THE EFFECT OF PARA-SUBSTITUENTS ON THE BACTERIOSTATIC PROPERTIES OF PHENYLACETIC ACID.*

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Many organic acids are known which possess some bacteriostatic and bactericidal action. Various investigators have attempted to correlate the structure and physical properties of these acids with this action. Alkyl substituents have been most widely investigated, particularly by Dohme, Cox and Miller (1, 2) and Coulthard, Marshall and Pyman (7) who found that as the length of the alkyl chain was increased, a peak was reached against *Eberthella typhi* but that solubility appeared to be the limiting factor against *Staphylococcus aureus*. Klarmann (17) and Suter (20), (27) studied the effect of alkoxy substituents. Browning (5) considered the effect of amino and substituted amino groups in the acridine dyes. A correlation between bactericidal power and oil-water distribution was found by Daniels and Lyons (9) in their study of an homologous series of acids. Baldinger and Nieuwland (2) found a correlation between the bactericidal action and the physical properties of the alpha-phenylalkanoic acids. The entire series of phenylalkanoic acids up to delta-phenylvaleric acid was investigated by Degering and Goshorn (14), who showed a distinct correlation between oil-water distribution, charcoal adsorption and the bactericidal action of this series of acids. Degering and Goshorn also found that these compounds were most effective over a p_H range of four to five.

Cheeseworth and Cooper (6) as well as others found that substituents in the para position were more effective than in the ortho or meta positions. Up to the present time few studies have been made of various substituents in one position of the same molecule. Such an investigation was made, however, by Labes and Jansen (19) on para derivatives of benzoic acid.

It was the purpose of this work to study the effect of para-substituents on the bacteriostatic action of phenylacetic acid and to investigate the relation of oil-water distribution to bacteriostatic properties.

EXPERIMENTAL.

A number of para-substituted derivatives of phenylacetic acid were prepared according to methods found in the literature.

Phenylacetic acid was prepared by hydrolysis of benzyl cyanide (1), conversion to the sodium salt, and purification before isolation as the free acid.

The nitro compound, *p*-nitrophenylacetic acid, was prepared by nitration of benzyl cyanide followed by hydrolysis to the acid (23). Reduction of *p*-nitrophenylacetic acid to *p*-aminophenylacetic acid was carried out by treatment with hydrogen sulfide in an ammoniacal solution of the acid (23).

Substituted amino compounds were prepared by acylation with the corresponding acyl halide. The acetyl compound, *N*-acetyl-*p*-aminophenylacetic acid, was prepared (13) by acylation of *p*-aminobenzyl cyanide and subsequent hydrolysis. Equally good results were obtained by direct acylation of *p*-aminophenylacetic acid. Orton's method (21) was used in the preparation of *N*-benzoyl-*p*-aminophenylacetic acid and the method of Jacobs and Heidelberger (15) was used to prepare *N*-chloroacetyl-*p*-aminophenylacetic acid.

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p-Hydroxyphenylacetic acid was prepared (18) by diazotization and hydrolysis of *p*-aminophenylacetic acid. This was then converted to the alkoxy derivatives, *p*-methoxyphenylacetic acid and *p*-ethoxyphenylacetic acid, by treatment with the corresponding alkyl sulfates in alkaline solution (8).

The halogen substituted derivatives, *p*-bromophenylacetic acid and *p*-iodophenylacetic acid, were prepared from phenylacetic acid by the action of the appropriate halogen and nitric acid in glacial acetic acid (10). *p*-Chlorophenylacetic acid was prepared by diazotization of *p*-aminophenylacetic acid (22), followed by a Sandmeyer reaction (24). The copper was removed by precipitation with hydrogen sulfide, the acid extracted with ether, and the product recrystallized from water.

The alkyl derivative, *p*-ethylphenylacetic acid, was prepared by (4) condensing ethylbenzene with trioxymethylene (paraformaldehyde) and moist hydrogen chloride in the presence of anhydrous zinc chloride (3), (26). The chloride was then converted to the nitrile by the reaction of potassium cyanide with an alcoholic solution of *p*-ethylbenzyl chloride. This nitrile was hydrolyzed by dilute sulfuric acid to *p*-ethylphenylacetic acid.

PREPARATION OF SOLUTIONS.

Solutions for the bacteriostatic tests were prepared and adjusted to a p_H of five. Due to lower solubility, samples of *p*-bromophenylacetic acid and *p*-iodophenylacetic acid were prepared in a concentration of one Gm. in one L. of solution. The *N*-benzoyl-*p*-aminophenylacetic acid was prepared in a concentration of 0.5 Gm. in one L. of 10% ethylene glycol. Samples of the other acids were prepared in a concentration of two Gm. in one L. of solution.

The proper amount of acid was weighed, transferred to a 100-ml. volumetric flask, and dissolved in 80–90 ml. of distilled water. The p_H of these solutions was then adjusted by addition of the necessary amount of 0.12 normal sodium hydroxide or 0.12 normal hydrochloric acid. A "universal" potentiometer assembly with a glass electrode was used in measuring the hydrogen-ion concentration. The electrode was checked against a buffer solution of known hydrogen-ion concentration before and after each period of use.

After adjustment, the solution was again poured into the volumetric flask and diluted to the mark with distilled water which had also been adjusted to a p_H of 5.0 ± 0.1. The p_H of this solution was re-checked with the glass electrode, a drop or two of alkali or acid being added if necessary, and the solution returned to the flask and stoppered.

BACTERIOSTATIC TESTS.

Escherichia coli and *Staphylococcus aureus* were used as test organisms. One ml. of the sample solution which had been made up to a given dilution with sterile distilled water of the same p_H was added to nine ml. of sterile nutrient broth which was also adjusted to a p_H of 5, thereby diluting the original concentration of the acid tenfold. The tubes of acidified nutrient broth were then inoculated with a standard loopful of a twenty-four-hour culture of *Staphylococcus aureus* or *Escherichia coli*. The tubes were incubated at 37° C. for forty-eight hours and read at the end of this period. Controls of inoculated nutrient broth were run for each bacterium tested.

All of the bacteriostatic tests were made at a p_H of five in order to minimize variations caused by the hydrogen-ion concentration of the medium. Degering, Tetrault and Goshorn (11) have shown that the small concentration of sodium and chloride ions present in the test solutions due to adjustment of the p_H is ineffective. Thus it is apparent that the results are not complicated by extraneous factors. The effect noted in each case is therefore a result due only to the presence of the test compound. By comparing a substituted compound with the parent acid, the relative effect of the substituent group may be determined. The results of these tests are presented in Table I.

DETERMINATION OF THE OIL-WATER DISTRIBUTION COEFFICIENTS.

The oil-water distribution coefficients were determined using U. S. P. cotton seed oil and one two-hundredth normal solutions of the acids. Equal volumes of oil and the acid solution were mixed in a rotatory agitator for eighteen hours at room temperature. After standing about an hour, centrifugation was employed to complete the separation.

Samples of the aqueous layer were then pipetted out and titrated by one-hundredth normal sodium hydroxide solution which was standardized against the original acid solution, phenolphthalein being used as the indicator. From the results of the known normality of the original aqueous solutions, the oil-water distribution coefficient was calculated. The results are given in Table I.

TABLE I.—BACTERIOSTATIC TESTS.

Name of Compound.	Highest Dilution Negative Growth. *Staph. Aureus.*	*Esch. Coli.*	Lowest Dilution Positive Growth. *Staph. Aureus.*	*Esch. Coli.*	Oil-Water Distribution Coefficient.
p-Bromophenylacetic acid	1/5,000	*	1/6,000	1/1,220	73.1
p-Iodophenylacetic acid	1/4,000	*	1/5,000	1/1,220	42.9
p-Chlorophenylacetic acid	1/3,000	1/1,000	1/4,000	1/1,500	23.7
N-Chloroacetyl-*p*-aminophenylacetic acid	1/3,000	1/3,000	1/4,000	1/4,000	0.25
p-Ethylphenylacetic acid	1/2,500	1/1,000	1/3,000	1/1,500	36.1
p-Nitrophenylacetic acid	1/2,000	1/610	1/2,500	1/720	2.71
p-Aminophenylacetic acid	1/1,500	1/2,000	1/2,000	1/2,500	0.13
Phenylacetic acid	1/1,000	1/1,500	1/1,500	1/2,000	1.85
p-Methoxyphenylacetic acid	1/1,000	1/1,500	1/1,000	1/1,500	2.79
p-Ethoxyphenylacetic acid	1/1,000	*	1/1,500	1/610	8.28
p-Hydroxyphenylacetic acid	*	*	1/610	1/610	0.09
N-Acetyl-*p*-aminophenylacetic acid	*	*	1/610	1/610	0.28
N-Benzoyl-*p*-aminophenylacetic acid (solution in 10% ethylene glycol)	*	*	1/610	1/610	...

* The lowest dilution gave positive growth. No killing action was observed in the dilutions possible.

CONCLUSIONS.

Interpretation of Bacteriostatic Tests.—The results of the bacteriostatic tests are shown for *Staphylococcus aureus* and *Escherichia coli* in Table I. There appears to be some variance between the two types of bacteria. The differences can usually be attributed to the varying resistance and composition of the species of bacteria.

No apparent explanation has been found for the fact that *p*-bromophenylacetic acid gives a higher maximum effective dilution than the corresponding iodo compound. This behavior is anomalous on the basis of the work of others. An explanation may be that the competing effects of decreasing solubility and increasing bacteriostatic action show a maximum for the bromo derivative, while the peak is not quite reached for the chloro compound and has been passed for the iodo compound. Another possible explanation is the "quasi-specific" activity noted by Klarmann and co-workers (17).

Moore, Day and Suter (20) found that 4-bromo-6-*n*-butylresorcinol was more effective toward *Staphylococcus aureus* than toward *Eberthella typhi*. A similar effect is noted in this research in that all three of the halogen compounds showed a greater effect with *Staphylococcus aureus* than with *Escherichia coli*.

A striking contrast results from a comparison of the data on *N*-acetyl-*p*-aminophenylacetic acid and *N*-chloroacetyl-*p*-aminophenylacetic acid. The substitution of one chlorine atom on the terminal carbon atom evidently has a very pronounced effect. No explanation of this has been offered other than the normal effect of halogen substitution. The general behavior of amino substituents

closely follows that noted by Browning and others (6) in their study of the bacteriostatic action of the acridine dyes against *Staphylococcus aureus* and *Escherichia coli*. The amino group by itself in *p*-aminophenylacetic acid increases the effective dilution over that of the free acid. When one of the amino hydrogen atoms is substituted by an acetyl group, a marked lowering in the bacteriostatic action is noted. This same lowering occurs when the amino group is replaced by a hydroxyl group as shown by the relative ineffectiveness of *p*-hydroxyphenylacetic acid.

The low effectiveness of *N*-benzoyl-*p*-aminophenylacetic acid may be attributed to the fact that it also is an acylated amino compound, and to decreased solubility caused by the large benzoyl radical. This is not in accord with the conclusion of Johnson and Lane (16) who found that the increase in bactericidal properties is a function of the size of the group introduced, since *N*-benzoyl-*p*-aminophenylacetic acid contained the largest substituent group of the compounds tested.

The results obtained from the tests on the two alkoxy compounds do not check with those of other investigators. Klarmann and others (17) found an increase in the effectiveness of alkoxy compounds with an increase in the number of carbon atoms when the tests were made on *Staphylococcus aureus*. Methoxy- and ethoxy-phenylacetic acids against *Staphylococcus aureus* gave the same maximum effective dilutions. Against *Escherichia coli*, *p*-methoxyphenylacetic acid was more effective than *p*-ethoxyphenylacetic acid. Klarmann found an increase in activity with an increase in the length of the alkoxy chain when he compared the action of his compounds against *Eberthella typhi*.

The alkyl compound gave satisfactory results, based on the comparison of aliphatic chain substituents investigated by numerous workers. The nitro group seems to increase the effectiveness against *Staphylococcus aureus* but to be of little value against *Escherichia coli*.

In their study of the substituted benzoic acids, Labes and Jansen (19) arranged their substituent groups in the following decreasing order: iodo, bromo, chloro, nitro, methyl and hydroxy. The same groups in the phenylacetic acid series, according to this study, take the order: bromo, iodo, chloro, ethyl, nitro and hydroxy.

The introduction of the methoxy group does not seem to inhibit nor enhance the bacteriostatic action of phenylacetic acid since the results against both *Staphylococcus aureus* and *Escherichia coli* were the same for each compound.

Both the bacteriostatic tests and the oil-water distribution coefficient for phenylacetic acid check with those obtained by Degering and Goshorn (14) for the same compound.

Interpretation of Oil-Water Distribution Coefficients.—The results of the oil-water distribution tests are shown in Table I. These coefficients do not coincide, in many instances, with the results from the bacteriostatic tests, but this is not an indication that there is no relationship between the two. However, it is an indication that correlation is best shown by members of an homologous series. The halogen derivatives bear this out in that the results check for the two tests. The alkoxy compounds show an increase in the distribution coefficient with an increasing number of carbon atoms in contrast to their behavior as bacteriostats.

The amino and substituted amino groups gave particularly anomalous oil-water distribution coefficients as compared with their bacteriostatic action. For instance, *p*-amino-phenylacetic acid was the lowest and *N*-acetyl-*p*-aminophenylacetic acid the highest even though the highest maximum effective dilution was given by *N*-chloroacetyl-*p*-aminophenylacetic acid.

If the phenylacetic acid molecule is considered as a constant and the solubility class of the compounds predicted on the basis of the tables of Shriner and Fuson (25), a very interesting result is obtained. The bromo, iodo, ethyl and chloro substituent groups fall in Class I, the ethoxy and methoxy groups are border-line cases between Class N_2 and N_1, the nitro, *N*-acetylamino and *N*-chloroacetylamino groups fall in Class M, the amino group falls in Class B, and the hydroxy group falls in Class A_2. The groups arranged in the order of decreasing oil-water distribution coefficients are found also to be in the order of their increasing solubilities in water. Although the assumption made in predicting these solubilities may seem erroneous, the result is entirely in accord with the relationship between oil-water distribution and solubility.

SUMMARY.

1. Twelve para-substituted derivatives of phenylacetic acid have been prepared, and bacteriostatic tests made against *Staphylococcus aureus* and *Escherichia coli*.

2. Oil-water distribution coefficients have been determined and their relationship to bacteriostatic activity has been discussed.

3. The para-bromo derivative was the most effective of those studied.

4. Substitution of a chlorine atom on the acetyl group of *N*-acetyl-*p*-aminophenylacetic acid caused a very marked increase in bacteriostatic action.

5. The substitution of hydroxy and *N*-acetylamino groups decreased bacteriostatic action, while all other substituents studied enhanced it by varying amounts.

6. Both alkoxy derivatives gave the same maximum effective dilution against *Staphylococcus aureus*, but *p*-methoxyphenylacetic acid was more effective than *p*-ethoxyphenylacetic acid against *Escherichia coli*.

7. *N*-Chloroacetyl-*p*-aminophenylacetic acid, *p*-aminophenylacetic acid, *p*-methoxyphenylacetic acid and phenylacetic acid were found to be more effective against *Escherichia coli* than against *Staphylococcus aureus*.

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INVESTIGATIONS ON THE CHEMISTRY AND PHYSIOLOGY OF THE VENOM OF THE HONEY BEE (*APIS MELLIFICA*).*

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The recent investigations of Hahn and collaborators (1, 2, 3, 4), Reinert (5) and Essex, Markowitz and Mann (6) have thrown considerable light upon the chemical and physiological properties of bee-venom. The earlier work by Flury (7), Langer (8) and Phisalix (9) described methods of obtaining the venom which soon were shown to be less efficient than those adopted by later investigators. Beck (10) gives a description of obtaining the venom for laboratory purposes which appears to be best suited to obtain as little contaminated a venom solution as is possible.

The venom is produced by the insect in the so-called poison-sac by mixing the secretions of three glands. Two of these are known as the acid glands while the third one is called the alkaline gland, because of the p_H of their secretions. Carlet's (11) experiments seemed to show that only the mixture of the two liquids has the typical toxic properties of bee-venom. Hesselhaus (12) believes that the acid glands alone furnish the toxin, and that the secretion of the alkaline gland serves to neutralize the acid that remains in the sting.

Recent investigations, however, seem to indicate that the venom is secreted only in one gland. This gland is in the average 14 mm. long and has a diameter of 0.19 mm. The gland is forked at its end, and this division into two parts has been the reason for earlier investigators to arrive at the conclusion that two different glands are present. The so-called third venom gland which enters just below the poison-sac seems to be merely a producer of a lubricating substance and does not contribute at all to the venom itself.

Most of the work, although this is not especially mentioned, has obviously been done on the northern European variety of bees, the so-called British Black (*Apis Mellifica Mellifica*), an insect which differs somewhat in color and other characteristics from the so-called Italian race of bees (*Apis Mellifica Ligustica*), which is practically the only variety obtainable in the

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