gm of free paraoxybenzoic acid were recovered from the urine. This corresponds to about 17% of theoretical.

Section diagnosis (Dr Wanger -4-)

No particular diagnosis as to brain and chest cavities. Liver unusually fatty. Several pin-head sized petechias on the surface. MIliary spots on section. Tumors... Spleen almost slightly shrunk. Kidneys very fatty. Urine bladder shows numerous scattered submucous bleedings. Intestinal wall slightly inflamed. Small intestine much inflamed. Tumors with scurf. Several lentil-sized bleedings (submucous) in the large intestine, partly necrotic.

MIcroscopic examination: Kidney: several, partly accumulated, fresh leucocyte foci. Glomerules strongly hemorrhaged; small drop adiposis of the straight urine canal, uneven adiposis of the winding one, where partly lacking. Occasional fat drops in glomerule epithelia

Inflamable infiltrates lacking in heart.

Multiple miliary hemorrhaging tumors in liver. General leucocyt particularly in capillaries. Small-drop dinosis of almost all liver cells, particularly strong azino-peripherally. Kupffer's stellate cell are normal. Hyperemia of vessels.

Lungs are somewhat heremious. They contain small, unfolding tumors.

A series of spleen pulp cells contains fine-grained fat, giant bone marrow cells dragged and scattered in the vessels.

Leucocytose in intestine is in the form of cell decomposition. Vessels show very strong leucocytose. Tumors with strong hemorrhage. Submucus is edematously penetrated. Burst tumor (exulceration). Strong perivascular leucocyte infiltrates.

Urine bladder shows bleedings with mucous membrane destruction

No pathological changes in eyes.

Kidney, heart, liver, lung and spleen fats are not doubly-refracting in polarized light.

Another male dog of 11.5 kg weight was given 1.5 g/kg of paraoxybenzoic acid methyl ester the first day, 2 g/kg the second day, 2 g/kg the 3rd day, a total of 63.25 gm. The urine mostly shows a positive Nylander test, the specific weight fluctuates from 1012 to 1038. The animal vomited once, showed no appetite, was always apathet and somnolent. Two days after the last feeding the animal continuousl bled from the mouth. Blood also in stool. However the animal ultimate recovered gradually.

3475 gm of urine were evaporated under vacuum and 7.8 gm of paraoxybenzoic acid were recovered therefrom. This is about 15% of theoretical.

This test shows that 2 g/kg of paraoxybenzoic acid methyl ester may act toxically.

In another test, the doses were daily increased by 1 gm/kg.

Male dog MINKO; weight: 8 kg; pinscher; (methyl ester).

Date (1929)	g/kg	urine amount in cc	albumin	Nylander	Fehling	pola-risation	spec. weight	reacti
13 Mar	1(8gm)	180	-	-	-	-	1040	alka.
14 "	2(16")	90	cloudy	+	-	left rotat.	1034	neut.
15 "	3(24")	320	-	++	+	left rot.	1020	"

Notes: 1. Is all right: 2. ditto: 3. Even after precipitation with lead acetate, urine still strongly rotates towards left. Upon addition of HCl, oxybenzoic acid precipitates.
In all three cases, sediment: -

16 March: 7 AM: dizzy, but still quite upright. 8 AM: lies on side, breathes deeply. Starting with 8:15 AM, clonic-tonic irregular cramps occur, lasting each about one minute. Cramps frequently are tetanus-li opisthotonose. Intervals between cramps are about 10-15 minutes, followed by deep breathing. After 10 AM cramps are fewer, The poisonin much reminds of uremia.

Section: miliary, submucous, diffuse bleedings in stomach. Liver reminds of human nutmeg oil liver. Kidneys very bloodrich.

URINE EXAMINATION FOR INDIVIDUAL ANIMALS

The urine of each animal was collected and for as low as possible a temperature was evaporated to a small volume under vacuu in order to avoid decomposition. Then several samples were purified if possible with lead acetate and the excess lead was removed with hydrogen sulfide. However this lead purification led to losses as regards free paraoxybenzoic acid, even though it is not precipitated by lead acetate. But because of adsorption and lead acetate precipitation, part of the paraoxybenzoic acid is carried along. Therefore lead precipitation was abandoned. The condensed urine first was shaken 6-10 times with ether in abundant amounts. P-oxybenzoic acid being very soluble in ether, it may easily be shaken out of the urine. Thereafter the urine was reacted with sulfuric or hydrochloric acid until a strongly acid reaction had been obtained. We had engured previously that p-oxybenzoic acid combines only slightly with sulfuric acid and amino acetic acid in the organism. The paired sulfuric acid is but slightly increased. Thus we could corroborate the findings of Baumann and Herter (5). Recently Power & Sherwin (6) claimed furthermore that p-oxybenzoic acid fed (to animals) would be eliminated uncombined. If this strongly acid urine is heated for some time in a water bath, the oxybenzoic acid combined with sodium is released; possibly too there occurs a hydrolysis of the organically bound acid. Soon a deep red dye appears. The reaction mixture smells of phenol and of cresols. After cooling, the "bound" paraoxybenzoic acid may be shaken out with ether. The attempt was made to shake out quantitatively the free and bound p-oxybenzoic acid with ether. Excrement samples were tested for ester and acid contents. The excrements were carefully mixed with water and heated in the water bath and then evaporated to dryness. Neither ester nor its separation product, the free acid, could be found. This indica that the esters of the paraoxybenzoic acid in the stomac intestinal tract probably were wholly reabsorbed. It is only of minor significanc

whether they are saponified in the intestine wall or only in the blood

Recently the Japanese Kyoji Kase (7) investigated the physiological effects of salicyl derivatives. On the basis of his work, he assumes that paraoxybenzoic acid methyl ester is more toxic than salicylic acid. When determining the minimum lethal dose for mice, he did however not test at all the paraoxybenzoic acid methyl ester. Salicylic acid sodium salt was injected in rabbits intravenousl while paraoxybenzoic acid methyl ester on the other hand was administered per os, and then in much larger doses, namely 0.14 gm/kg for the salicylic acid sodium salt and 0.68 g/kg for paraoxybenzoic acid methyl ester. Appreciable disorders in behavior could be observed for neither case. Because of the different methods of ingestion, both methods cannot be compared, even aside from the question of different dosages; it makes a difference whether a substance is administrated per os or intravenously. In the latter case, an organic lipoid-solubl substance will show a much different effect when being used directly than when being used as a lipoid insoluble sodium compound, as was don by Kyoji Kase for salicylic acid. He provides no indication as to the dosis used for the pregnant rabbit which died after being given paraoxybenzoic acid methyl ester. In any event, Kyoji Kase's assumption that paraoxybenzoic acid methyl ester is more toxic than salicyli acid is unquestionably erroneous, it is less so. However his indicatio are correct in one respect, namely that methyl ester is more toxic than its particular free acid, and this because of its higher lipoid-solubility.

II TEST WITH P-OXYBENZOIC ACID METHYL ESTER

a) Experiments with cats.

A cat was given for 8 days 4 g/kg per os of ethyl ester daily, and once 3/4 g/kg, for a total of 11.7 gm. Besides a one-time choking or retching and saliva flow, no toxic symptoms were observed. Occasio ally there was a positive Uhlander sample for the urine, but no Trommer reduction, and always acid reaction. 204 cc of urine were boiled off under vacuum down to a small volume (25 cc) and yielded 2.3 gm of free and 0.6 gm of combined p-oxybenzoic acid by means of shaking with ether. This corresponds to about 33% of the theoretical amount.

b) Test with rabbits (ethyl ester)

black female rabbit: weight: 2.8 kg

	date (1929)	g/kg	urine volume in cc	albumin	saccha-rum	colori-sation	spec. weight	reaction	total amount in gm
(1)	16 Jan.	4	128	-	+	-	1025	neutr.	11.2
(2)	17 "	5	86	-	+	rotates left	1040	acid	14.0
	18 "	receives no preparation							

Notes. (1): cough soon after administration for a few minutes.

(2): loss of appetite, fairly somnolent.

Exitus letalis in the night of 18-19.

Section : a white layer forms on the blood when standing. (Ester?). Minor bleeding in the submucus of the stomach. Otherwise only miliary knots in gall bladder.

2 gm of p-oxybenzoic acid were shaken out of 230 cc of urine concentrated under vacuum. This corresponds to 10% of theoretical. The rabbit had been administered a total of 25.2 gm of ethyl ester.

c) Tests with dogs.

A dog weighing 6.2 kg for 5 days was administered daily 4 gm/kg and for two subsequent days 1 gm/kg of ethyl ester, a total of 27.0 gm. Once there was vomiting after 1g/kg, otherwise there were no particular disorders. 10.7 gm of p-oxybenzoic acid could be recovered from 2232 cc of urine boiled off under vaccum, or 49% of theoretical.

A second dog weighing 12.5 kg received 1 gm/kg on the first day, 2gm/kg on the second day, 3 gm/kg on the third day and 4gm/kg on the fourth day. No egg white occurred in the urine. The Nylander test most often was positive. The urine rotated several times left. For the administration of 3g/kg and for 4g/kg, vomiting occurred only after 10-12 hours. After the large doses* of 4gm/kg, a certain sleepiness was observed and little appetite. The dog was normal again after 2 days. He was then administered 3 gm/kg and 2 gm/kg for two da

The animal was administered the huge amount of 187.5 gm of ethyl ester without serious toxic symptoms appearing. 5.0 gm of free and 4.0 gm of combined p-oxybenzoic acid could be isolated from 7603 cc of urine that was boiled off under vacuum, or about 7% of theoretic

One may conclude from these test results that p-oxybenzoic acid ethyl ester indeed is less toxic than the methyl ester of that acid.

Thereupon propyl ester was tested for comparison.

III TESTS WITH PARAOXYBENZOIC ACID PROPYL ESTER

a) Tests with dogs.

A male dog weighing 7.5 kg was administered daily and in increasing dosages 1,2,3,4 gm/kg. Only for 4gm/kg did the animal show minor sleepiness and diarrhea. The dog would not eat on the 5th day and therefore was administered nothing. On the 6th day he received 4gm/kg and on the 7th 3 gm/kg of polyester per os. In general the animal was all right. Occasionally the urine reduced the Fehling/Nylander solution. The dog had received a total of 127 gm of propyl ester. 5 gm of free and 13.5 gm of combined p-oxybenzoic acid, i.e., a total of 18.5 gm, could be recovered from 3124 cc of urine that was boiled off under vacuum. This corresponds to about 21% of the theoretically possible amount.

* sic.

b) Rabbit test

gray female rabbit, wighing 2.25 kg

p-Oxybenzoic acid propyl ester

date (1928)	g/kg	total amount ester in gm	urine volume in cc	albumin	saccharum	polarisation	spec. weight	reaction
8 Nov.	½	1.125	67	-	-	l=0.3	-	alk.
9 "	½	"	50	-	-	-	-	"
10 "	½	"	54	-	-	-	-	"
11 "	½	"	81	-	-	-	-	"
12	½	"	90	cloudy	-	-	-	"
13	½	"	74	"	-	-	-	"
14	1	2.225	102	+	-	-	-	acid
15	2	4.5	39	+	-	l=1.0	-	neut.
16	3	6.75	218	cloudy	-	-	1023	acid
17	-	-	-	-	-	-	-	-
18	-	-	98	+	-	-	1035	acid
19	3	6.75	60	-	-	-	1037	"
20	-	-	100	+	-	-	1035	"
21	-	-	29	cloudy	-	-	1034	neut.
22	-	-	66	-	-	-	1022	acid
23	-	-	106	+	-	-	1020	alk.
24	3	6.72	152	cloudy	-	-	1022	neut.
25	-	-	40	dubious	-	-	1025	"
26	4	7.76	170	cloudy	-	-	1030	"
27	-	-	-	-	-	-	-	-
28	-	-	9	-	-	-	1025	acid
29	5	11.05	73	cloudy	-	-	1007	neut.
30	6	13.26	60	-	+	-	1023	acid

7,8:

Notes: 1. OK; 2. No findings; 3,4,5,6: no findings; 9: does not eat; 10. No urine, loss of appetite; 11: does not eat; 12: eats, weighs 1.9 kg; 13: sick; 14:-; 15: normal; 16: no findings; 17,18,19: -; 20: does not eat; 21: does not eat; 22: OK; 23:-

11 hours after administering ester, animal shrunk together; did no eat all day; deep breathing and slowed. Head bowed forward. Terminal exitus letalis.

Section: two stomach ulcers the size of lentils and numerous bleedings in their vicinity (submucous); liver very adipose, also kidneys.

The rabbit received a total of 65.79 gm of p-oxybenzoic acid propyl ester. 1787 cc of urine were boiled off in vacuum to syrup consistency. However only 0.6 free and 1.1 gm combined p-oxybenzoic acid could be recovered therefrom, a total of 1.7 gm, corresponding to about 4% of theoretical.

Concerning parcoxybenzoic acid.

As already mentioned, p-oxybenzoic acid could be obtained from all the urines of the test animals in free and combined form by shaking with ether. Most of the time the raw acid was brown or red. By repeated boiling with animal charcoal and by means of five or six-fold recrystallisation from boiling water, this acid finally is obtained in beautiful, silk-shiny crystals with a melting point of 210-212°C. The recrystallisation mother lyes will color deep green with iron chloride, like pyrocatechin. It is likely those are decomposition products of p-oxybenzoic acid, since this latter does not color in the pure state when reacted with iron chloride. At 12°C, 0.3 gm will dissolve in 100 parts water; at 34°C, 0.9 gm. P-oxybenzoic acid is very soluble in ether. At 17°C, 100 cc ether will dissolve 9.43 gm (Beilstein). The acid behaves monobasically with respect to litmus and Congo-red; p-oxybenzoic acid is termed non toxic in the literature. Baumann & Herter (8) have shown, that in the dog's organism, the acid is partly converted into 4-oxyhippuric acid and 4-carboxy-phenyl sulfuric acid. According to Fribrom (9), p-oxybenzoic acid is characterized by a diuretic effect. . The pancreatic juice decomposes it into phenol and carbon dioxide (Baumann -10). This finding may explain why only a smaller or larger part of the p-oxybenzoic acid expected actually is found in the urine. Even though Kolbe claims that this acid is not acting antiseptically, Sabalitschka recently found reliabl that its effect on micro-organisms is stronger than phenol's. By introducing a carboxyl group into the carbolic acid, one also achieves a reinforcement of the bactericidal effect. The toxicity on the human organism supposedly is eliminated. In order to prevent yeast fermentation, three times as much p-oxybenzoic acid and 7 times as much carbolic acid is required as salicylic acid.

ELEMENTARY ANALYSES AND DETERMINATIONS OF MOLECULAR WEIGHTS

Substance I, obtained by shaking the urine with ether, and substance II, obtained after boiling with concentrated hydrochloric acid and renewed shaking with ether, were repeatedly recrystallized from water and dried over phosphorus pentoxide. One thus obtains th melting point, the lye consumption during titration and the carbon and hydrogen contents by means of elementary or ultimate analysis.

SUBSTANCE I:

melting point: 214-215°C (corrected)

equal parts mixture of strongly dried p-oxybenzoic acid melting point, corrected: 214-215°C

0.1652 gm of the substance were used up during titration with alizarin yellow as indicator 24.05 n/10 lye, corresponding to 0.1659 gm of p-oxybenzoic acid

0.1986 gm of the substance yielded 0.4430 gm of CO_2 and 0.0778 gm of H_2O.

Computed: C=60.9%; H= 4.3%; found: C= 60.8%; H= 4.38%.

Substance II:

melting point: 213-214°C (corrected);

equal parts mixture of strongly dried p-oxybenzoic acid meltin point: 213.5 - 214.5°C (corrected):

0.1001 gm of the substance were used for titration with alizar yellow as indicator 15.85 n/10 lye, corresponding to 0.1094 gm of p-oxybenzoic acid:

0.1782 gm of the substance yielded 0.3072 gm of CO_2 and 0.0691 gm of H_2O;

Found: C=60.8 %: H=4.34

Computed: C=60.9 : H=4.3

From the elementary or ultimate analyses, from the molecular weight determinations by means of titration, finally from the melting points, particularly after mixing the isolated p-oxybenzoic acid obtained from the urine with pure one, these perfuce results that both substances are identical and consist of pure p-oxybenzoic acid.

SUMMARY

The methyl, ethyl and propyl esters of parooxybenzoic acid were analyzed in the organisms of the cat, rabbit and dog. It is likely that saponification occurs for the alkaline small intestine reaction. This causes parooxybenzoic acid formation which is eliminated as such and in the form of salts through the kidneys. A small part of this acid seems to combine with sulfuric acid and glycocoll. Detorificatio due to saponification occurs to the extent that, while the esters do indeed form a bactericidal acid, it seems non-toxic for human and ani organisms. It seems that part and probably the largest part of this acid is decomposed in the organism. Perhaps the pancreas is involved. One should assume that phenol and carbon dioxide are formed, so that decomposition proceeds further yet and that the benzol ring is broken

It was shown that the toxicity decreases with the magnitude of the alkyl in the parooxybenzoic acid esters, while on the other hand the bactericidal effectiveness increases. Of the three esters, methyl ester is the most toxic, propyl ester is the compound most effective against microbes. Very likely the toxicity in the animal organism depends on the distribution coefficient, i.e., on the ratio of water solubility to lipoid solubility. The esters are easily soluble in fats, but not their resultant p-oxybenzoic acid. We do no know whether the esters as such may be reabsorbed without saponification. In any event, the alcohols formed upon ester splitting may al be of significance toxically. In that respect the saponification rat is of great significance. If for instance methyl ester is being saponified slowly, the resultant methyl alcohol slowly enters the blood stream and the cells. Methyl alcohol can be formed rapidly and and therefore there may be flooding and poisoning of the organism, or the alcohol released may be created gradually and not reach the toxic threshold because failing to reach the toxic concentration on account of elimination or combustion. This viewpoint should be of the highest significance as regards methyl alcohol. 1 gm of methyl ester yields o.2 gm, or 1/5 of the ester mass, in methyl alcohol. When incorporating 5 gm of ester, the human organism would thus form 1 gm of methyl alcohol: when ingesting 10 gm of methyl ester, this would be 2gm of methyl alcohol. These quantities do not act toxicall

and in practice it is unlikely that when eating from cans, 10 gm of ester should be ingested at once. 1 gm of ethyl ester will yield 0.28 gm of ethyl alcohol, and 1 gm of propyl ester will yield 0.33 gm of propyl alcohol. These alcohols are so little toxic that they canno play a poisonous role. Only the formation of methyl alcohol might be dangerous (11), but there is none such in this case. Apparently slight more prone damages are incurred in the kidneys and liver at higher doses of methyl ester than for the other esters. No formic acid ever could be found in the urine after administering larger quantities of methyl ester. This acid must be formed from methyl alcohol by oxidation. However after administering the three esters and particularly when in high doses, varying amounts of egg white could be found in the urine, also reducing substances and left-rotation. The latter persists after boiling the urine with hydrochloric acid. Hence the substance causing left-rotation is not easily decomposed by acids, since normall the rabbit urin reacts alkaline. The urine, however, because of the elimination of the p-oxybenzoic acid, may become neutral, even acid.

Chronic feeding of the three esters showed that accumulation do not occur.

15-40% of the computed amount of p-oxybenzoic acid are eliminat through the kidneys for methyl ester ingestion, 7-49% for ethyl ester ingestion, and 4-21% for propyl ester ingestion. Neither ester nor fre p-oxybenzoic acid could be found in the droppings. Toxic and lethal doses of methyl ester for both dog and rabbit are resp. 2g/kg and 3 gm/kg, while for ethyl ester they are resp. 4gm/kg and 5gm/kg, and for propyl ester 3-4 gm/kg and 6 gm/kg.

If salicylic acid methyl ester is compared, there results that it is more toxic than p-oxybenzoic acid methyl ester, since the former will be reportedly lethal already for 2-2.1 gm/kg in dogs. The free salicylic acid also seemes more toxic, since 1.1-1.6 gm of this acid are lethal to rabbits, and 0.4 - 0.5 gm of the sodium salt of this acid are lethal to dogs, and 1.2-1.68 gm of the sodium salt of this acid are lethal to rabbits (12).

In any event the paraoxybenzoic acid esters are less toxic thar benzoic acid and its sodium salt, about which, according to Rost etal (13) already 1 gm/kg and 2gm/kg act resp. toxically and lethally.

There is insufficient explanation for the problem of kidney damage and transient occurrence of egg white in the case of toxic doses, also for the urine's left-rotating compound. It is unlikely that kidney damage may occur from the paraoxybensoic acid being formed which generally is considered as non-toxic. It is unlikely furthermo that non-saponified esters reach the kidneys. Perhaps the latter become diseased when large doses are being administered because they actively participate in the detoxification as an elimination organ.

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(11) Dr. Schmeltzer examined histologically the eyes of dogs dead p-oxybenzoic acid methyl ester doses. No pathological finding ever was made. I thank Dr. Schmeltzer...

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RECENT DEVELOPMENTS IN THE PRESERVATION OF PHARMACEUTICALS*

By H. Sokol**

From the earliest times, pharmacists and pharmaceutical chemists have been troubled by the development of microbial contamination in preparations which are subjected to prolonged periods of storage or are packaged in multiple dose containers and used in small portions. It was soon discovered that certain preparations, because of high ionic concentrations, acidity or alkalinity, presence of alcohol, etc., remained resistant to bacterial contamination in spite of repeated exposure to contaminating conditions. Developments in the fields of microbiology and antisepsis ultimately revealed the presence of certain chemical agents which had the ability to inhibit the growth of a wide variety of microorganisms at relatively low concentrations. One of the earliest and most widely used type of compound in this category was phenol.

As experience was gained in this field, the development of new preservatives and their increasing importance in the marketing of pharmaceuticals combined to produce a situation such as we encounter today. Most manufacturers of pharmaceutical preparations would not contemplate the introduction of a new product without devoting considerable time and effort to the study of the shelf life of the product and the type of preservative which will be compatible with the product and provide ample antimicrobial protection during this period.

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PREPARATIONS REQUIRING PRESERVATIVES.—The types of pharmaceutical preparations which may require preservatives are given in Table I. This tabulation encompasses almost all the types of pharmaceutical products on the market today. It is further evident that the work of the pharmaceutical chemist is complicated by the wide variety of vehicles, active principles, and inert ingredients which may be contained in these groups of pharmaceuticals, and the development of a finished preparation must give consideration to the compatibility of the preservative with the other components of the formulation.

TABLE I.—TYPES OF PHARMACEUTICAL PREPARATIONS REQUIRING PRESERVATIVES

Parenteral solutions and suspensions	Suppositories and jellies
Oral solutions, suspensions, and emulsions	Gums and alginates
Ointments	Syrups
Drug extracts	Mouth wash and toothpaste
Eye lotions	Lotions and creams

As a case in point, there can be mentioned the difficulties which many members of the pharmaceutical industry have experienced, and are still experiencing, with microbial contamination of a pharmaceutical type included in the first category in Table I, namely, the aqueous suspension of procaine penicillin. Manufacturers of this particular formulation have devoted considerable energy to the selection of a preservative which will neither act to increase the rate of decomposition of the active ingredient nor permit the development of color during the life of the product. The problems with such a product are considerably different from those encountered in the preservation of eye lotions, for example, where entirely different factors with regard to use, irritation, and stability must be considered. The preservative to be included in each different formulation must be chosen independently on the basis of the particular set of characteristics possessed by the formulation and the use to which it is to be put.

PRESERVATIVE CRITERIA.—In general, regardless of the type of formulation under consideration, certain criteria must always be borne in mind when selecting a suitable preservative. The characteristics of the ideal preservative were first set down in a formal manner by Gershenfeld and Perlstein (1). These are reproduced in Table II. Several of these characteristics are of such a nature as to prohibit complete fulfillment under any circumstances. For example, it is doubtful if any single chemical agent will ever be found to be wholly effective in inhibiting all types of microorganisms causing decomposition at sufficiently low concentrations considered to be completely nontoxic in all the various preparations outlined in Table I.

PHARMACEUTICAL PRESERVATIVES.—As a consequence of the wide variety of conditions which must be met in this field, it is not surprising that a relatively large number of preservatives has been suggested and used at one time or another. Table III presents a partial listing of the most popular members of this group. Consideration of the properties of these preservatives

TABLE II.—CHARACTERISTICS OF AN IDEAL PRESERVATIVE

1. Effective against all types of micro-organisms causing decomposition.
2. Soluble in the concentration used.
3. Not toxic where used and in concentration employed.
4. Compatible; must not alter the character of the preparation as far as objectionable odor, taste, etc., and must be practically neutral so that it will not alter the *pH* of the preparation.
5. Cost should not increase the price of the preparation to any marked extent.
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.

TABLE III.—PHARMACEUTICAL PRESERVATIVES

Alcohol	Boric acid
Phenol or cresol	Chloroform water
Benzoic acid and its sodium salt	Mercurials
Salicylic acid and derivatives	Mercuric cyanide
Esters of *p*-hydroxybenzoic acid	Phenyl mercuric acetate (borate, nitrate, chloride)
Chlorobutanol	Merthiolate (Sodium ethylmercurithiosalicylate)
Quaternary ammonium salts (Zephiran, Phemerol)	Metaphen (Anhydride of 4-nitro-5-hydroxymercuri-*o*-cresol)
Formaldehyde	
Sodium bisulfite	

in the light of the ideal characteristics proposed by Gershenfeld and Perlstein will immediately relegate certain of these materials to a position of questionable utility. For example, most of us would not be very enthusiastic over the incorporation of formaldehyde or chloroform water in pharmaceutical products to be marketed under present day conditions. In a similar manner, alcohol, salicylic acid and its derivatives, and boric acid are useful only under very narrow circumstances and at concentrations which make them unattractive in comparison with other available preservatives. The esters of parahydroxybenzoic acid, on the other hand, were proposed by Gershenfeld and Perlstein (1) in 1939 as the most useful preservatives available at that time, when evaluated on the basis of their criteria. These esters had been first reported to be useful in preserving pharmaceuticals, cosmetics, and foods by Sabalitschka (2) in Germany in 1924, and in the years that followed many articles on the subject appeared in European journals.

PHYSICAL PROPERTIES.—Intensive investigation of the efficacy, safety, and compatability of these esters has substantiated the contention of Gershenfeld and Perlstein so that today the esters of parahydroxybenzoic acid have become popular and useful preservatives in the pharmaceutical field. Commercial production of these compounds began in the United States

in the early 1930's; they now are available to the industry under a variety of trade names including the Parabens (the nomenclature used in the U.S.P. XIV), the Parasepts, the Tegosepts, Nipasols, Nipagins, etc. These esters now are officially recognized by the pharmacopoeias of Switzerland, Great Britain, and the United States. Table IV lists certain of the physical properties of the five commercially available esters of parahydroxybenzoic acid.

TABLE IV.—PROPERTIES OF SOME ESTERS OF P-HYDROXYBENZOIC ACID

p-Hydroxybenzoate	Methyl	Ethyl	Propyl	Butyl	Benzyl
Molecular Weight	152.14	166.17	180.20	194.23	228.24
Melting Range	125-127°C	116-118°C	97-100°C	68-71°C	108-113°C
Solubility - Gm./100 Gm., 25°					
Acetone	64	84	105	240	102
Benzene	0.7	1.65	3	40	2.6
Carbon Tetrachloride	0.1	0.9	0.8	1	0.08
Ether	23	43	50	150	42
Methanol	59	115	124	220	79
Ethanol	52	70	95	210	72
Peanut Oil	0.5	1	1.4	5	0.5
Propylene Glycol	22	25	26	110	13
Water	0.25	0.17	0.05	0.02	0.006
Water*	2	0.86	0.30	0.15	0.09

*At 80°C.

PRESERVATIVE LEVELS.—A tabulation was prepared some time ago by Suess (3) (Table V) and, during the past fifteen years, has served as a guide to most pharmacists in selecting the proper level of preservative for incorporation in any desired type of pharmaceutical product. In general, it may be concluded from this presentation that the greater the molecular weight of the ester in the alkyl series the smaller the quantity recommended for preservation of any of these types of pharmaceutical preparations.

During the past few months, a considerable body of new evidence has been accumulated which tends to offer additional substantiation to the conclusions reached by the early workers with the esters of parahydroxybenzoic acid regarding their efficacy and safety. It is the purpose of the remainder of this presentation to offer the results of some of the more recent work with this interesting series of esters. More complete reports are now in the process of being written and will shortly be submitted for publication in the Scientific Edition of the *Journal of the American Pharmaceutical Association.*

Most of the information which appears in the literature of the efficacy, of the Parasepts and other agents recommended as pharmaceutical preservatives

is fragmentary, contradictory and, by and large, virtually impossible to correlate in a manner necessary to give satisfactory comparative information regarding these various agents. This is due in part to the fact that various investigators have used different tests organisms as well as different testing procedures.

TABLE V.—EFFECTIVE PRESERVATIVE LEVELS

Type of Preparations	Parahydroxybenzoic Acid Esters, % Required				
	Methyl	Ethyl	Propyl	Butyl	Benzyl
Creams					
Free from oil	0.15	0.05	0.02	0.01	0.01
Low oil content	0.3	0.1	0.03	0.02	0.01
Medium oil content	0.5	0.15	0.1	0.07	0.05
High oil content	1.0	0.3	0.2	0.15	0.1
Containing cholesterol and lecithin	0.3	0.1	0.03	0.02	0.01
Lotions					
Low oil content	0.3	0.1	0.03	0.02	0.01
High oil content	0.5	0.15	0.1	0.07	0.05
Jellies					
Acacia, tragacanth, pectin, starch, gelatin, agar, Iceland moss	0.15	0.05	0.02	0.01	0.01
Syrups	0.5	0.15	0.1	0.07	0.05
Aqueous solutions and mixtures	0.15	0.05	0.02	0.01	0.01

ANTIFUNGAL AND ANTIBACTERIAL PROPERTIES.—In order to clarify certain of the conflicting reports regarding the antimicrobial activities of the Parasepts, experiments were designed to screen the four alkyl esters of parahydroxybenzoic acid against a group of typical fungi which may be encountered as contaminants as well as a group of some of the more common bacteria. Results obtained in the evaluation of the antifungal properties of the four Parasepts are tabulated in Table VI. The antifungal tests were performed by dissolving the esters in methanol at such concentrations that when 0.5 ml. was added to 25 ml. of broth, the flask contained the desired final concentration of preservative. The medium was made up by dissolving 85 grams of honey and 10 grams of peptone in enough water to produce one liter of broth. This was dispensed into 125-ml. Erlenmeyer flasks which were plugged and autoclaved for twenty minutes at 20 lb. steam pressure. These flasks were inoculated with one drop of spore suspension made by adding 25

TABLE VI.—ANTIFUNGAL PROPERTIES OF FOUR ESTERS OF P-HYDROXYBENZOIC ACID

	Methyl, %	Ethyl, %	Propyl, %	Butyl, %
Aspergillus niger NRRL 337 (ATCC 10254)	0.1	0.04	0.02	0.02
Penicillium digitatum ATCC 10030	0.05	0.025	0.00625	<0.003125
Rhizopus nigricans ATCC 6227A	0.05	0.025	0.0125	0.00625
Trichoderma lignorum ATCC 8678	0.025	0.0125	0.0125	0.00625
Chaetomium globosum ATCC 6205	0.05	0.025	0.00625	<0.003125
Trichophyton mentagrophytes ATCC 9533	>0.008	0.008	0.004	0.002
Trichophyton rubrum ATCC 10218	>0.008	0.008	0.004	0.002
Candida albicans ATCC 10231	0.1	0.1	0.0125	0.0125
Saccharomyces cerevisiae ATCC 9763	0.1	0.05	0.0125	0.00625
Saccharomyces pastorianus ATCC 2366	0.1	0.05	0.0125	0.00625

ml. of sterile water to one well grown out slant of the organism. Each concentration was run in triplicate for a period of seven days at room temperature. At the end of this time the quantity of growth was read. The numbers indicated in Table VI represent the percent concentration of the water in the final test medium producing complete inhibition of the organism. It will be noted that all four esters exhibit considerable activity against this rather broad spectrum of fungi. Further, it should be pointed out that the conditions employed are those which are quite conducive to the growth of these fungi and are quite different from those normally encountered in the standard type of pharmaceutical preparation where the substrate is not as rich as that used in this test. It may be stated with a reasonable degree of assurance that the concentrations of esters inhibiting the fungi in this test represent maximal concentrations for use in pharmaceutical preparations where fungus contamination would cause most serious difficulties.

Further study of Table VI reveals quite strikingly that antifungal activities increase sharply with increase in molecular weight of the alkyl group. However, it must be recalled that solubility in water of these esters decreases in the same manner in progressing from the methyl through the butyl compound; hence it is necessary to compromise in the selection of the ester and concentration to be employed under any given set of circumstances.

In a similar manner, the antibacterial properties of these same four esters of parahydroxybenzoic acid were evaluated against a broad spectrum of both Gram-positive and Gram-negative organisms. The results of this investigation are presented in Table VII. In this study, the plate streak method was employed. The dilutions of the sample were made in water using sufficient sodium hydroxide for solubility. The medium was so prepared that, when 5.0 ml. of the sample was added to 10 ml. of medium, the proper concentration of Parasepts and ingredients was obtained. The medium was composed of glucose 22.5 Gm., peptone 15.0 Gm., sodium chloride 2.25 Gm., beef extract 4.5 Gm., agar 22.5 Gm., and water 1 liter. This medium was dispensed into test tubes and sterilized. After addition of the samples of Parasepts, the contents of the tubes were poured in plates. Inoculations were made by dipping a loop in the suspension from 10 ml. of water on a 24-hour slant of the organism and streaking on one sector of the plate. Readings were made after 24-hour incubation at 37°C. The numbers listed in Table VII again indicate the per cent Parasept in the final medium providing complete inhibition

TABLE VII.—ANTIBACTERIAL PROPERTIES OF FOUR ESTERS OF P-HYDROXYBENZOIC ACID

	Methyl, %	Ethyl, %	Propyl, %	Butyl, %
Bacillus subtilis ATCC 6633	0.2	0.1	0.025	0.0125
Bacillus cereus var. *mycoides* ATCC 6462	0.2	0.1	0.0125	0.00625
Staphylococcus aureus F.D.A., strain 209P (*Micrococcus pyogenes* var. *aureus* ATCC 6538P)	0.4	0.1	0.05	0.0125
Sarcina lutea[a], Mount Sinai	0.4	0.1	0.05	0.0125
Klebsiella pneumoniae F.D.A., strain PC 1602 (ATCC 10031)	0.1	0.05	0.025	0.0125
Escherichia coli ATCC 9637	0.4	0.1	0.1	0.4
Salmonella typhosa[b]	0.2	0.1	0.1	0.1
Salmonella schottmulleri[b]	0.2	0.1	0.05	0.1
Proteus vulgaris ATCC 8427	0.2	0.1	0.05	0.05
Aerobacteraerogenes ATCC 8308	0.2	0.1	0.1	0.4
Pseudomonas aeruginosa ATCC 9027	0.4	0.4	0.8	0.8
Pseudomonas fluorescens ATCC 9721	0.4	0.2	0.2	0.4

[a]Culture obtained from Mount Sinai Hospital, New York, New York.
[b]Culture obtained from Dr. Geoffrey Rake, E. R. Squibb and Sons, New York, New York.

of the test organisms. The same considerations regarding the order of magnitude of the concentrations necessary to inhibit completely these bacteria, as were mentioned for the fungicidal values, apply in this case; that is, these concentrations probably represent maximal values for pharmaceutical preparations because of the optimum condition of the substrate.

Several interesting points become evident from a study of these data. One is, that somewhat greater concentrations of the Parasepts are required to inhibit completely bacteria than fungi, although it should be borne in mind that these two tests were performed in somewhat different manners. In addition, it is interesting to note that the Parasepts are, by and large, more active against the Gram-positive organisms tested than the Gram-negative ones. In this latter class of organisms, we also note a failure of the alkyl substituent-molecular weight principle; that is, in certain cases the butyl ester is apparently less active than the lower esters.

On the basis of these inhibiting values presented in Tables VI and VII, it is certainly evident that the optimum concentrations of Parasepts recommended by Suess (3) as presented in Table V do indeed have a real basis in fact according to these microbiological data, particularly with regard to aqueous systems.

In order to compare the activities of a Parasept with those of two other popular preservatives, the aforementioned test techniques were applied to phenol and to sodium benzoate, as well. The results of the comparative activities against six bacteria and one fungus, namely, *A. niger*, one of the most resistant members of this group, are presented in Table VIII. From these numbers, it is evident that sodium benzoate is somewhat inferior to phenol, whereas Propyl Parasept by comparison certainly appears quite favorable. It is of further interest to note that approximately the same concentration of phenol (0.2%) is required to inhibit all the bacteria in this test.

Extension of the testing with the Parasepts to include a variation of *pH* demonstrated quite conclusively that there was no significant difference in antimicrobial activities of any of the four alkyl esters over the range of *pH* from 4 to 8. Since most pharmaceutical preparations possess a *pH* which falls in this range, it is evident that the Parasepts should have broader applicability than a preservative like sodium benzoate, for example, whose antimicrobial activity falls off sharply above *pH* 6.

As a logical outgrowth of the obvious trend demonstrated by these esters with regard to their relationship of antimicrobial activities and chemical structure, it was, of course, desirable to prepare and evaluate other esters of parahydroxybenzoic acid. Table IX lists certain of these materials together with their minimal concentrations required to inhibit *A. niger*. These data indicate the propyl or butyl esters to be the most effective antimicrobial agents of the series, since the lower esters are less active and the higher ones so insoluble that a sufficient quantity for inhibition will not go into solution.

TABLE VIII.—COMPARATIVE ANTIMICROBIAL EFFECTIVENESS OF PROPYL PARASEPT, PHENOL, AND SODIUM BENZOATE

	Propyl Parasept	Phenol	Sodium Benzoate
B. subtilis	0.025	0.2	0.2
S. aureus	0.05	0.2	0.4
K. pneumoniae	0.025	0.2	0.2
E. coli	>0.4	0.2	0.2
S. typhosa	0.1	0.2	0.1
Ps. fluorescens	>0.4	0.2	>0.4
A. niger	0.02	>0.04	>0.04

TABLE IX.—MINIMAL CONCENTRATIONS OF VARIOUS ESTERS OF P-HYDROXYBENZOIC ACID REQUIRED TO INHIBIT A. NIGER

Methyl	0.1
Ethyl	0.04
Propyl	0.02
Iso-propyl	0.05
Butyl	0.02
Iso-amyl	>0.015 - <0.1
Hexyl	>0.015 - <0.1
Cyclohexyl	>0.015 - <0.1
2-Ethylhexyl	>0.1
o-Chlorobenzyl	>0.1
β-Hydroxyethyl	>0.1
Tetra-hydrofurfuryl	>0.1
p-Chlorobenzyl	insoluble
Glyceryl	>0.1
Pentaerythrityl	>0.1

Turning now to the aspect, of toxicity, data have been recently collected and are still being obtained which, it is anticipated, will provide for the first time a sound basis for judging the safety of these compounds. Examination of the available literature pertaining to the toxicity of the esters of parahydroxybenzoic acid reveals a dearth of information obtained under closely controlled experimental conditions, or at least a failure of the authors to report completely their experimental details. One of the earliest studies of this subject was performed by Schubel and Manger (4), the results of which are summarized in Table X. The most evident conclusion from these numbers is that, via the oral route in these larger animals, the esters of parahydroxybenzoic acid are quite nontoxic, particularly in comparison with benzoic acid, a material which has been used for many years as a food perservative without any apparent untoward effects.

TABLE X.—ACUTE ORAL TOXICITIES OF SOME ESTERS OF P-HYDROXYBENZOIC ACID CATS, DOGS, AND RABBITS

	Dose Gm./Kg., Body Wt.	
Compound	Toxic	Lethal
Methyl *p*-hydroxybenzoate	2	3
Ethyl *p*-hydroxybenzoate	4	5
Propyl *p*-hydroxybenzoate	3-4	6
Benzoic Acid	1	2

TOXICITY.—Additional acute toxicity data recently obtained on the Parasepts to be published shortly are presented in Table XI. It is interesting to note that in mice it is virtually impossible to demonstrate acute toxicity via the oral route. The quantities presented here are upper figures which are limited only by the bulk density of the ester and the capacity of the mice for receiving this volume of material. Regarding LD-50's via the intraperitoneal route, it is significant to note that the four alkyl esters are less toxic than the free parahydroxybenzoic acid, with the methyl ester having approximately one-fourth the toxicity of the free acid and the propyl

TABLE XI.—ACUTE TOXICITIES OF P-HYDROXYBENZOIC ACID AND SOME OF ITS ESTERS IN MICE

	Paralytic $Dose_{50}$ Mg./Kg.		Lethal $Dose_{50}$ Mg./Kg.	
Compound	Oral	I.P.	Oral	I.P.
p-Hydroxybenzoic acid	--	--	2200	210
Methyl Parasept	>8000	370	>8000	960
Ethyl Parasept	>8000	275	>8000	520
Propyl Parasept	>8000	400	>8000	640
Butyl Parasept	>5000	ca. 200	>5000	230

ester being less toxic than the ethyl or butyl esters. All the esters produced a degree of paralysis, from which some animals recovered, prior to death. Parahydroxybenzoic acid, on the other hand, produced no paralysis. These data suggest poor absorption from the gastrointestinal tract although, as will be pointed out later, this is not necessarily the case, since acute toxicity appears to be closely related to the rate of administration of these agents. When the esters and free parahydroxybenzoic acid were prepared in the form of sodium salts and administered intravenously in both anesthetized and unanesthetized dogs, an important relationship was observed between the speed of administration and fatal effects. For example, in the case of the propyl ester, the fatal dose was of the order of 85 mg./Kg. when administered at the rate of 20 mg./Kg./min. However, when the same ester was administered at the rate of 5 ml./Kg./min., the fatal dose was in excess of 600 mg./Kg. The toxic effects produced by intravenous injection are extremely rapid and either kill the animal within a few minutes or permit complete recovery without any apparent subsequent toxic manifestation.

Turning now to the chronic toxicity of the alkyl esters of parahydroxybenzoic acid, various investigators have reported on the innocuous nature of these products. For example, Cremer (5) in 1935 reported that 2 to 20 mg./Kg. per day of the lower esters fed to rabbits, guinea pigs, and rats for a period of 120 days appeared to be entirely harmless. Three times this quantity fed to rats for thirty days also produced no ill effects.

When the ethyl and propyl esters were fed to rats over an 18-month period at a level of 150 mg./Kg. per day, no ill effects were observed. As a matter of fact, at this level there was some evidence of growth stimulation. When fed at 10 times this level there was a decrease in growth rate, but no irregular pathological changes could be found.

Additional recent data on the chronic toxicity of the Parasepts are shortly to be published. This work indicates that, when Methyl and Propyl Parasepts were incorporated in the diets of rats at a 2 per cent level, no apparent ill effect was observed over a period of eighteen months. Under these conditions, ester intake averaged approximately 1 Gm./Kg. of body weight per day. Similarly, no untoward effects have been observed in dogs subjected to this same regimen for a period of one year.

From these acute and chronic toxicity data, it is readily apparent that the esters of parahydroxybenzoic acid exhibit a very low order of toxicity and must certainly be considered safe by even the most rigid criteria for pharmaceutical use in the usual quantities employed as a preservative.

ALLERGENICITY.—Because of the desirability of incorporating the esters of parahydroxybenzoic acid in ointments, creams, etc., for topical use, the question of the allergenicity of these compounds has been raised. Recent efforts toward the elucidation of this question have revealed some interesting results.

Following the Food & Drug Administration's recommended procedure for the determination of allergic reactions in quinea pigs (6), solutions of the four lower alkyl esters of parahydroxybenzoic acid at a concentration of

0.1 per cent in physiological saline were prepared, using sodium hydroxide to solubilize the esters when necessary. One-tenth ml. portions of these solutions were injected intracutaneously into the backs and upper flanks of male guinea pigs weighing approximately 400 grams at intervals of three times weekly. No reaction was noted twenty-four hours after the first injection. Two weeks after the tenth injection a retest injection of a freshly prepared solution of the Parasept was made in the area adjacent to the site of the sensitizing injection. The guinea pigs were closely observed for a reaction during the next forty-eight hours. In no case did any allergic response appear.

As an extension of this work, 10 per cent pastes of the methyl and propyl esters in a hydrophilic ointment base were applied to the skin of the backs of white rabbits. There was no noticeable irritation detected after forty-eight hours. Upon sacrificing the animals, no Parasepts or degradation products thereof were detected in the kidneys.

Irritation and sensitivity studies were also carried out in humans. In order to determine the primary irritant effect of the four alkyl esters of parahydroxybenzoic acid, solutions of these esters in propylene glycol were prepared at concentrations of 5, 7, 10, 12, and 15 per cent. Sterile cotton gauze was impregnated with these solutions and applied to the backs of 50 human subjects for a period of five days. At the end of this period the patches were removed and the degree of irritation noted. Table XII indicates the results of this test. At the ester levels indicated none of the 50 subjects exhibited any irritation whatsoever. In each case, at the next higher level, some manifestations of irritation were observed. Considering the order of magnitude of these concentrations, it may be safely concluded that the lower alkyl esters of parahydroxybenzoic acid are relatively non-irritating.

Utilizing the repeated insult method for the determination of sensitization, cotton patches impregnated with the propylene glycol solution of the Parasepts at the maximum non-irritating level indicated in Table XII were applied to the skin of the backs of 50 human subjects for a period of from four to eight hours every other day for 10 applications. At the end of this time, a period of three weeks was allowed to elapse and the materials reapplied for a period of 24 to 48 hours. In no case was there observed any evidence of sensitization toward the Parasepts. Thus, it is evident that the testing in humans confirms the results obtained in animals, both indicating the lower alkyl esters of parahydroxybenzoic acid to be remarkably free from undesirable allergenic behavior.

TABLE XII.—PRIMARY IRRITANT EFFECT PATCH TESTS - 50 HUMAN SUBJECTS

Parasept	Conc. (%, w/v) in Propylene Glycol Not Causing Irritation
Methyl	5
Ethyl	7
Propyl	12
Butyl	5

TABLE XIII.—SUMMARY OF ABSORPTION AND EXCRETION OF PARA-HYDROXYBENZOIC ACID AND ITS CARBOXYLIC ESTERS

		Average of Three Dogs Given 1.0 Gm./Kg. Orally for Each Compound							Average of Three Dogs Given 50 Mg./Kg. I.V. for Each Compound						
		γ/cc. Plasma Levels			Dose Excreted (Accumulative), %				γ/cc. Plasma Levels			Dose Excreted (Accumulative), %			
Drug	Time	Parasept	Free PHBA	Total	Parasept	Free PHBA	Gluconic Acid Conjugate	Total	Parasept	Free PHBA	Total	Parasept	Free PHBA	Gluconic Acid Conjugate	Total
PHBA	5 min.	...	...	...	...	...	...	...	...	112	122	...	...	...	...
	15 "	...	46	59	...	...	...	...	...	80	100	...	...	...	...
	30 "	...	...	...	...	...	...	...	...	59	93	...	...	...	...
	1 hr.	...	249	260	...	...	0.2	2.0	...	28	73	...	5.5	3.8	18.0
	2 "	...	399	420	...	...	1.1	6.0	...	1.3	56	...	20.0	18.2	43.1
	4 "	...	603	703	...	...	4.2	19.1	...	0	13	...	24.0	18.2	74.2
	6 "	...	660	660	...	...	8.2	30.3	...	0	6	...	25.0	18.2	80.3
	24 "	...	4	10	...	...	30.0	87.1	...	0	0	...	25.0	18.2	83.0
	48 "	...	0	4	...	...	30.0	94.0	...	0	0	...	25.0	18.2	83.0
Methyl Parasept	5 min.	...	...	...	...	...	...	...	0	82	115	...	...	...	...
	15 "	0	15	17	...	...	...	...	0	68	120	...	...	...	...
	30 "	...	...	...	...	...	...	...	0	48	115	...	...	...	...
	1 hr.	0	110	273	0.003	0.7	0.5	8.1	0	19	113	0.03	8.4	4.3	14.1
	2 "	0	293	437	0.005	2.3	2.1	14.4	0	12	61	0.03	12.4	9.5	28.6
	4 "	0	553	827	0.005	6.2	4.9	15.5	0	4	27	0.08	18.1	28.5	67.3
	6 "	0	630	863	0.014	11.0	28.8	32.0	0	0	12	0.10	20.6	28.9	80.0
	24 "	0	19	77	0.014	21.2	33.1	66.1	0	0	0	0.58	20.6	32.0	85.0
	48 "	0	0	3	0.014	21.3	35.1	89.0	0	0	0	0.58	20.6	32.0	85.0
Ethyl Parasept	5 min.	...	...	...	...	...	...	...	5	51	85	...	...	...	...
	15 "	2.3	4.3	32	...	...	...	...	5	62	71	...	...	...	...
	30 "	...	...	...	...	...	...	...	0	16	66	...	...	...	...
	1 hr.	0	99	134	0.001	0.31	0.26	2.0	0	12	54	0.03	5.7	2.8	14.0
	2 "	7.0	130	228	0.005	1.5	1.1	4.5	0	3	36	0.24	13.0	9.2	37.0
	4 "	7.0	375	480	0.005	3.1	2.4	7.4	0	0	30	0.34	17.0	14.0	56.0
	6 "	0	427	648	0.006	4.5	7.7	18.5	0	0	3	0.34	17.0	15.0	61.0
	24 "	0	0	17	0.034	12.5	32.5	64.0	0	0	0	0.34	17.0	15.0	70.0
	48 "	0	0	0	0.034	12.3	32.5	66.0	0	0	0	0.34	17.0	15.0	70.0
Propyl Parasept	5 min.	...	...	...	...	...	...	...	3	85	93	...	...	...	...
	15 "	2	5.5	16	...	...	...	...	0	62	101	...	...	...	...
	30 "	...	...	...	...	...	...	...	0	47	83	...	...	...	...
	1 hr.	0	32	79	0.003	0.09	0.11	0.37	0	17	49	0.01	12.5	7.1	20.3
	2 "	0	48	142	0.006	0.81	0.68	2.9	0	5	32	0.06	19.0	11.6	47.0
	4 "	0	95	232	0.001	2.3	1.8	9.0	0	0	13	0.06	23.0	17.5	75.5
	6 "	0	205	370	0.006	3.1	3.0	12.2	0	0	0.3	0.10	23.6	19.7	83.7
	24 "	0	13	43	0.042	12.1	12.0	53.2	0	0	0	0.10	26.0	22.6	94.0
	48 "	0	0	0	0.042	12.7	16.5	57.6	0	0	0	0.10	26.0	22.6	94.0
Butyl Parasept	5 min.	...	...	...	...	...	...	...	0	47	64	...	...	...	...
	15 "	5	0	0.7	...	...	...	...	0	39	54	...	...	...	...
	30 "	...	...	...	...	...	...	...	0	26	36	...	...	...	...
	1 hr.	14	0	15	0.002	0.0	0.10	0.06	0	12	26	0.20	5.0	17.0	11.0
	2 "	3	1.3	34	0.018	0.09	0.40	0.87	0	5	14	0.30	9.0	17.0	26.3
	4 "	0	5.5	119	0.021	0.32	1.9	2.6	0	1	7	0.31	10.1	19.3	37.1
	6 "	0	15	141	0.075	1.05	4.3	6.9	0	0	1	0.31	10.1	20.1	40.1
	24 "	0	1.7	25	0.47	4.9	27.5	47.0	0	0	0	0.31	11.3	20.1	40.1
	48 "	0	0	0	0.54	4.9	27.5	48.2	0	0	0	0.31	11.3	20.1	40.1

METABOLISM.—One important aspect of considerable interest which it is desirable to know about any chemical agent recommended for human use is the mechanism of metabolism of this agent and its ultimate fate in the body. No thorough work of this type has ever been reported in the literature for the esters of parahydroxybenzoic acid. There will, however, appear in the near future results of an investigation which has been in progress for several years designed to clarify these points. It may be appropriate to mention in a general manner at this time some of the more interesting aspects of this study.

The chemical test used to follow the esters and free parahydroxybenzoic acid in the animal body was based on the amino-antipyrine test for phenols unsubstituted in the para position. Under the conditions of this test, a free carboxyl group para to the phenol position is removed to produce the unsubstituted phenol. An ester group para to the phenol will not react directly in the test but must first be hydrolyzed to the free acid. This difference in behavior may be used to indicate the percentage of free acid and ester in any given mixture.

In order to understand how the body handles these drugs, a comparison was made of oral and intravenous administration of the Parasepts and parahydroxybenzoic acid. Intravenous injections of 50 mg./Kg. of the four Parasepts and the free acid were each given to a series of 3 or more fasted dogs and blood and urine samples taken at frequent intervals following the injection until levels fell to 0. Determination of the compound and its various metabolic products were then made on the samples. A similar study was carried out in which 1 Gm./Kg. of the drugs was given orally in capsules to the Dogs. Summaries of these experiments are presented in Table XIII. Excellent recoveries of total material were noted from the urine of dogs having received both intravenous and oral doses of the free acid and the three lower esters. These ranged from 60 to 95 per cent, indicating that most of the drug as it is metabolized and excreted by the body retained its phenolic structure. Most of the material is excreted by the body six hours after injection, whereas appreciable amounts are still detected thirty hours after oral ingestion. These results seem to indicate that absorption of these compounds from the gastro-intestinal tract is essentially complete, since recovery from urine is equally complete after either method of administration. Recoveries of Butyl Parasept after oral and intravenous doses were only 48 and 40 per cent, respectively. It was felt that this might indicate a relatively less effective hydrolysis of the butyl ester by the body, which would allow other degradation processes such as oxidation and reduction to take place, thus destroying the structure necessary for coupling with amino-antipyrine.

Detoxication mechanisms seem to proceed efficiently immediately after injection of the drugs. The concentration of the Parasept in the plasma only rarely exceeds the lower limits of sensitivity of the determination even immediately following intravenous injection. Significant levels were found only in the plasma of some dogs a few hours after oral administration. The amount of free Parasept excreted never exceeded 0.6 per cent, regardless of the method of dosage. The bulk of the total material seems to be excreted in the conjugated form. This conjugation may take place before or after hydrolysis, and the biochemical methods employed can not distinguish between the two metabolic routes. However, rapid hydrolysis of all but the butyl ester linkage is indicated since, after intravenous doses of the compounds, as much free parahydroxybenzoic acid is excreted as when the acid itself is injected. After intravenous administration, the values for free parahydroxybenzoic acid and total material in the plasma are similar, but the total values remain high for a long time. From this, it would seem that the first detoxication mechanism is one of hydrolysis.

An experiment was designed to study the distribution of parahydroxybenzoic acid and its esters in animal tissue. Dogs were perfused at the rate of 2 mg./Kg./min. until a total of 100 mg./Kg. of the compound under study was delivered. When 95 mg./Kg. had been given, blood samples were taken. The dog was then killed with intravenous air and the tissues immediately isolated. Analyses were made on the various tissues and the plasma to determine the levels of parahydroxybenzoic acid, Parasepts, and total material present in them at the time of death. Results of this study are shown in Table XIV. Appreciable Parasept was found only in the brain, spleen, and pancreas. High total values were found in the liver and kidney, and high values for free parahydroxybenzoic acid were detected in the kidney. Other than these instances, the levels in various organs were below plasma levels. Apparently, this rate of administration was slow enough to overcome most enzymatic differences in the handling of these compounds, for other than higher Parasept values, the butyl compound does not differ appreciably from the others.

In vitro studies of normal tissue mashes mixed with the various Parasepts revealed that esterases in liver and kidneys were extremely efficient in hydrolyzing the Parasepts to free parahydroxybenzoic acid. These data are presented in Table XV. It is interesting to note that these mashes were capable of almost complete hydrolysis after three minutes of contact with all the Parasepts except Butyl and perhaps Isopropyl, in which case thirty to sixty minutes of contact was necessary. Muscle enzymes were capable of this hydrolysis to a much smaller degree, while the other tissues, plasma, and whole blood showed no *in vitro* ability to break the ester linkage. Dilutions of liver up to 1:200 still showed this enzymatic activity. *In vivo* experiments showed no free Parasept in the tissues which exhibit this ability to destroy the ester linkage. The liver, having low parahydroxybenzoic acid levels in the intact animal, presumably conjugates, or otherwise metabolizes, most of the free parahydroxybenzoic acid to further degradation products.

A study in which 70 mg./Kg. of Methyl Parasept was given orally to a fasted man revealed that the drug is handled similarly in human metabolism. No detectable Parasept levels were found in the plasma and urine. Fifty per cent of the administered dose was recovered from the urine after 12 hours as total material, of which 11 per cent was accounted for as free parahydroxybenzoic acid.

No accumulation of products was noted in a dog which had been given 1 Gm./Kg. of Methyl Parasept orally every day for a period of one year. The rate of urinary excretion in this dog had increased to such an extent that

TABLE XIV.—DISTRIBUTION OF PARAHYDROXYBENZOIC ACID, ITS ESTERS, AND METABOLIC PRODUCTS IN TISSUES OF DOGS EACH COMPOUND INFUSED INTRAVENOUSLY AT A RATE OF 2 MG./KG./MIN. FOR 50 MINUTES

Concentration In Tissues (γ per Gm. or ml.)

Drug Given	pHBA					Parasept					Total				
	Butyl	Propyl	Ethyl	Methyl	PHBA	Butyl	Propyl	Ethyl	Methyl	PHBA	Butyl	Propyl	Ethyl	Methyl	PHBA
Liver	28	19	28	74	56	0	0	0	0	...	240	245	280	520	540
Kidney	186	116	150	190	192	0	0	0	0	...	520	330	330	520	540
Brain	0	0	3	14	20	50	23	30	10	...	36	25	36	32	64
Spleen	14	35	20	36	64	32	0	0	6	...	76	53	80	56	108
Pancreas	2	10	16	...	30	70	0	16	...	...	80	50	132	...	124
Muscle	9	23	18	12	24	0	0	0	0	...	30	48	120	34	42
Plasma	72	72	47	155	225	25	5	0	0	...	193	275	177	265	300

TABLE XV.—PER CENT HYDROLYSIS AT 3, 30 AND 60 MINUTES OF 100 γ/CC. OF THE PARASEPTS IN VARIOUS TISSUE MASSES AT ROOM TEMPERATURE

Tissue	Min.	Ester Hydrolyzed			
		Methyl	Ethyl	Propyl	Butyl
Liver	3	100	96	100	64
	30	100	96	100	100
	60	100	96	100	100
Brain	3	0	0	0	0
	30	0	0	0	0
	60	0	0	0	0
Kidney	3	72	60	18	14
	30	100	100	100	74
	60	100	100	100	100
Muscle	3	0	0	0	0
	30	16	16	0	0
	60	18	16	8	1.4
Spleen	3	0	0	0	0
	30	0	0	0	0
	60	0	0	0	0
Pancreas	3	0	0	0	0
	30	0	0	0	0
	60	0	0	0	0
Plasma	3	0	0	0	0
	30	0	0	0	0
	60	0	0	0	0
W. Blood	3	0	0	0	0
	30	0	0	0	0
	60	0	0	0	0

after twenty-four hours 96 per cent of the dose was recovered in the urine, whereas in a dog given a single oral dose it was not until forty-eight hours that the entire amount was accounted for.

From these studies, it appears that parahydroxybenzoic acid and its methyl, ethyl and propyl esters are efficiently absorbed, metabolized and excreted by the body. Hydrolysis of the ester linkage and metabolic conjugation provide adequate degradation mechanisms in these instances, whereas Butyl Parasept, with a slower enzymatic hydrolysis rate, requires some destruction of the phenolic structure to accomplish sufficient detoxication when large amounts are introduced rapidly into the animal.

SUMMARY

The necessity for including preservatives in a wide variety of pharmaceutical preparations has been indicated and a listing of the most important agents of this type has been given. The characteristics of an ideal pharmaceutical preservative as originally presented by Gershenfeld and Perlstein have been reviewed, and factors have been discussed regarding their proposal of the esters of parahydroxybenzoic acid as the agents most closely approaching the ideal preservative. Recent experimental data on the antifungal and antibacterial activities of these esters have been presented, as well as a comparison between them and several of the other more popular preservatives. Further, new data on the acute and chronic toxicities of these esters have been discussed and the results of irritation studies in animals and humans described. Finally, the general aspects of the metabolism and fate of these esters of parahydroxybenzoic acid have been indicated.

On the basis of this information, it may be stated that recent additional evidence regarding the efficacy and safety of the Parasepts has now been obtained which indicates more strikingly the close similarity between the Parasepts and the so-called "ideal pharmaceutical preservative."

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178. Hisao Tsukamoto*1 and Seisuke Terada*2: Metabolism of Drugs. XXIII.*3 Metabolic Fate of *p*-Hydroxybenzoic Acid and its Derivatives in Rabbit. (1).

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The alkyl *p*-hydroxybenzoates have been widely used as a preservative for galenicals, foods, and cosmetics. In the studies on the biotransformation of *p*-hydroxybenzoic acid in the animal body, unchanged *p*-hydroxybenzoic acid and *p*-hydroxyhippuric acid[1-3] have been isolated from human and dog urine, and 3,4-dihydroxybenzoic acid[4] has been detected in the urine of rabbit as the metabolites. On the occurrence of conjugated glucuronic acid, Quick[3] also demonstrated the diglucuronide excretion in the urine of man and dog receiving *p*-hydroxybenzoic acid, but monoglucuronide of *p*-hydroxybenzoic acid has not yet been isolated as a urinary metabolite of *p*-hydroxybenzoic acid or its esters.

As *p*-hydroxybenzoic acid and its esters possess two functional groups which would conceivably be metabolized, two monoglucuronides are possible, the ether, *p*-carboxyphenyl glucuronide, and the ester, *p*-hydroxybenzoyl glucuronide. In the present paper is described the glucuronide formation in the metabolism of methyl *p*-hydroxybenzoate using

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Both forms of p-hydroxybenzoate glucuronides were identified by paper chromatography in the urine and the ether-type was isolated as methylacetyl derivative whose structure has been established as methyl (*p*-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate.

Experimental

Separation of Glucuronide from the Urine of Rabbits—Separation of glucuronide from the urine used the $(AcO)_2Pb$ method, which is a general procedure of preparing glucuronides described by Williams, *et al.*[5]

The animals used were male rabbits weighing 2.4~2.8 kg. They were housed in metabolism cages and fed 'Okara' (soybean curd residue) only. Methyl *p*-hydroxybenzoate Na salt (0.8 g./kg. body wt. as methyl *p*-hydroxybenzoate) was administered by stomach tube as a 12% (w/v) solution and a total dose of 8.2 g. of methyl *p*-hydroxybenzoate was used for 4 rabbits. The decomposition of urinary metabolites was prevented by the addition of toluene.

The collected 24-hr. urine was filtered through cotton wool, adjusted to pH 4 with glacial AcOH, and then treated with saturated lead acetate solution until precipitation was complete. The precipitate was removed by filtration. The filtrate was adjusted to pH 8 with NH_4OH and saturated basic lead acetate solution was added in excess. The basic lead precipitate was collected and washed several times with H_2O. This was made into a fine suspension in MeOH and the lead salt was decomposed by treatment with H_2S. After removal of PbS by filtration, MeOH solution was evaporated to dryness at 25~30° under a reduced pressure. The residue was dissolved in a small volume of H_2O and extracted with Et_2O to remove unchanged methyl *p*-hydroxybenzoate and *p*-hydroxybenzoic acid (extract A). The aqueous solution was evaporated again to dryness at 30° under a reduced pressure and a red gum (gum B) was obtained.

This crude gum (B) was dissolved in about 30 cc. of EtOH and Et_2O was added until precipitation no longer occurred. The precipitate was filtered off and the filtrate was evaporated to dryness under a reduced pressure, giving a reddish gum (gum C).

This gum (C) was dissolved in EtOH and saturated $(AcO)_2Ba$ solution was added until precipitation was complete. The precipitate was collected, washed repeatedly with EtOH, and dried over anhyd. $CaCl_2$ *in vacuo*. The dried powder was dissolved in H_2O and filtered. The filtrate was adjusted to pH 7 and saturated basic lead acetate solution was added in excess. The basic lead precipitate was collected, washed with H_2O, made into a fine suspension in MeOH, and the lead salt was decomposed by treatment with H_2S. After removal of PbS by filtration, MeOH solution was evaporated to dryness under a reduced pressure at 20° and the residue was dried over P_2O_5 *in vacuo*. A pale yellow glucuronide (1.3 g.) was obtained.

Isolation of the Derivative of Ether-type Glucuronide—To 1 g. of the purified glucuronide in 25 cc. of MeOH was added Et_2O solution of CH_2N_2, freshly prepared from 5 g. of nitrosomethylurea, under cooling in ice, and the mixture was allowed to stand overnight in a refrigerator. The solvent was evaporated to dryness under a reduced pressure and the pale yellow residue was dissolved in 6 cc. of pyridine and 4 cc. of Ac_2O. The mixture was allowed to stand at room temperature for 2 days and poured into 50 cc. of ice-water with stirring. After the mixture had been allowed to stand overnight in a refrigerator, the pale brown precipitate produced was collected and dissolved in Et_2O. Et_2O solution was washed successively with 2% HCl and H_2O, dried over anhyd. Na_2SO_4.

Fig. 1.
Isolated Crystals

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Chart 1. Separation of Ether-type Glucuronide of *p*-Hydroxybenzoic Acid

Urine

filtd. through cotton

Filtrate

added with satd. $(AcO)_2Pb$ at pH 4 (adjusted with AcOH)

Filtrate — ppt.

added with satd. basic lead acetate at pH 8 (adjusted with NH_4OH)

ppt. — Filtrate

treated with H_2S in MeOH

Filtrate — PbS

evapd. to dryness

Residue

dissolved in H_2O, extd. with Et_2O

aq. soln. — Et_2O soln. Extract (A)

evapd. to dryness

Red gum (B)

dissolved in EtOH, added with Et_2O

Filtrate — ppt.

evapd. to dryness

Reddish gum (C)

dissolved in EtOH, added with satd. $(AcO)_2Ba$

Ba salt — Filtrate

dissolved in H_2O, added with satd. basic lead acetate at pH 7

ppt. — Filtrate

treated with H_2S in MeOH

Filtrate — PbS

evapd. to dryness

Pale yellow glucuronide

dissolved in MeOH, methylated with CH_2N_2, evapd. to dryness

Residue

acetylated with pyridine and Ac_2O, poured into H_2O

ppt.

dissolved in Et_2O, washed with HCl and H_2O

Et_2O soln.

evapd. to dryness

White solid

crystallized from Et_2O-petr. ether

Needles (Fig. 1)

and evaporated to dryness under a reduced pressure. The residue was crystallized from EtOH. The white powder (m.p. 147~152°) so obtained was recrystallized from Et_2O-petr. ether as colorless needles, m.p. 157~158°; $[\alpha]_D^{23}$ −42.0° (in $CHCl_3$). Yield, 0.35 g., having the crystal form shown in Fig. 1. *Anal.* Calcd. for $C_{21}H_{24}O_{12}$: C, 53.85; H, 5.16. Found: C, 53.85; H, 5.24. IR (in Nujol) cm^{-1}: $\nu_{C=O}$ 1760, 1718, ν_{C-O} 1230, 1083, 1044. UV: λ_{max}^{EtOH} 245 mμ.

When mixed with synthesized methy (*p*-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate, the m.p. was not depressed. This compound was non-reducing and was positive to both naphthoresorcinol reaction and Millon reaction after acid-hydrolysis. The infrared spectrum of this compound also indicated that it was identical with authentic sample. The course of separation of the glucuronide is shown in Chart 1. After hydrolysis of crude red gum with alkaline solution, the derivative of ether-type *p*-hydroxybenzoic acid glucuronide was also obtained by a method similar to the above.

Paper Chromatography of the Metabolites—Ascending development was employed with Toyo Roshi No. 50. Solvent system used was BuOH-AcOH-H_2O (4:1:5). Urinary metabolites were detected on paper chromatograms by spraying with the following reagents: (1) 1% $NaIO_4$, 1% $KMnO_4$, followed by benzidine reagent;[6] (2) Lintner's reagent[7] (after spraying with 10% $Hg(NO_3)_2$ solution, the paper is heated for a few min. at 60°, then sprayed with a mixture of equal volumes of 5% $NaNO_2$ and 5% H_2SO_4, and again heated at 60°); (3) diazotized sulfanilic acid[8] and *p*-nitroaniline;[9] and (4) aniline hydrogenphthalate.[9]

The red gum (B) obtained by $(AcO)_2Pb$ separation indicated 5 blue spots (Rf 0.88, 0.74, 0.57, 0.18~0.24, 0.10~0.14) and 3 pink spots (Rf 0.88, 0.74, 0.57) on paper strips on spraying the reagents (1) and (2), respectively. The spot of Rf 0.10~0.14 was also obtained from the gum of normal urine. Paper chromatography of the purified gum (C) showed the presence of a new substance whose Rf value was 0.42~0.46, and the spots of 0.18~0.24 and 0.10~0.14 all disappeared. A new spot of Rf 0.42~0.46 was also detected with the reagents (1) and (2), and the spots of 0.88, 0.74, 0.57 and 0.42~0.46 colored with the reagent (3).

To detect the glucuronides, MeOH solution of the gum (B) was developed on a large filter paper (40×40 cm.) using the same solvent. The sections of Rf 0.88, 0.74, 0.57 and 0.42~0.46 spots were cut out, each section was eluted with hot H_2O, and the eluate was filtered. The filtrate was concentrated to a small volume under a reduced pressure.

When the extract of Rf 0.88 was developed using BuOH saturated with 5*N* NH_4OH, it was identical with *p*-hydroxybenzoic acid indicating a spot at Rf 0.08~0.10, and the extract (A) also gave the same result. In this case, unchanged methyl *p*-hydroxybenzoate (corresponding to Rf 0.56~0.58) was not detected.

The naphthoresorcinol test was positive in both extracts of Rf 0.57 and 0.42~0.46.

The section of Rf 0.57 was resistant to alkaline-hydrolysis. After hydrolysis with 5% HCl in a steam-bath for 20 min., paper chromatography using BuOH-AcOH solvent system showed 2 spots corresponding to *p*-hydroxybenzoic acid and glucuronolactone (Rf 0.30~0.32) with reagents (2) and (4).

After developing repeatedly on a filter paper, the section of Rf 0.42~0.46 separated into Rf 0.50 and 0.40. When the extract of Rf 0.50 was hydrolyzed with 0.2% NaOH at 60° for 1 hr., 2 spots corresponding to *p*-hydroxybenzoic acid and glucuronic acid (Rf 0.12~0.14) were revealed by paper chromatography using the same solvent system. On the other hand, after hydrolysis with 5% HCl at 80° for 1 hr., the section of Rf 0.50 was partially decomposed into *p*-hydroxybenzoic acid and glucurone, and the spot of unchanged Rf 0.50 still remained. The amount of the extract of Rf 0.40 was so small compared to Rf 0.57 and 0.50, that its structure has not been clarified.

The section of Rf 0.74 may be considered a conjugated glycine, because it was positive to Ninhydrin test after acid-hydrolysis.

Synthesis of Methyl (*p*-Methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (III)—The method of Lunsford and Murphy[10] was applied to the preparation of derivative of ether-type *p*-hydroxybenzoic acid glucuronide, in which quinoline was used instead of isoquinoline.

A mixture of 1.2 g. of methyl (tri O-acetyl-α-D-glucopyranosyl bromid)uronate (I), 0.91 g. of methyl *p*-hydroxybenzoate (II), and 1.5 cc. of quinoline was triturated with a pestle in a mortar, cooling the mortar in an ice-bath. To this mixture, 1.5 g. of freshly prepared Ag_2O was added in small portions with continuous mixing. After complete addition, mixing was continued for 15 min., the viscous mixture was allowed to stand in a desiccator for 2 hr., and then was extracted several times with 20-cc. portions of Et_2O. Et_2O solution was washed successively with 2% H_2SO_4 and H_2O, dried over

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anhyd. Na_2SO_4, and evaporated under a reduced pressure. The residue was dissolved in EtOH and treated with activated charcoal. The precipitate produced was collected and recrystallized from Et_2O-petr. ether as colorless large needles, m.p. 167–153; $[\alpha]_D$ 42.0 (in $CHCl_3$). Yield, 0.4 g. *Anal.* Calcd. for $C_{20}H_{24}O_{10}$: C, 53.85; H, 5.16. Found: C, 53.81; H, 5.16. IR (in Nujol) cm^{-1}: $\nu_{C=O}$ 1758, 1715, ν_{C-O} 1227, 1082, 1044.

This compound was non-reducing and positive to naphthoresorcinol reaction and Millon reaction after acid-hydrolysis. The course of its synthesis is shown in Chart 2.

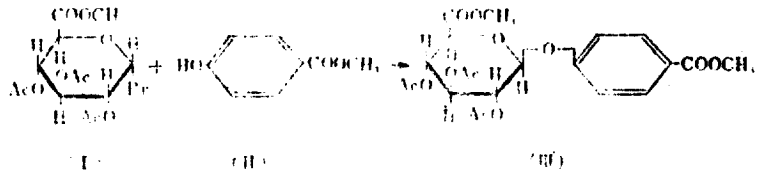

Chart 2. Preparation of Ether-type *p*-Hydroxybenzoic Acid Glucuronide

Discussion

In the present study, excretion of unchanged methyl *p*-hydroxybenzoate was not identified qualitatively by paper chromatography. Previous to this investigation, quantitative study on the metabolism of methyl *p*-hydroxybenzoate in rabbit showed that, after a dose of 0.4 g./kg. body wt. of methyl *p*-hydroxybenzoate, the excretion was concluded in almost 6 hr., about 1/3 portion being excreted as free *p*-hydroxybenzoic acid and the rest was discharged as conjugated form, without the unchanged methyl *p*-hydroxybenzoate. Schotten[2] also found that after rabbit received *p*-hydroxybenzoic acid, during 24 hr., 35% of it was excreted as unchanged compound. This fact shows that the ester decomposition is very easy in the animal body.

Robinson and Williams[11] isolated a derivative of the ether-type glucuronide of salicylic acid from the urine of rabbits receiving methyl salicylate. In the present experiment using methyl *p*-hydroxybenzoate, although the result could not be quantitated, the ether-type glucuronide of *p*-hydroxybenzoic acid appeared to be present in greater amount than the ester-type on paper chromatogram. Therefore, it could be generally supposed that the ether-type occurs in larger amount in the urine of rabbit receiving alkyl esters of hydroxybenzoic acid.

From the glucuronide fraction obtained by lead acetate method, the ether-type glucuronide was successfully isolated as methylacetyl derivative and its structure was established as methyl (*p*-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate with the synthetic sample, but the isolation of the ester-type has so far been unsuccessful and now further investigation is under way.

The authors are indebted to Mr. H. Matsui for determination of infrared spectra and to Miss S. Tada for the elemental analyses.

Summary

The ether-type glucuronide of *p*-hydroxybenzoic acid was isolated as methylacetyl derivative from the urine of rabbits receiving methyl *p*-hydroxybenzoate and its structure was established as methyl (*p*-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)-uronate with the synthetic sample. Ester-type glucuronide was detected in the urine by paper chromatography.

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M. Mano Tadanoto[*1] and Seizaho Terada[*2]: Metabolism of Drugs. XXXIV. Metabolic Fate of *p*-Hydroxybenzoic Acid and its Derivatives in Rabbit. (2).[*4]

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In the previous paper[3] it was reported that the ether-type glucuronide of *p*-hydroxybenzoic acid was isolated as the methylester derivative on methylation and acetylation of the glucuronide fraction which was separated by lead acetate[1] from the urine of rabbits receiving methyl *p*-hydroxybenzoate, and its structure had been established as methyl (*p*-methoxycarbonylphenyl 2,3,4,6-tri-O-acetyl-β-D-glucopyranosid)uronate (I).

In the present investigation, five metabolites; *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid, *p*-carboxyphenyl glucuronide(ether-type glucuronide), *p*-hydroxybenzoyl glucuronide(ester-type glucuronide), and *p*-carboxyphenyl sulfate, were identified in the urine of rabbits receiving ethyl *p*-hydroxybenzoate. The three main metabolites, the ether-type glucuronide, *p*-hydroxyhippuric acid, *p*-hydroxybenzoic acid, were isolated as crystalline materials. In the present series of work, structure of the ether-type glucuronide was established as *p*-carboxyphenyl-β-D-glucopyranosiduronic acid by identification with the authentic sample prepared by saponification of (I).

Experimental

Separation of the Metabolite Fraction from the Urine of Rabbits——The animals used were male rabbits weighing [illegible] kg. They were housed in metabolism cages and fed "Okara" (soybean curd residue). A total dose of 7.6 g. of methyl *p*-hydroxybenzoate (0.8 g./kg. body wt.) was administered [illegible] solution in the form of Na salt to 3 rabbits by a stomach tube.

* Author's [illegible]
* Nagasaki [illegible]
*4 [illegible] Bulletin, 9, 584 (1961).
*5 [illegible]
1) [illegible] J. Biochem. J., 50, 293 (1951).

The 24-hr. urine collected was filtered through cotton, the filtrate was adjusted to pH 4, and treated with saturated $(AcO)_2Pb$ solution. The filtrate separated from the precipitate was adjusted to pH 8 and saturated basic lead acetate solution was added in excess. The basic lead salt was collected, decomposed with H_2S in MeOH, and the filtrate was evaporated to dryness in a reduced pressure. A small amount of H_2O was added to the residue and the mixture was extracted with Et_2O to remove *p*-hydroxybenzoic acid (2.4 g.) excreted as a metabolite. The aqueous solution was evaporated to dryness in a reduced pressure and a crude gum (3.5 g.) was obtained.

Isolation of *p*-Hydroxyhippuric Acid and Ether-type Glucuronide of *p*-Hydroxybenzoic Acid——The crude gum was dissolved in about 30 cc. of H_2O and the solution was continuously extracted with Et_2O in a Soxhlet liquid extractor for 30 hr. Et_2O solution was dried by evaporation and the residue was treated with activated charcoal in MeOH. The filtrate was evaporated to dryness in a reduced pressure and the residual solid was recrystallized from hot H_2O to colorless needles, m.p. 235~236°. Yield, 0.35 g. *Anal.* Calcd. for $C_9H_9O_4N$: C, 55.38; H, 4.65; N, 7.18. Found: C, 55.58; H, 4.75; N, 7.16.

This was non-reducing and developed no color with naphthoresorcinol reagent, but was positive to the Millon and Ninhydrin tests after acid hydrolysis. From the result of elemental analyses and color reactions, this compound was identified as *p*-hydroxyhippuric acid.[2]

The mother liquor, left after removal of the above *p*-hydroxyhippuric acid, was evaporated to dryness in a reduced pressure. The dried residue was dissolved in a minimum of MeOH and (iso-$Pr)_2O$ was cautiously added dropwise to the solution until a slight turbidity developed. After the filtrate was allowed to stand overnight, the crystalline mass produced was collected and recrystallized repeatedly from MeOH-(iso-$Pr)_2O$ to colorless needles, m.p. 193~195° (decomp.). Yield, 0.1 g. *Anal.* Calcd. for $C_{15}H_{17}O_8$: C, 49.39; H, 4.49. Found: C, 49.78; H, 4.31.

This compound showed no depression of m.p. when mixed with the authentic *p*-carboxyphenyl-β-D-glucopyranosiduronic acid (II). The infrared absorption spectrum, behavior to hydrolyses, and color reactions were also the same as those of the authentic sample. The course of separation of the metabolites is shown in Chart 1.

Urine
- separated by $(AcO)_2Pb$ method

Separated substance
- mixed with H_2O, extd. with Et_2O

aq. soln.
- continuously extd. with Et_2O for 30 hr.

Et_2O soln.
- evapd. to dryness, crystallized from hot H_2O

Mother liquor
- evapd. to dryness

Residue
- crystallized from MeOH-(iso-$Pr)_2O$

Needles
p-Carboxyphenyl-β-D-glucopyranosiduronic Acid

Et_2O soln.
- evapd. to dryness

p-Hydroxybenzoic acid

Needles
p-Hydroxyhippuric acid

Chart 1. Separation of Metabolites from Urine

The aqueous solution, left after removal of *p*-hydroxyhippuric acid and the ether-type glucuronide by continual extraction with Et_2O, was purified by the same procedure as previously reported[3] and gave a purified gum. Attempt to isolate the ester-type glucuronide as the methylacetyl derivative from this gum was unsuccessful, this derivative being obtained only in the case of ether-type glucuronide.

Preparation of *p*-Carboxyphenyl-β-D-glucopyranosiduronic Acid (II)——To 0.5 g. of methylacetyl compound (I) 20 cc. of $C_{15}H_{17}O_8$ solution was added and the mixture was heated at 70° for 2 hr. with occasional shaking. The clear hydrolyzed mixture was adjusted to pH 8 with AcOH and saturated basic lead acetate solution was added in excess. The basic lead precipitate produced was

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3) H. Tsukamoto, S. Terada: This Bulletin, 3, 1035 (1960).

collected and washed thoroughly with H_2O. This was made into a fine suspension in MeOH and the lead salt was decomposed by treatment with H_2S. After removal of PbS by filtration, MeOH solution was evaporated to dryness in a cold room and the white residue was dried in a desiccator. The dried solid was extracted in a [illegible] and the [illegible] was cautiously added dropwise to the solution [illegible] stirring. A white precipitate that formed was removed by filtration and the filtrate was allowed to stand overnight in a refrigerator. A crystalline material (decomp. 200 [illegible]) produced was collected and recrystallized twice from MeOH-(iso-Pr)$_2$O to colorless needles, m.p. [illegible] (decomp.), [illegible] (DMSO, $CHCl_3$). Yield, 0.2 g. *Anal.* Calcd. for $C_{12}H_{14}O_8$: C, 49.03; H, 4.80. Found: C, [illegible]; H, 4.88.

This compound was [illegible] positive to [illegible] and Et_2O extract after its acid hydrolysis gave a red color with the Millon reagent.

Conversion of (I) to (II): [illegible] of (I) [illegible] of MeOH was cooled in ice and an Et_2O solution of CH_2N_2, freshly prepared from [illegible] of nitrosomethylurea, was added. The mixture was allowed to stand overnight in a refrigerator, the solvent was removed by evaporation, and the residue was dissolved in 1.0 cc. of pyridine and 0.5 cc. of Ac_2O. The reaction mixture was allowed to stand overnight at room temperature and poured into 10 cc. of ice-water with stirring. The precipitate produced was collected and washed successively with 2% H_2SO_4 and H_2O. The dried white solid was recrystallized from H_2O-petr. ether to colorless needles, m.p. 157~158°. Yield, 0.1 g. The compound showed no depression of m.p. with the authentic (I).

The infrared absorption spectra of (I) and (II) are shown in Fig. 1 and the route of preparation correlative to these is illustrated in Chart 2.

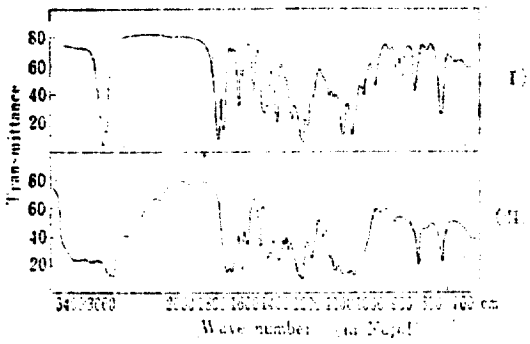


Fig. 1. Infrared Absorption Spectra

(I) Methyl (o-Methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate

(II) p-Carboxyphenyl-β-D-glucopyranosiduronic acid

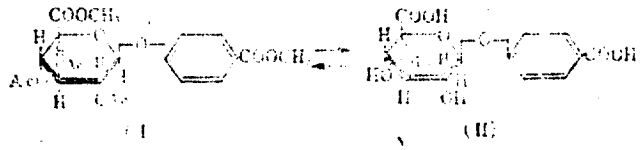


Chart 2.

Paper Chromatography of the Metabolites——It has previously been shown[3] that the gum, obtained from the urine of rabbits receiving methyl p-hydroxybenzoate, gave five spots at Rf 0.88, 0.74, 0.57, 0.48, and 0.38 on the paper chromatogram using the solvent system of BuOH-AcOH-H_2O, and that the spots of Rf 0.88, 0.57, and 0.48 are those of p-hydroxybenzoic acid, the ether-type glucuronide and the ester-type glucuronide, respectively.

For the chromatography, ascending development was employed with Toyo Roshi No. 50 and the solvent system used was BuOH-AcOH-H_2O [illegible]. The spot of metabolites was detected with the following reagents: (1) 1% $NaIO_4$, then 1% KMnO$_4$ followed by benzidine reagent;[4] (2) $Hg(NO_3)_2$ reagent: 20 parts of 10% $Hg(NO_3)_2$ solution mixed with 1 part of conc. HNO_3 just before spraying. The chromatogram sprayed with this reagent is placed in an oven at 90~100° for 5~10 min. and p-hydroxybenzoic acid derivative appears as blue or pink spot on the paper; (3) diazotized sulfanilic acid or p-nitroaniline; (4) aniline hydrogenphthalate reagent; and (5) Ninhydrin reagent.[9]

4) M.L. Wolfrom, J.E. Miller: Anal. Chem., 28, 1097 (1956).
5) T. Mann, E. Leone: Biochem. J., 53, 140 (1953).
6) H.C. Bray, W.V. Thorpe, K. White: *Ibid.*, 46, 271 (1950).
7) S.M. Partrige: Nature, 164, 443 (1949).
8) E.F. McFarren, J.A. Mills: Anal. Chem., 24, 650 (1952).

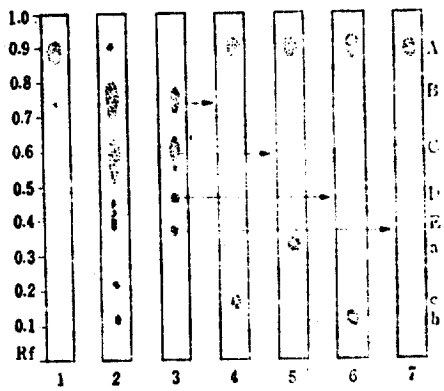
Paper chromatography of the crude gum separated from the urine revealed 2 large spots at Rf 0.74 and 0.57, and a small spot at Rf 0.42~0.46 by spraying with reagents (1), (2), and (3). However, in the case of the gum obtained after continuous extraction with Et_2O, the spots became small zones at Rf 0.74 and 0.57, and new spots appeared at Rf 0.48 and 0.33.

To detect unknown metabolites, a solution of this gum was developed on a large filter paper (40×40 cm.), each zone whose position was tested with color reagents, was cut out and eluted with 50% MeOH. The eluate was filtered and the filtrate was concentrated to a small volume in a reduced pressure.

The extract from Rf 0.74 section, which came in a large quantity, was resistant to alkaline hydrolysis. After hydrolysis with 5% HCl in a steam-bath for 60 min., the paper chromatogram showed 2 spots corresponding to *p*-hydroxybenzoic acid (Rf 0.88) and glycine (Rf 0.15) with reagents (2) and (5). Consequently, this substance may be considered as *p*-hydroxyhippuric acid.

In the case of a section corresponding to Rf 0.33, the original spot disappeared after acid hydrolysis under a similar condition as above and a new spot appeared at Rf 0.63. The fraction of Rf 0.38 was identified with the synthetic *p*-carboxyphenyl sulfate[9] in points of the chromatographic behavior and the acid hydrolysate being obviously positive to SO_4 test with Ba rhodizonate.

The foregoing evidence shows that a rabbit receiving methyl *p*-hydroxybenzoate excretes three main metabolites in the urine, *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid, and *p*-carboxyphenylglucuronide, and two minor metabolites, *p*-hydroxybenzoyl glucuronide and *p*-carboxyphenyl sulfate. Chromatographic data of urinary metabolites are summarized in Fig. 2.



Solvent System: BuOH-AcOH-H_2O(4:1:5)
1) Et_2O extract
2) Gum before continuous extraction with Et_2O
3) Gum after continuous extraction with Et_2O
4) Acid hydrolysate of Rf 0.74 section
5) Acid hydrolysate of Rf 0.57 section
6) Alkaline hydrolysate of Rf 0.43 section
7) Acid hydrolysate of Rf 0.33 section
A, *p*-hydroxybenzoic acid
B, *p*-hydroxyhippuric acid
C, *p*-carboxyphenyl glucuronide
D, *p*-hydroxybenzoyl glucuronide
E, *p*-carboxyphenyl sulfate
a, glucurone b, glucuronic acid c, glycine

Fig. 2. Paper Chromatograms of the Gum obtained from the Urine of Rabbits.

Discussion

Since methyl *p*-hydroxybenzoate possesses two functional groups which could be metabolized, eight different metabolites are theoretically possible. The hydroxyl group may remain free or be combined with glucuronic acid or sulfuric acid, while the methoxycarbonyl group may be unconverted or become free carbonyl group on saponification, and in some case this free carboxyl group might combine with glycine or glucuronic acid. However, this compound was not excreted in the urine of rabbits receiving methyl *p*-hydroxybenzoate, as already reported[9] and this fact shows that the ester-decomposition would probably be very easy *in vivo*. It would be reasonable to suspect that methyl *p*-hydroxybenzoate will be converted to five metabolites in the body of rabbit.

On the metabolic fate of *p*-hydroxybenzoic acid using a dog, Quick[3] stated that a large quantity of *p*-hydroxyhippuric acid was isolated from the urine.

In the present experiment on feeding rabbits with methyl *p*-hydroxybenzoate, *p*-hydroxyhippuric acid and the ether-type glucuronide were successfully isolated on continual extraction with Et_2O from a gum separated by the lead acetate method from the urine. This ether-type glucuronide was structurally identified with the authentic *p*-car-

9) K. Yamaguchi: Nippon Kagaku Zasshi, 80, 171 (1959).

boxyphenyl-β-D-glucopyranosiduronic acid. This compound was converted by methylation and acetylation to the methylester derivative; methyl (*p*-methoxycarbonylphenyl 2,3,4-O-acetyl-β-D-glucopyranosiduronate.

By the isolation of the ether-type glucuronide, there is no doubt that saponification of the methoxycarbonyl group in methyl *p*-hydroxybenzoate and the glucuronic acid conjugation with hydroxyl group take place.

In the study on the metabolism of methyl salicylate,[10] the ether-type glucuronide of salicylic acid has been isolated as methylacetyl derivative from the urine of rabbits, but the ester-type has not been obtained.

Although a portion of the ether-type glucuronide was isolated as crystalline material in the present work on the metabolism of methyl *p*-hydroxybenzoate, it was not possible to isolate the derivative of ester-type glucuronide from the gum obtained after separating the ether-type, contrary to the expectation.

The above facts suggest that the main metabolites of methyl *p*-hydroxybenzoate in rabbit are *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid and the ether-type glucuronide.

The present investigation showed that the ester-type glucuronide and *p*-carboxyphenyl sulfate occur in the urine only to a small amount. Further observation on metabolic fate of *p*-hydroxybenzoic acid will be discussed in a later paper.

The authors are indebted to Mrs. H. Mazume of Pharmaceutical Faculty, University of Nagasaki, for the determination of infrared spectra and for elemental analyses.

Summary

The ether-type glucuronide of *p*-hydroxybenzoic acid was isolated from the urine of rabbits receiving methyl *p*-hydroxybenzoate and its structure was established as *p*-carboxyphenyl-β-D-glucopyranosiduronic acid. These main metabolites, *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid, and the ether-type glucuronide, were identified in the urine, and the ester-type glucuronide and *p*-carboxyphenyl sulfate were proved as minor metabolites by means of paper chromatography.

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107. Hisao Tsukamoto*1 and Seisuke Terada*2: Metabolism of Drugs. XLVII.*3 Metabolic Fate of *p*-Hydroxybenzoic Acid and its Derivatives in Rabbit. (4).

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Alkyl *p*-hydroxybenzoates are widely used as a preservative for drugs, foods, and cosmetics. Nevertheless, there is only a few literature on the metabolism and excretion of these materials.

p-Hydroxybenzoic acid and its alkyl esters which possess two functional groups might undergo various metabolic changes in the animal body. Particularly in the ester, a functional carboxyl group could be formed on hydrolysis *in vivo*.[1,2]

It has been reported by some workers that the metabolic products of *p*-hydroxybenzoic acid detected and isolated from the urine of animals or man receiving this drug were *p*-carboxyphenyl sulfate (sulfuric acid conjugate)[3,4] the free acid, *p*-hydroxyhippuric acid (glycine conjugate),[5] *p*-carboxyphenyl glucuronide (ether-type glucuronide), and *p*-hydroxybenzoyl glucuronide (ester-type glucuronide).[6]

The authors reported previously that the above five metabolites also occurred in the urine of rabbits administered orally methyl *p*-hydroxybenzoate, but the excretion rate of the free or conjugated *p*-hydroxybenzoic acid differed when given *p*-hydroxybenzoic acid, though the result could not be quantified.[7]

Metabolism of *p*-hydroxybenzoic acid has already been observed quantitatively by several workers. Schotten[8] found that after feeding man with *p*-hydroxybenzoic acid, 16% of its dose was excreted in the urine during 24 hours, as *p*-hydroxyhippuric acid and 35% unchanged. Hartles and Williams,[4] and Bray, *et al.*,[9] reported that, in a rabbit, *p*-hydroxybenzoic acid was largely unchanged and converted into the glucuronide (10~20%), ethereal sulfate (5~7%), and glycine conjugate (about 20%).

On the other hand, Jones, *et al.*[2] described the result of quantitative experiment on the metabolic fate of alkyl *p*-hydroxybenzoates and stated that after 1.0 g./kg. of these esters was given orally to dogs, most of the material was metabolized and excreted during 6~24 hours and that the recoveries from the urine of total material ranged 50~90%.

Biological interest in the fate of *p*-hydroxybenzoic acid and its esters has been reported but individual excretion rate of the above-mentioned five metabolites in rabbit has not yet been observed in detail.

The present investigation is a comparative study on the metabolism of various alkyl *p*-hydroxybenzoates and of *p*-hydroxybenzoic acid, which is the major metabolic product of its esters. Metabolism of the esters is very rapid and excretion of the free

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*2 Nakagawa-cho, Nagasaki (長崎市).
*3 Part XLVI. K. Kato, K. Yoshida, H. Tsukamoto: This Bulletin, 12, 6[illegible] (1964).
1) H. Tsukamoto, S. Terada: This Bulletin, 8, 1056 (1960).
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3) E. Baumann, F. Herter: Z. physiol. Chem., Hoppe-Seyler's, 1, 244 ([illegible]).
4) R.L. Hartles, R.T. Williams: Biochem. J., 43, 293 (1948).
5) A.J. Quick: J. Biol. Chem., 97, 403 (1932).
6) H. Tsukamoto, S. Terada: This Bulletin, 10, 91 (1963).
7) *Idem*: *Ibid.*, 10, 86 (1962).
8) C. Schotten: Z. physiol. Chem., Hoppe-Seyler's, 7, 23 (1883).
9) H.G. Bray, B.E. Ryman, W.V. Thorpe: Biochem. J., 41, 212 (1947).

acid in the urine after administration of the esters is much less than that in the case of *p*-hydroxybenzoic acid, but output of the acids conjugated with glucuronic acid or glycine is much larger.

Experimental

Materials——Methyl *p*-hydroxybenzoate, m.p. 125°, ethyl *p*-hydroxybenzoate, m.p. 117°, propyl *p*-hydroxybenzoate, m.p. 95.5°, and butyl *p*-hydroxybenzoate, m.p. 71° were purchased, isopropyl *p*-hydroxybenzoate, m.p. 86°, isobutyl *p*-hydroxybenzoate, m.p. 77°, and *sec*-butyl *p*-hydroxybenzoate, m.p. 61°, were supplied by the courtesy of Nipa Seiyaku Co., and *p*-hydroxybenzoic acid, m.p. 214° was prepared by alkaline hydrolysis of the methyl ester.

Animal, Diet, and Dosage——Male rabbits (2.00~3.00 kg. body wt.) kept on a fixed diet as previously described,[7] were used. The materials were administered by a stomach tube as a 12% solution in the form of Na salt and 2 dose levels were used, 0.4 and 0.7 g./kg.

Procedure——The sample solution containing 50~100% of *p*-hydroxybenzoic acid in 10 ml. was placed in a glass-stoppered tube and added with 1 ml. of Millon's reagent with heating in a boiling water bath.[10] The tube was immersed in a boiling water bath for 10 min. and added with 8 ml. of H_2O immediately, and then cooled under running H_2O. The reaction mixture was diluted to 20.0 ml. with H_2O, mixed well, and absorbance measured at 510 mμ against a reagent blank within 5 min. Simultaneously, the absorbance was controlled with the use of a solution of *p*-hydroxybenzoic acid of known concentration.

Estimation of Free and Conjugated *p*-Hydroxybenzoic Acid——The method for the separation described by Bray, *et al.*[9] was applied in this work. Urine (10 ml.), acidified with $2N H_2SO_4$ (2 ml.), was extracted with three 15 ml. portions of Et_2O. The extract was evaporated to dryness under a reduced pressure, the residue was extracted successively with three 10 ml. portions of boiling toluene, and the toluene solution was evaporated to dryness under a reduced pressure. The solid residue was dissolved in H_2O and an aliquot of this solution was used for the determination of free *p*-hydroxybenzoic acid by the above procedure.

The residue (toluene-insoluble part) was combined with the aqueous solution remaining after the above Et_2O extraction. To this solution, 8 ml. of $10N H_2SO_4$ was added and the mixture was hydrolyzed by boiling for 60 min. The hydrolyzate was extracted with three 15 ml. portions of Et_2O, the extract was dried over anhyd. Na_2SO_4, and evaporated to dryness. The residue was dissolved in H_2O and an aliquot of this solution was used for the determination of conjugated *p*-hydroxybenzoic acid by the above procedure.

Control experiments, in which a mixture of known amounts of *p*-hydroxybenzoic acid (10 mg.), *p*-hydroxyhippuric acid (corresponding to 5 mg. as *p*-hydroxybenzoic acid), *p*-carboxyphenyl glucuronide (2.5 mg., ditto) and *p*-carboxyphenyl sulfate (2.5 mg., ditto) were extracted from normal rabbit urine, gave average recoveries corresponding to 97% of free *p*-hydroxybenzoic acid and 96% of the conjugated acid.

Estimation of Individual Metabolic Product in the Urine——It has previously been shown[6,7] that the urine of rabbits receiving *p*-hydroxybenzoic acid and its ester, gave five spots on the paper chromatogram using the solvent system of BuOH-AcOH-H_2O, and that the spots of Rf 0.88, 0.74, 0.57, 0.48, and 0.38 are those of *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid, *p*-carboxyphenyl glucuronide, *p*-hydroxybenzoyl glucuronide, and *p*-carboxyphenyl sulfate, respectively. Because of good separation, this technique was used for the estimation of each individual metabolite. The 24 hr. urine adjusted to pH 5 with AcOH, was evaporated at 35° under a reduced pressure to one-half the original volume, mixed with an equal part of EtOH, and centrifuged. An aliquot of the supernatant (0.05~0.10 ml.) was chromatographed on several sheets of filter paper (Toyo Roshi No. 50, 2×40 cm., solvent BuOH-AcOH-H_2O=4:1:5). Each zone of metabolites, whose position was tested with color reagent, was cut out and eluted with three 10 ml. portions of hot $0.2N$ NaOH. The eluate was evaporated on a boiling water bath to about 10 ml. and 8 ml. of $10N H_2SO_4$ added. The solution was hydrolyzed by boiling for 60 min., except that of Rf 0.88 fraction. The acidic solution was extracted with three 15 ml. portions of Et_2O. Et_2O solution was dried over anhyd. Na_2SO_4 and evaporated to dryness. The solid residue was dissolved in H_2O and subjected to quantitative analysis by the above procedure.

Control experiments, in which mixtures of known amounts of the various metabolites were added to normal rabbit urine, showed average recoveries corresponding to 97% of the free acid, 89% of glycine conjugate, 90% of glucuronic acid conjugate (the ether-type), and 86% of H_2SO_4 conjugate.

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10) F. D. Snell, C. T. Snell: "Colorimetric Methods of Analysis," 3, 413 (1953), D. Van. Nostrand Co., Inc., New York.

Results and Discussion

It has previously been confirmed by paper chromatography that the unchanged ester was present only in a small amount during 24 hours in the urine of rabbit orally administered methyl *p*-hydroxybenzoate.[1] In the present investigation, in order to mark quantitatively the excretory unchanged ester, an aliquot of 24 hour urine, acidified with diluted sulfuric acid, was extracted with ether. The extract was treated with sodium hydrogencarbonate solution to remove free *p*-hydroxybenzoic acid and majority of other acidic substances, and then dried over anhydrous sodium sulfate. After removal of impurities with activated alumina, the ether solution was filtered and the filtrate was evaporated to dryness. The unchanged ester thus obtained was dissolved in ethanol and its absorbance measured at 252 mμ. When the unchanged ester in the urine was analyzed by this method, present recoveries were only about 0.2~0.9% of the dose of various alkyl esters. It may, therefore, be considered that alkyl *p*-hydroxybenzoates are easily decomposed *in vivo* and essentially all of those was converted into the free and conjugated *p*-hydroxybenzoic acid.

Table I, gives summarized results the free and conjugated *p*-hydroxybenzoic acids, found in the urine after oral administration of various alkyl *p*-hydroxybenzoates and

TABLE I. Excretion Rate of Metabolites in Urine of Rabbits receiving *p*-Hydroxybenzoic Acid and its Alkyl Esters

Drug	Rabbit body wt (kg.)	Dose (mg./kg.)	Time (hr.)	*p*-Hydroxybenzoic acid: Free (mg.)	Conjugated (mg.)	Total (mg.)	Metabolic rate (%)
Methyl *p*-hydroxybenzoate	2.30	400	0~6	295	280	575	69
			6~10	45	40	665	80
			10~24	23	12	700	84
	2.80	800	0~9	504	647	1151	57
			6~10	[illegible]	256	1649	[illegible]
			10~24	47	93	1794	88
Ethyl *p*-hydroxybenzoate	2.30	400	0~9	293	289	582	76
			9~24	18	33	628	82
			24~48	10	21	659	83
	2.80	800	0~9	454	530	993	90
			9~24	115	194	1302	78
			24~48	47	72	1412	85
Propyl *p*-hydroxybenzoate	2.60	400	0~9	202	358	560	70
			9~24	43	73	676	85
			24~48	13	12	701	88
	2.80	800	0~9	413	552	965	56
			9~24	139	192	1296	76
			24~48	33	67	1396	81
Butyl *p*-hydroxybenzoate	2.80	400	0~9	172	258	440	55
			9~24	66	142	647	81
			24~48	23	31	702	88
	3.20	800	0~9	381	525	907	50
			9~24	186	279	1372	73
			24~48	32	46	1450	80
p-Hydroxybenzoic acid	2.25	400	0~6	444	225	669	74
			6~10	56	52	797	89
			10~24	23	7	827	92
	2.25	800	0~6	824	319	1143	63
			6~10	227	121	1491	83
			10~24	31	22	1544	86

p-hydroxybenzoic acid, estimated at timely intervals. In the case of *p*-hydroxybenzoic acid, some 90% of the dose may be excreted in the urine as a metabolites during 24 hours, while about 70~90% of alkyl esters appeared as the excretory products. The present results approximately agree with observations made by Bray, *et al.*,[9] and by Jones, *et al.*[4] However, when the alkyl esters were administered, excretion was rather slower than that of *p*-hydroxybenzoic acid and became slightly slower with increase in the carbon number of alkyl group of the esters. Moreover, in the case of *p*-hydroxybenzoic acid, the output of unchanged acid was usually greater than that of the conjugated acid, while in the alkyl esters, the output of conjugated acid was considerably increased and was occasionally equal to or greater than that of the free acid.

Table II. Excretion Rate of Major Metabolites in 24 hour Urine of Rabbits receiving *p*-Hydroxybenzoic Acid and its Alkyl Esters

Drug	Rabbit body wt. (kg.)	Dose (mg./kg.)	*p*-Hydroxybenzoic acid (%) Free	Conjugated with Glycine	Glucuronic acid (ester-type)	Glucuronic acid (ether-type)	Sulfuric acid	Metabolic ratio (%)
Methyl *p*-hydroxybenzoate	2.60	800	43	7	4	11	9	74
	2.65	800	33	16	8	21	7	85
	2.25	400	45	14	9	14	9	91
	2.50	400	31	22	8	16	14	94
	means		39	15	7	15	10	86
Ethyl *p*-hydroxybenzoate	2.50	800	32	14	8	13	12	79
	2.50	800	47	17	7	19	4	94
	2.60	400	37	22	7	9	13	88
	2.60	400	29	27	9	9	6	80
	means		36	20	8	12	9	85
Propyl *p*-hydroxybenzoate	2.30	800	26	26	7	10	4	71
	3.10	800	28	30	5	12	9	84
	2.30	400	32	14	8	16	10	80
	3.20	400	33	27	6	14	5	85
	means		30	24	7	13	7	81
Isopropyl *p*-hydroxybenzoate	3.20	800	30	29	4	17	5	84
	3.10	800	24	31	7	20	4	83
	2.90	400	32	19	9	24	7	91
	3.50	400	25	37	4	10	12	86
	means		26	29	6	18	7	86
Butyl *p*-hydroxybenzoate	2.80	800	37	18	4	8	10	77
	2.60	800	29	23	7	9	4	72
	2.80	400	24	28	5	16	10	83
	3.00	400	34	27	6	12	5	84
	means		31	24	6	11	7	79
Isobutyl *p*-hydroxybenzoate	3.00	800	33	16	8	14	8	79
	3.40	800	26	21	6	8	6	77
	2.70	400	25	23	9	9	17	83
	3.40	400	32	22	6	10	15	85
	means		29	23	7	10	12	81
sec-Butyl *p*-hydroxybenzoate	3.10	800	24	28	5	10	8	75
	3.40	800	28	20	5	12	10	75
	3.10	400	28	30	5	9	9	81
	3.40	400	19	38	3	8	14	82
	means		25	29	4	10	10	78
p-Hydroxybenzoic acid	2.25	800	55	17	9	2	4	87
	2.30	800	63	8	6	5	6	88
	2.60	400	58	18	7	5	5	93
	2.70	400	45	23	7	10	4	94
	means		55	18	7	6	5	91

The individual metabolic product, excreted in 24 hour urine after oral administration of *p*-hydroxybenzoic acid and its alkyl esters, was separated by paper chromatography and estimated by spectrophotometric method using Millon's reagent. The separation of metabolites by this technique was satisfactory for the present purpose because control experiments with known materials showed average recovery of about 90%. It was found that, after the administration of *p*-hydroxybenzoic acid 50% of the dose was excreted in the urine during 24 hours as the unchanged acid, 10% as conjugated with glycine, 7% as the ester-type glucuronide, 6% as the ether-type glucuronide, and 5% as conjugate with sulfuric acid. When the alkyl esters were administered, it was converted into the free acid (25~32%), glycine conjugate (15~33%), ester-type glucuronide (5~8%), ether-type glucuronide (10~18%), and the sulfate (7~12%).

These results are summarized in Table II, from which it is observed that the excretion of free acid is much less in the case of the alkyl esters than that *p*-hydroxybenzoic acid and that this tendency becomes marked with increase of carbon number in the esters.

When the alkyl esters were given, glucuronic acid conjugation occurs marked as compared to the administration of *p*-hydroxybenzoic acid, and above all, a difference in the ether-type conjugation is especially great. In the case of alkyl esters, output of the acid conjugated with glycine is occasionally equal to or greater than that of *p*-hydroxybenzoic acid.

The major metabolic products of alkyl *p*-hydroxybenzoates were tested except *p*-hydroxybenzoyl glucuronide for the preventive effect on the growth of *Staphylococcus aureus* 209-p, *Pseudomonas aeruginosa*, *Escherichia coli* 0111, *Aspergillus oryzae*, and *Saccharomyces sake*. Methyl *p*-hydroxybenzoate was used as the control material. Their activity was excessively weak and was less than 1/10 of that to methyl *p*-hydroxybenzoate.

The authors express their gratitude to Ueno Seiyaku Co. for supplying various alkyl *p*-hydroxybenzoates.

Summary

Metabolism of various alkyl *p*-hydroxybenzoates was investigated in rabbits using oral dose levels of 0.4 and 0.8 g./kg., and compared with that of *p*-hydroxybenzoic acid, which is a major metabolite of their esters. Metabolism of the esters was very rapid and excretion of the free *p*-hydroxybenzoic acid in the urine after administration was much less than that of *p*-hydroxybenzoic acid but output of the acid conjugated with glycine and glucuronic acid was much larger. When various alkyl esters were administered, those were converted into the free acid (25~32%), glycine conjugate (15~33%), ester-type glucuronide (5~8%), ether-type glucuronide (10~18%), and the sulfate (7~12%) during 24 hours.

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Stimulation of the Growth of Organ Cultures by Methyl and Propyl Parabens.* (32514)

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Some years ago we were studying the effects of hydrocortisone hemisuccinate on organ cultures of embryonic chick bones. We noted that certain levels appeared to stimulate the growth of these cultures, (as measured by dry weight), which was contrary to previous reports(1,2,3). We found that this growth stimulation was difficult to reproduce. When pure hydrocortisone hemisuccinate was substituted for the pharmaceutical preparation we previously used (Solu-Cortef, Upjohn) only inhibitory effects were obtained. The pharmaceutical preparation, in addition to the steroid, contained methyl paraben and propyl paraben as preservatives. These are the methyl and propyl esters of p-hydroxybenzoic acid. When the effects of these compounds upon growth were studied, we found definite stimulatory effects.

Material and methods. The femora were dissected from 10-day White Leghorn chick embryos. They were cultured in medium MB 752/1a containing 25% embryo extract, using the roller tube system described by Teaford and White(4). Paired cultures were used, in which the femur from one leg is grown in the

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TABLE I. Effects of Methyl and Propyl Parabens, and EDTA on Growth of Chick Embryo Femora *in vitro*.

A. *Effect of methyl paraben*						
Methyl paraben concentration (μg/ml)		.025	.25	2.5	25	250
(M) 1.64x		10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Dry wt difference (μg)	$\bar{D}$	25.6	81.5	75.3	10.4	224.6
	$S\bar{d}$	19.6	21.4	18.6	22.3	31.9
% Change		−2.7	+9.2	+7.7	−1.1	−19.6
Significance (P)		>.05	<.01	<.01	>.05	<.01
B. *Effect of propyl paraben*						
Propyl paraben concentration (μg/ml)		.0025	.025	.25	2.5	
(M) 1.38x		10^{-8}	10^{-7}	10^{-6}	10^{-5}	
Dry wt difference (μg)	$\bar{D}$	17.7	65.0	99.8	88.4	
	$S\bar{d}$	14.6	17.0	16.8	19.1	
% Change		−1.8	+6.8	+11.7	+8.8	
Significance (P)		>.05	<.01	<.01	<.01	
C. *Effect of methyl and propyl parabens combined*						
Methyl paraben concentration (μg/ml)		.025	.25	2.5	25.0	
Propyl paraben concentration (μg/ml)		.0025	.025	.25	2.5	
Dry wt difference (μg)	$\bar{D}$	78.5	57.7	54.5	1.4	
	$S\bar{d}$	42.3	47.9	5.8	1.0	
% Change		−10.1	+7.6	+6.8	+1.6	
Significance (P)		<.01	<.01	<.01	>.05	
D. *Effect of EDTA*						
EDTA concentration (M) 1.64x		10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Dry wt difference (μg)	$\bar{D}$	54.9	2.8	2.2	10.0	216.6
	$S\bar{d}$	17.8	20.2	21.5	25.4	15.9
% Change		−7.6	+0.4	+0.3	−1.1	23.0
Significance (P)		.01<.05	>.05	>.05	>.05	<.01

control medium, while the other femur is grown in the same medium, to which has been added the compound in question. Generally 12 pairs were grown for each level of paraben. After 2 days in culture, the bones were dried in an oven and weighed on a micro balance. The differences in weights between the control and experimental cultures were averaged and the data were analyzed statistically by the paired variate method using the Student T-test(5).

Results and discussion. Table I, A, shows that at 0.25 and 2.5 μg/ml of methyl paraben there resulted a significant increase in dry weight over the controls (9.2 and 7.7% respectively). The propyl paraben is less water soluble than the methyl ester, and therefore the concentration levels assayed were more restricted. However there was a significant growth stimulation at 0.025, 0.25, and 2.5 μg/ml of the propyl paraben (Table I, B). When the two compounds were combined in the same ratio occurring in the pharmaceutical preparation originally used, the growth stimulatory effect was not found to be additive (Table I, C). The stimulatory range was also more restricted. An interesting development was the significant growth inhibition resulting in the lowest levels used (10.1% inhibition at 0.025 μg/ml methyl paraben plus 0.025 μg/ml propyl paraben).

Whitehouse and Boström have studied the action of salicylate analogues in inhibiting the biosynthesis of mucopolysaccharides(6,7,8,9). However these salicylates were only inhibitory when the ortho phenolic group was present, and Whitehouse found that m- and p-hydroxybenzoates did not inhibit mucopolysaccharide synthesis(10). No mention was made of stimulatory effects. Whitehouse pointed out that the compounds he studied were able to form complexes with metal ions,

and that they were lipophilic in character.

To determine whether the parabens might be complexing with a metal ion, EDTA (disodium ethylenediaminetetraacetate) was added to the medium, in molar concentrations equivalent to the molar concentration used for methyl paraben (Table I, D). No stimulatory effect was noted, although there was an inhibitory effect at the lowest level (10^{-7} M) which may be significant. The second criterion Whitehouse suggested for biological activity of these compounds, a lipophilic character, was not fulfilled by EDTA. Although the para structure of the parabens would appear to exclude the possibility of chelate formation with metal ions, it would not exclude the possibility of other types of coordination complexes. Whitehouse notes that the m- and p-hydroxybenzoates do not form complexes with ferric ion, however this may not be the case with other ions, *e.g.*, cupric ion.

Another possible explanation relates to the effects of the salicylate analogues on glucose metabolism. Sturman and Smith found that p-hydroxybenzoic acid and a series of related compounds inhibited glucose utilization by the human erythrocyte(11). Both glycolysis and the pentose phosphate pathway was affected. The salicylate analogues *inhibited* glucose utilization from 5×10^{-3} M to 20×10^{-3} M which is in the concentration range we find growth inhibition. These authors do not mention if lower concentrations of the salicylates stimulate glucose utilization. One would expect that an increase in growth rate would be correlated with an increase in glucose utilization rather than an inhibition.

A third approach to the mechanism of action of the parabens relates to the observation of Miller and Smith that rat liver lysosomes were stabilized by acetylsalicylic acid at concentrations of 10^{-3} to 10^{-5} M(12). We obtained growth stimulation at 10^{-5} M with both parabens, however the methyl ester was active at 10^{-6} M while the propyl ester was active at 10^{-6} and 10^{-7} M. This may reflect the relatively greater lipid solubility of these esters at neutral pH as compared to acetylsalicylic acid.

In other studies we have observed that there is a period of "adaptation" of the femora to *in vitro* conditions. During this period, which may last from 6 to 12 hours, there is a lag period during which little growth occurs. This is followed by a rapid increase in dry weight. It is not unreasonable to conclude that there is a labilization of lysosomes during the initial part of the adaptation period, after which the lysosomal integrity is restored. There are a variety of factors present during the early culture period which might labilize lysosomes. These include rapid changes in pH, oxygen tension and temperature, and the trauma involved in the removal of the femur from the chick. An attractive hypothesis would be that the parabens stabilize lysosomes, and therefore prevent a partial autolysis during the early period of the culture. This stabilization would result in a more rapid adaptation to culture, and consequently a greater final weight.

While the mechanism of action of the parabens is unknown, the significance of these observations is considerable. Anomalous results have been reported by others when pharmaceutical preparations of hydrocortisone were utilized(13). In this Department, Caster, Garner and Luckey noted an increase in the size of lymph nodes when mice were injected with saline containing the parabens(14). This had actually been done as a control for another experiment, and for convenience, hospital saline was used. It appears that most hospital saline solutions contain the parabens, as do pharmaceutical preparations of many drugs. The possible biological effects of the parabens should not be ignored.

Summary. When 10-day-old chick embryo femora were cultured for 2 days in medium containing methyl or propyl paraben, there resulted an increase in dry weight as compared with femora cultured in the control medium. The methyl paraben concentrations found to stimulate growth were 10^{-5} and 10^{-6} M. Propyl paraben stimulated at 10^{-5} to 10^{-7} M. The effects of the two were not additive. It is suggested that these compounds may stabilize lysosomes.

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