however, that both the sulfonyl and sulfamylureas are stable when heated alone in pure hexyl acetate but that prior washing of the hexyl acetate with dilute hydrochloric acid causes the reaction to proceed smoothly. The requirement of both acid and water becomes clear from the preceding discussion of the reactions involved.

The analytical method described for the fluorine containing sulfamylureas is novel because direct use is made of the fluorine substituent in the molecule. The electronegativity of the pentafluoropropyl group increases the acid strength of the series, evidenced by a decrease in pKa [from 7.5 to 8.3 in the cycloalkyl series to 6.5 in the pentafluoropropyl series (3)], enhancing the acid stability of these compounds to the point where they are resistant to hydrolysis under the assay conditions described for the sulfamylureas of the cycloalkyl series. The plasma and urine blank values in the combustion assay are low (*ca.* 1 mcg./ml.), being limited more by the cleanliness of the analytical apparatus than by biological contaminants.

The DNFB assay has been most extensively studied in the sulfamylurea series, but is also applicable to the sulfonylureas (tolbutamide, chlorpropamide,[1] acetohexamide, and cycloheptolamide). Plasma and urine blanks are low (about 5 mcg./ml.) and the assay has a wide range (10–200 mcg./ml.).

SUMMARY

Assays for sulfamylurea hypoglycemic agents and their metabolites in blood plasma and urine have been described. Sulfamylureas were determined by degradation to the corresponding primary amine which was determined spectrophotometrically after reaction with 2:4-dinitrofluorobenzene. The compounds in a series of fluorine-containing sulfamylureas were determined as fluoride ion after Schöniger combustion. One class of metabolites, the sulfamides, was hydrolyzed under basic conditions to give a secondary amine, which was then determined by the methyl-orange-dye–complex technique. The remaining amine metabolites were assayed directly by the methyl orange procedure. Although the assay has been primarily studied for the sulfamylureas, it is equally applicable to the determination of the hypoglycemic sulfonylureas. The high blanks caused by hemolyzed plasma samples in currently used sulfonylurea assays have been eliminated in the assay described.

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Relation of pH to Preservative Effectiveness II

Neutral and Basic Media

By BILLIE WICKLIFFE and DURWARD N. ENTREKIN

Liquid trypticase soy broth media was buffered with Prideaux and Ward's universal buffer to neutral and basic pH levels (pH 7 to 10 inclusive). Preservative solutions of various concentrations were added to tubes containing the media. After inoculation of the tubes with a slurry obtained from soil samples suspended in water, these preparations were examined for preservative effectiveness over a 6-month period. In most cases preservative effectiveness varied with pH alteration. The preservative activity ranged from negligible with cinnamic acid and some of its derivatives, the amides of bromal and dichloroacetaldehyde, sorbic acid and dehydroacetic acid-sodium, to fair in the case of parabens and salicylanilide, to good with cetrimide, chlorophenesin, vanillic acid esters, hexylresorcinol, hexachlorophene, and phenylethanol.

THE DEGREE of dissociation and the pH have been shown to influence the ability of preservatives to act in a chemical or physical way to prevent multiplication of organisms (1–3). The acidity or alkalinity of a product is just one of the many factors involved in the selection of effective preservatives for pharmaceutical preparations (1, 4–6). Antiseptics are said to be effective

at certain pH ranges, and this pH range is different for each antiseptic or group of antiseptics (2, 3).

Some preservatives active in acid pH are not effective at neutral or basic pH levels (7). Studies concerning the parabens as preservatives showed greater concentrations were needed for alkaline than for acid or neutral syrups (8). Antifungal activities of many preservatives have been observed at acid and basic pH levels (9–12).

Bacterial growth has been shown to be inhibited at pH 2 without the use of additional preservative material (13); it has been stated that asepsis also occurs above pH 9 (14).

The presence of buffers in test preparations has been shown to have great influence on the activity of the preservatives employed (15–17). Preservatives incorporated in different buffer mixtures of approximately the same pH value will exhibit varying germicidal activity (17).

Previous work in this series gave results of preservative effectiveness in acid media (13). The work presented here expands this study to include an investigation of the effects of neutral and alkaline pH levels.

EXPERIMENTAL

The procedure as outlined in previous work (13) was followed in this test with the following exceptions:

(*a*) Prideaux and Ward's universal buffer was used in this experiment as it would yield pH values over the entire range to be tested, *i.e.*, pH 7, 8, 9, and 10 (18). This buffer alone has no bactericidal effect at any pH level used in this test (19).

(*b*) The soil sample used for the inoculum had been checked and contained Gram-positive and Gram-negative spore forming rods, cocci, yeast, and mold. To insure the inoculum containing some organism which would multiply at all pH levels included in this investigation, a suspension of *Alcaligenes* was added to the soil sample. The *Alcaligenes* was isolated from a milk of magnesia suspension with pH approximately 10. A pure culture of *Alcaligenes* was grown on an agar slant for several days and washed off with normal saline to prepare the fortifying suspension.

(*c*) Buffered media was autoclaved at 15 p.s.i. for 15 minutes to effect the sterilization. The media buffered to pH 8, 9, and 10 resulted in lower pH readings after this procedure. Therefore, to obtain media with the desired pH values, solutions were prepared at correspondingly higher pH levels which upon autoclaving fell into the desired pH range.

It was noted that the media darkened during the process of sterilization. Media at pH 7 turned yellow, and the color change increased with the increase in alkalinity to pH 10 which turned a light brown. A slight precipitate was formed in the pH 10 media.

Many preservatives included in the previous work (13) and several preservatives not included

were used in this experiment (Table I). Solid preservatives were prepared by weight/volume per cent and liquid preservatives by volume/volume per cent. Results were recorded as: visible growth (+), static condition ($\oplus$), and complete killing of organisms (. .).

RESULTS AND CONCLUSIONS

Cinnamic Acid.—In this study no preservative activity was observed with the aromatic materials tested. Cinnamyl acetate, cinnamic alcohol, and methyl, ethyl, and propyl cinnamate at concentrations of 0.0375, 0.075, and 0.15% were included in the test. These indicated no preservative activity

TABLE I.—PRESERVATIVES SELECTED FOR USE AND THEIR SOURCES

Preservatives	Source
p-HYDROXYBENZOATES	
Methyl parasept	Heyden Newport Chemical Corp.
Ethyl parasept	Heyden Newport Chemical Corp.
Propyl parasept	Heyden Newport Chemical Corp.
Butyl parasept	Heyden Newport Chemical Corp.
CHEMOCIDES	
Cetremide (Chemocide CETB)	Chemo Puro Manufacturing Corp.
Chlorophenesin (Chemocide PCPD)	Chemo Puro Manufacturing Corp.
Salicylanilide (Chemocide SA)	Chemo Puro Manufacturing Corp.
CINNAMYL DERIVATIVES	
Cinnamyl acetate	Fritzsche Brothers, Inc.
Cinnamyl alcohol	Fritzsche Brothers, Inc.
Methyl cinnamate	University of Georgia Research Laboratories
Ethyl cinnamate	University of Georgia Research Laboratories
Propyl cinnamate	University of Georgia Research Laboratories
VANILLIC ACID DERIVATIVES	
Vanillic acid	E. R. Squibb & Sons
Vanillic acid, ethyl ester	E. R. Squibb & Sons
Vanillic acid, propyl ester	E. R. Squibb & Sons
Vanillic acid, isobutyl ester	E. R. Squibb & Sons
AMIDES OF BROMALS AND DICHLOROACETALDEHYDES	
Dichloroacetaldehyde chloroacetamide	W. D. Easterly, Jr., University of Arkansas
Bromal chloroacetamide	W. D. Easterly, Jr., University of Arkansas
Dichloroacetamide phenylacetamide	W. D. Easterly, Jr., University of Arkansas
Dichloroacetamide benzamide	W. D. Easterly, Jr., University of Arkansas
MISCELLANEOUS	
Dehydroacetic acid-sodium	The Dow Chemical Co.
Sorbic acid	Union Carbide Chemical Co.
Hexachlorophene	K & K Laboratories, Inc.
Hexylresorcinol	Winthrop Laboratories
Phenylethanol	Mann Research Laboratories

over the test period, with the ethyl and propyl cinnamate showing no activity at the end of the second month. It should be noted that the solubility of these preservatives in the media was exceeded in most cases; therefore, the material available to exert the preservative action in these tubes was less than the amounts indicated. These materials maintained a static condition in many of the tubes during the earlier readings, particularly at the neutral or weakly basic levels. However, they lost their activity fairly rapidly and eventually exhibited no preservative action. Although these esters have been shown to be effective antifungals (20), they were ineffective as preservatives against mixed organisms at neutral and alkaline pH values.

Chemocides (Table II).—The chemocides as a

TABLE II.—SIX-MONTH READINGS OF CHEMOCIDES

pH	Blank	Concentration, % w/v		
Chlorophenesin (Chemocide PCPD)		0.05	0.1	0.5
7	+	+	+	..
8	+	+	+	..
9	+	+	+	..
10	+	+	+	..
Salicylanilide (Chemocide SA)		0.06	0.1	0.14
7	+	.., +[a,b]	⊕[a]	.., +[a,b]
8	+	..[a]	..[a]	..[a]
9	+	..[a]	..[a]	..[a]
10	+	+	..[a]	..[a]
Cetremide (Chemocide CETB)		0.025	0.05	0.1
7	+	..	..	..
8	+	..	..	..
9	+	..	..	..
10	+	..	..	..

[a] Precipitate formed in tubes on the addition of the preservative solution; therefore, apparently a saturated solution at this temperature has been produced. [b] Duplicate tube did not produce identical readings.

TABLE III.—SIX-MONTH READING OF *p*-HYDROXY-BENZOATES

pH	Blank	Concentration, % w/v		
Methyl Paraben		0.1	0.2	0.3
7	+	+	⊕, ..[b]	..
8	+	+	+	..
9	+	+	+	⊕
10	+	+	+	+
Ethyl Paraben		0.05	0.1	0.2
7	+	+	..	..[a]
8	+	+	⊕, +[b]	..[a]
9	+	+	+	..[a]
10	+	+	+	+
Propyl Paraben		0.075	0.1	0.125
7	+	..[a]	⊕, ..[a,b]	..[a]
8	+	..[a]	..[a]	..[a]
9	+	⊕[a]	..[a]	..[a]
10	+	+	⊕	⊕
Butyl Paraben		0.03	0.04	0.05
7	+	..	..	..
8	+	..	..	..
9	+	..	..	..
10	+	+	+	..

[a] Precipitate formed on the addition of the preservative solution. This precipitate floated on the media. Apparently a saturated solution at this temperature has been produced. [b] Duplicate test did not produce identical readings.

group showed varying degrees of preservative activity. Chlorophenesin was effective only at the maximum concentration (0.5%) used. Similar results have been recorded at these concentrations in media buffered at pH levels 3 through 6 (13). Salicylanilide gave inconsistent results at the lower concentration and neutral pH. At pH 8 and 9 salicylanilide was effective at all concentrations used in this experiment when examined at the end of the test period. It should be noted, however, that conditions of stasis were observed in earlier readings, indicating that this material is not an immediate destroyer of all microorganisms. At pH 10, conditions of stasis developed in tubes at the 3-month reading which at earlier readings showed effective preservative activity. This static condition was observed because growth was evident in the subculture, but no growth was seen in the preserved tube. It is believed that the tubes were contaminated during addition of sterile water used to keep the volume of the test media constant. However at the 6-month reading, preservative effectiveness was observed at 0.1 and 0.14% concentrations. This indicates that at pH 10 salicylanilide is an effective preservative even after 6-months' storage at this extreme pH. It has been shown (13) that salicylanilide is more effective at pH 5 or below in tests run on media buffered to acid levels (pH 3 to 6).

Cetremide, a cationic quaternary detergent, was effective in all concentrations and all pH levels tested throughout the 6-month test period. Cetremide is therefore valuable as a preservative in these concentrations in both acid (13) and basic media, pH 3 and 10 inclusive.

p-Hydroxybenzoates (Table III).—The preservative activity of the parabens increased as the length of the side chain increased. These results agree with previous reports on their effectiveness as preservative or antifungal agents at the pH levels included in this test (6, 9, 13, 21). However, it has been reported that this relationship between chain length and activity does not exist at pH 6 since methyl and ethyl esters were more active at pH 6 than the propyl and butyl esters (13). Propyl and butyl parabens were effective at pH 9; however, all parabens studied, with one exception, were ineffective at pH 10, probably because of hydrolysis of the esters. The exception noted is the butyl ester at its maximum concentration. While the ethyl and propyl esters were not completely soluble in the concentrations used at pH 7, 8, and 9, these were soluble in media buffered to pH 10. This may be explained by the formation of a salt at the more alkaline level. At the lower concentration and higher pH levels, these esters became less active as time elapsed and many cultures changed from static conditions to those of visible growth. This, too, was probably due to hydrolysis of the chemical. At the lower pH levels, however, these static conditions improved to the point of showing effective preservative action.

Vanillic Acid (Table IV).—Vanillic acid gave negative results similar to that of the cinnamic acid derivatives. The esters of vanillic acid, however, showed preservative action; this activity increased as the length of the side chain increased. Vanillic acid was prepared at lower concentrations than its esters due to its decreased solubility. The solubility

of the esters in the media was exceeded in many instances resulting in an "oil-like" substance floating on the test media and red-brown "oil-like" sediment precipitating in the tubes. Therefore, percentages given in the table were not always true values.

The propyl ester was the most effective of those studied; its activity at pH 10 was slightly decreased, possibly due to the formation of alkali salts (22) and/or hydrolysis. This loss of effective-

TABLE IV.—SIX-MONTH READING OF VANILLIC ACID DERIVATIVES

pH Vanillic Acid	Blank	Concentration, % w/v		
		0.5	0.1	0.2

Growth was evident in all tubes before the end of the test period

Vanillic acid, ethyl ester

		0.1	0.4	0.8
7	+	..	..	..[b]
8	+	+	..	..[b]
9	+	+	..	..[b]
10	+	+	+	..[b]

Vanillic acid, propyl ester

7	+	..	..[b]	..[b]
8	+	..	..[b]	..[b]
9	+	..	..[b]	..[b]
10	+	+	..[b]	..[b]

Vanillic acid, isobutyl ester

7	+	+	..[b]	..[b]
8	+	..	..[b]	..[b]
9	+	..	..[b]	.., +[a,b]
10	+	⊕	..[b]	..[b]

[a] Duplicate test did not produce identical readings. [b] Oily precipitate formed on addition of preservative stock solution to media. Apparently a saturated solution at this temperature has been produced.

TABLE V.—SIX-MONTH READING OF AMIDES OF BROMAL AND DICHLOROACETALDEHYDE

pH Bromal chloroacetamide	Blank	Concentration, % w/v		
		0.1	0.2	0.3
7	+	+	.., ⊕[a]	⊕
8	+	+	+	+
9	+	+	+	+
10	+	+	+	+

Dichloroacetaldehyde chloroacetamide

		0.1	0.2	0.4
7	+	+	+	⊕
8	+	+	+	+, ⊕,[a]
9	+	+	+	+
10	+	+	+	+

Dichloroacetaldehyde benzamide

		0.125	0.25	0.5
7	+	+	+	⊕
8	+	+	+	⊕
9	+	+	+	+
10	+	+	+	+

Dichloroacetamide phenylacetamide

7	+	+	+	⊕, ..[a]
8	+	+	+	+
9	+	+	+	+
10	+	⊕, +[a]	+	+

[a] Duplicate tubes did not produce identical readings.

TABLE VI.—SIX-MONTH READING OF MISCELLANEOUS PRESERVATIVES

pH Sorbic Acid	Blank	Concentration, % w/v		
		0.1	0.2	0.3
7	+	+	+	.., ⊕[b]
8	+	+	+	.., +[b]
9	+	+	+	+
10	+	+	+	+

Dehydroacetic Acid-Sodium

		0.01	0.2	0.5

Growth was evident in all tubes by the end of the test period

Hexylresorcinol

		0.02	0.05	0.1
7	+	..[a]	..[a]	..[a]
8	+	..[a]	..[a]	..[a]
9	+	+[a,c,d,f]	⊕[a,c,d,f]	..[a,c,d,f]
10	+	+[d,f]	+	+[a]

Hexachlorophene

		0.005	0.015	0.025
7	+	..	..[a]	..[a]
8	+	..	..	..
9	+	..	..	..
10	+	..	..	..

Phenylethanol

		1.0	2.0	3.0
7	+	..	..[e,f]	..[e,f]
8	+	.., +[b]	..[e,f]	..[e,f]
9	+	..	..[e,f]	..[e,f]
10	+	..	..[e,f]	..[e,f]

[a] Tubes appear cloudy on the addition of the preservative solutions. [b] Duplicate test did not produce identical readings. [c] Oily precipitate formed on addition of preservative stock solution to media. [d] Dark reddish precipitate formed on addition of preservative stock solution to media. [e] Liquid preservative floated on media. [f] Apparently a saturated solution at this temperature has been produced.

ness at pH 10 agrees with previous work (22). The activity of the ethyl ester was similar to that of the propyl ester, except that increased concentration of the preservative was required. The isobutyl ester was intermediate in its action. These esters produced stasis in many of their earlier readings; however, on standing this condition of stasis changed to completion preservation. These results showed some variation from results obtained in acid media where the ethyl ester was most active (13).

Amides of Bromals and Dichloroacetaldehyde (Table V).—The amides of bromals and dichloroacetaldehyde included in this test, although exhibiting antifungal activity in previous tests (23), showed negligible preservative action in these neutral and basic pH ranges. There was slight activity in the range of pH 7 and 8 at the highest concentrations used. As with many of the other materials used in this study, conditions of stasis in the earlier stages of the test reverted to that of visible growth at or before the 6-month reading. It is interesting to note that even though earlier workers (23) had encountered some difficulty with the solubility of the bromal amides, no difficulty was encountered concerning the solubility of any of these compounds in the test media and in the concentration used here.

Miscellaneous (Table VI).—The preservatives collected in a miscellaneous group gave varied results ranging from no action (sorbic acid and dehydroacetic acid-sodium) to complete action (hexachlorophene).

Sorbic acid, an unsaturated fatty acid, was not an effective preservative within the limitations of this test, showing slight activity only at the maximum concentration (0.3%) and the lower pH levels studied. Growth appeared within 2 weeks after inoculation in a majority of these tests. Earlier tests had shown this to be an effective preservative at pH 5 and below (13) but to exhibit no antifungal activity at pH 9 (11). As the pH increases, ionization increases, thus possibly explaining the loss of preservative activity in these tests.

Dehydroacetic acid-sodium gave negative results similar to those of sorbic acid. The highest concentration at pH 7 inhibited visible growth for only 2 weeks. The material was effective in earlier studies in the acid pH range (13).

Hexylresorcinol exhibited preservative action at the lower pH levels tested in all concentrations. A dark reddish-brown precipitate was formed on the addition of this preservative to the media. The quantity of this precipitate increased and darkened as the concentration of the preservative and the pH increased at all concentrations and at all pH levels. As alkalinity of the media increased, the effectiveness of the preservative decreased, showing slight activity at 0.05% at pH 9 and no activity at any concentration used at pH 10. This decline in preservative action developed late in the test period. At 2 weeks only one tube showed a condition of stasis, while all other tubes showed no microbes present. It is believed that these tubes, as those in the salicylanilide series, then became contaminated during the addition of the water used to restore the volume of the test media. These contaminants combined with the hexylresorcinol's loss of effectiveness at pH 10 resulted in the growth observed late during this test. Hexylresorcinol and hexachlorophene were both shown to be effective at acid pH levels in previous work (13).

Hexachlorophene was effective at all pH levels in all concentrations tested in this series. Therefore, hexachlorophene has been shown to be effective over a pH range from 3 to 10, while hexylresorcinol exhibited activity between pH 3 and 9 (13).

It should be noted that the solubility of phenylethanol was exceeded in all tests, except for the lowest concentration used. Since the solubility of phenylethanol in water is 2.0%, it may be assumed that the tubes containing higher percentages of this material will actually contain only this amount in solution, with the remainder of the preservative separating into a second phase. Phenylethanol showed preservative activity in all concentrations studied at all pH levels, except for 1.0% at pH 8.

This was not consistent and must be due to experimental error.

SUMMARY

The effectiveness of several preservatives, both those in present day usage and a few experimental materials, at neutral and basic pH levels was observed over a 6-month test period. It was shown that bacterial growth occurs at all pH levels tested between 7 and 10.

As in the previous work done in this series of tests (13), a fortified soil sample was used as the inoculum in an effort to check the preservative effectiveness against a large number of organisms. Trypticase soy broth was the test media for this investigation with Prideaux and Ward's universal buffer used to maintain the desired pH levels.

Many of the materials tested, including cinnamic acid and some of its derivatives, the amides of bromals and dichloroacetaldehyde, sorbic acid, and dehydroacetic acid-sodium, were ineffective as preservatives. Others, the parabens and salicylanilide, showed some preservative activity; while cetremide, chlorophenesin, vanillic acid esters, hexylresorcinol, hexachlorophene, and phenylethanol exhibited very good preservative action. Those in the latter group therefore warrant further study.

It was seen that pH is a factor which must be considered if judicious selection of preservative agents is to be made.

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