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Some Aggregate Analogs of *p*-Aminobenzoic Acid and Dimethyldiaminobenzene Possessing Unusual Biological Properties

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Methods for the synthesis of 9 new compounds have been developed. These substances were called aggregate analogs because they were constructed by union of appropriate analogs of 2 distinct essential metabolites, *viz.*, *p*-aminobenzoic acid and 1,2-dimethyl-4,5-diaminobenzene. These aggregate analogs were conceived as a means of realizing new pharmacological agents of high potency, and with a type of action which would not be antagonized by the metabolites to which they were structurally or functionally related. Some of the compounds were shown to have these properties when tested as inhibitors of the growth of *Staphylococcus aureus*. The poisonous action of these substances on mice was examined, and of those tested, the toxicity was either low or undetectable. This low order of animal toxicity was predicted from previously established theoretical considerations.

The suggestion has recently been made that antimetabolites of unusual and desirable properties may be made by combining in one molecule two analogs of metabolites concerned in closely interrelated and vital functions of living cells.[2] Two such aggregate analogs were formed by combination of the two antimetabolites sulfanilamide and dichlorodiaminobenzene. The two metabolites, *viz.*, *p*-aminobenzoic acid and dimethyldiaminobenzene, to which these latter two antimetabolites are related, are known to function as precursors of 3 vitamins (folic acid, riboflavin and vitamin B_{12}) of which at least 2 have been shown to be intimately associated in the process of cell division. Thus, both folic acid and vitamin B_{12} are involved in the biosynthesis of desoxyribosides, of biologically important purines and pyrimidines and of methionine.[3]

The purpose of the present paper is to report some additional explorations of such aggregate analogs. Several such compounds have been synthesized for the first time, and their abilities to inhibit the growth of *Staphylococcus aureus* have been examined. Two kinds of outstanding biological properties were observed: (1) high antibacterial potency in some of the substances, and (2) difficulty, or inability of the structurally related metabolites, and even of the products of the inhibited reactions, to overcome the antibacterial effect. This irreversible kind of action has previously been emphasized to be a very desirable, if not an essential, property of the therapeutic agents.[4]

(1) With the technical assistance of G. Schaffner and P. N. Townsend.

(2) D. W. Woolley and A. Pringle, *J. Biol. Chem.*, **194**, 729 (1952).

(3) W. Shive, *Ann. N. Y. Acad. Sci.*, **52**, 1212 (1950).

(4) D. W. Woolley, *J. Exp. Med.*, **93**, 13 (1951).

Experimental Part

The two known compounds, *viz.*, 1,2-dichloro-4-sulfanilamido-5-aminobenzene and 1,2-dichloro-4-(*p*-nitrobenzenesulfonylamido)-5-aminobenzene were synthesized as described by Woolley and Pringle.[2] A more satisfactory route to the first of these was developed, and will be described. These compounds will be called compounds 1 and 2, respectively, in the present paper.

1,2-Dichloro-4-(*p*-nitrobenzenesulfonylamido)-benzene (Compound 3).—A suspension of 50 g. of 3,4-dichloroaniline in 700 cc. of 1 *N* aqueous NaOH was heated until the compound melted (about 80°), and 75 g. of *p*-nitrobenzenesulfonyl chloride was added rapidly. The flask was stoppered, and immediately shaken violently for about 15 minutes. The mixture was cooled, filtered, and the filtrate was acidified with concentrated HCl to *p*H 2. The precipitate which formed was collected, washed well with water and recrystallized from acetic acid plus ethanol. The yield was 38 g. of crystals which melted at 178° after sintering from 174°. The portion of the reaction mixture which was insoluble in alkali yielded 17 g. of unchanged dichloroaniline. Analytical findings for compound 3 and for other members of the series are shown in Table I. All melting points were determined with a hot-stage microscope.

TABLE I

PROPERTIES OF NEW COMPOUNDS

Number	Substance	M. p., °C.	Found C	Found H	Found N	Calculated C	Calculated H	Calculated N
1	1,2-Dichloro-4-sulfanilamido-5-aminobenzene	237 and 214	43.5	3.3	12.6	43.3	3.3	12.7
3	1,2-Dichloro-4-(*p*-nitrobenzenesulfonylamido)-benzene	178			8.1			8.1
4	1,2-Dichloro-4-(*p*-nitrobenzenesulfonylamido)-5-nitrobenzene	168-170			10.5			10.7
5	1,2-Dichloro-4-(*p*-nitrobenzamido)-benzene	266			9.1			9.1
6	1,2-Dichloro-4-(*p*-nitrobenzamido)-5-nitrobenzene	167–168			11.9			11.7
7	1,2-Dichloro-4-(*p*-aminobenzamido)-5-aminobenzene	183–186			14.1			14.2
8	1,2-Dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene	283–285	66.6	5.2	10.6	66.6	5.2	10.4
9	1,2-Dimethyl-4-(*p*-carbethoxyphenylazo)-5-hydroxybenzene	144–146			9.5			9.4
10	1,2-Dimethyl-4-(*p*-hydrazidophenylazo)-5-hydroxybenzene	246			19.6			19.7
11	1,2-Dimethyl-4-(*p*-sulfonamidophenylazo)-5-hydroxybenzene	256[a]	55.2	5.2	13.8	55.2	4.9	13.8

[a] Began to sinter at 236°.

1,2-Dichloro-4-(*p*-nitrobenzenesulfonylamido)-5-nitrobenzene (Compound 4).—Fuming HNO_3 (sp. gr. 1.5, 50 cc.) was cooled to −15°, and 7 g. of compound 3 was added at such a rate that the temperature did not rise above −10°. Thirty minutes after completion of the addition, the solution was poured on 400 g. of ice. The precipitate was collected, washed thoroughly and recrystallized from glacial acetic acid. The yield was 5 g. The position of the nitro group was established by reduction of this substance to compound 1, which is of known structure.[2]

1,2-Dichloro-4-sulfanilamido-5-aminobenzene (Compound 1).—Compound 4 (15.2 g.) was dissolved in 100 cc. of hot acetic acid and 250 cc. of concentrated HCl was added. The mixture was heated on a steam-bath and slowly treated with 22 g. of mossy tin. About 30 minutes after the metal had dissolved, and when the color had disappeared, the solution was concentrated under reduced pressure to 100 cc., diluted with 200 cc. of water, and freed of tin with H_2S. The filtrate from the tin sulfide was con-

TABLE II

GROWTH-INHIBITORY POWER OF AGGREGATE ANALOGS FOR *Staphylococcus aureus*

Number	Compound	Amount to cause 1/2-maximal inhibiton,[a] γ per cc. No supplement	+ PAB	+ DMDA	+ all metabolites[b]
1	1,2-Dichloro-4-sulfanilamido-5-aminobenzene	7	100	7	100
2	1,2-Dichloro-4-(*p*-nitrobenzenesulfonylamido)-5-aminobenzene	26			26
3	1,2-Dichloro-4-(*p*-nitrobenzenesulfonylamido)-benzene	3			3
4	1,2-Dichloro-4-(*p*-nitrobenzenesulfonylamido)-5-nitrobenzene	1.4	1.4	1.4	0.8
6	1,2-Dichloro-4-(*p*-nitrobenzamido)-5-nitrobenzene	Inactive at 60			
7	1,2-Dichloro-4-(*p*-aminobenzamido)-5-aminobenzene	34	34	34	34
8	1,2-Dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene	5.4	5	5.4	4.6
9	1,2-Dimethyl-4-(*p*-carbethoxyphenylazo)-5-hydroxybenzene	Inactive at 100			
10	1,2-Dimethyl-4-(*p*-hydrazidophenylazo)-5-hydroxybenzene	Inactive at 30			
11	1,2-Dimethyl-4-(*p*-sulfonamidophenylazo)-5-hydroxybenzene	Inactive at 100			

[a] Final concentrations per cc. of medium were PAB 1γ, DMDA 10γ, riboflavin 0.1γ, vitamin B_{12} 0.01γ, pteroylglutamic acid 0.01γ. [b] This indicates a mixture of the substances of the preceding footnote, in the concentrations defined there.

centrated under reduced pressure to dryness. The residue was suspended in 200 cc. of water, and the suspension was adjusted to *p*H 7 with NaOH. The desired compound was then extracted into ethyl acetate. This solvent was removed under reduced pressure, and the crystals so obtained were recrystallized from 50% aqueous ethanol. The yield was 13 g.

The melting point behavior of this compound was unusual. The first several batches were needles which melted at 214°, and this value was unchanged when the substance was mixed with the authentic specimen prepared by a different route.[2] However, later preparations melted at 237°, after showing some sintering from about 210°. The synthesis of this substance from 1,2-dichloro-4,5-diaminobenzene[2] was repeated, and the high melting variety was obtained, instead of the product melting at 214°. It was then observed that a sample of the low melting form which had been stored for about 6 months exhibited the higher melting point. Furthermore, seeding of ethanolic solutions of the low melting substance with the high melting form gave only crystals of the higher melting point. Both high and low melting materials showed theoretical values for C, H and N. They were therefore concluded to be polymorphic forms.

1,2-Dichloro-4-(*p*-nitrobenzamido)-benzene (Compound 5).—The same technique as for compound 3 was used, except that *p*-nitrobenzoyl chloride replaced the sulfonyl chloride and the product was filtered from the alkaline reaction mixture. Because this product was not an acid, it was insoluble at that stage. Washing of this precipitate with 1 *N* HCl and recrystallization of the residue from acetic acid gave 81% of the theoretical yield.

1,2-Dichloro-4-(*p*-nitrobenzamido)-5-nitrobenzene (Compound 6).—Compound 5 was nitrated in the manner described for the preparation of compound 4. The crude product was freed from *p*-nitrobenzoic acid (formed by concomitant hydrolysis) by extraction with aqueous $NaHCO_3$, and finally recrystallized from glacial acetic acid. It was obtained in 40% yield.

The structure of this substance was established by hydrolysis with strong HCl which gave 1,2-dichloro-4-amino-5-nitrobenzene, melting at 178°.[5]

1,2-Dichloro-4-(*p*-aminobenzamido)-5-aminobenzene (Compound 7).—In order to produce this substance, the reduction of compound 6 must be conducted in a way to avoid hydrolysis, or ring closure to the benzimidazole. Compound 6 (1.8 g.) was dissolved in 180 cc. of acetic acid and 10 cc. of water. Two hundred mg. of platinum oxide catalyst was added. The mixture was shaken under a hydrogen pressure of 60 pounds per sq. in. until the theoretical amount of hydrogen had been absorbed. The catalyst was removed, and the filtrate was concentrated to dryness under reduced pressure, below 40°. The sirupy residue, which darkened rapidly, was caused to crystallize by addition of water, and was recrystallized several times by dilution of an ethanolic solution with water.

1,2-Dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene (Compound 8).—$NaNO_2$ (7 g.) and 13.7 g. of *p*-aminobenzoic acid were dissolved in 60 cc. of cold 2 *N* NaOH, and the solution was added to 50 cc. of concd. HCl with cooling and stirring, such that the temperature did not rise above 4°. The resulting suspension of diazonium salt was slowly added to a solution of 12 g. of 1,2-dimethyl-4-hydroxybenzene in 300 cc. of 2 *N* NaOH, which was cooled to 0° and stirred. The bright red solution was kept cold for 30 minutes, and then acidified with concentrated HCl. The precipitate was collected and thoroughly washed with water. It was then suspended in 600 cc. of boiling ethanol. When the suspension had cooled, the insoluble product was collected and recrystallized from butanol. The yield was 22 g. Diepolder has shown[6] that the azo group enters the 5-position in similar reactions with aniline. The present compound was highly insoluble in water and in various nonpolar organic solvents, and only moderately soluble in alcohols or acetic acid. It formed a red sodium salt which was soluble in water at *p*H 7.

1,2-Dimethyl-4-(*p*-carbethoxyphenylazo)-5-hydroxybenzene (Compound 9).—This substance was prepared as was compound 8 except that *p*-aminoethyl benzoate hydrochloride was used instead of *p*-aminobenzoic acid. Because of the greater solubility of the diazonium salt of this ester than of that derived from *p*-aminobenzoic acid, the diazotization was done in the usual fashion by solution of the amine in aqueous HCl and addition of $NaNO_2$. To reduce the danger of hydrolysis of the ester during the coupling, 400 cc. of 1 *M* Na_2CO_3 and 100 cc. of 2 *N* NaOH were used to dissolve the phenol. After the coupling, the suspension was adjusted to *p*H 7, and the product was filtered off in order to separate it from any compound 8 which might have been formed by hydrolysis. The substance was extracted with hot ethanol, as was described for compound 8, and recrystallized from butanol. The yield was 23 g. from 12 g. of the phenol.

1,2-Dimethyl-4-(*p*-hydrazidophenylazo)-5-hydroxybenzene (Compound 10).—Compound 9 (1.8 g.) was suspended in 20 cc. of hydrazine hydrate (100%) and the mixture was heated at 100° for 6 hours. The hydrazide was then precipitated by addition of 75 cc. of ethanol, and recrystallized from butanol. This compound melted at 246°, but as heating was continued a few degrees above the melting point, long needles formed. These did not melt at 300°.

1,2-Dimethyl-4-(*p*-sulfonamidophenylazo)-5-hydroxybenzene (Compound 11).—Sulfanilamide (17.5 g.) in 160 cc. of 3 *N* HCl was diazotized in the usual way with 7 g. of $NaNO_2$, and coupled with 12 g. of the dimethylphenol as was described for compound 8. The yield was 21 g.

Inhibition of the Growth of *Staphylococcus aureus* Caused by Some of the Compounds.—The test for potency of the analogs as inhibitors of the growth of *S. aureus* was the same as that described earlier,[2,4] with the exception that *p*-aminobenzoic acid, riboflavin, pteroylglutamic acid, vitamin B_{12} and dimethyldiaminobenzene were omitted from the medium. The conditions previously outlined, especially the size of the inoculum and the time of incubation were strictly followed. The aggregate analogs were added in the form of sterile neutral aqueous solutions to the assay tubes after these tubes had been autoclaved. The relative potencies can be seen from the data in column 3 of Table II.

The ability of each of the metabolites, *p*-aminobenzoic acid, 1,2-dimethyl-4,5-diaminobenzene, riboflavin, vitamin B_{12} and pteroylglutamic acid, and of combinations of them, to reverse the antibacterial action of the aggregate analogs

(5) F. Beilstein and A. Kurbatow, *Ann.*, **196**, 229 (1879).

(6) E. Diepolder, *Ber.*, **42**, 2916 (1909).

was examined by determination of the growth-inhibiting potency in the presence of these substances. With dimethyldiaminobenzene and *p*-aminobenzoic acid, which were the metabolites related structurally to the inhibitors, several concentrations were examined in order to establish whether or not the antagonism was competitive. This, however, was only done in those cases in which evidence was found for antagonism (*e.g.*, compound 1). With riboflavin, pteroylglutamic acid and vitamin B_{12}, which are the products of the reactions presumed to be inhibited by the substances studied, a level sufficient to meet adequately all the needs for growth was employed without variation. The data in Table II will show that except for compound 1, all the analogs which showed growth-inhibiting powers were not antagonized by these metabolites. Compound 1, however, at concentrations below 100 γ per cc. was reversed by *p*-aminobenzoic acid. This antagonism was competitive, as the data in Table III will show. Nevertheless, growth in the presence of this aggregate analog was never maximal even in the presence of excess *p*-aminobenzoic acid. This retardation could not be overcome significantly by a mixture of all the metabolites here indicated. There was thus a small but readily discernible non-competitive inhibition with it. The character of this incomplete reversal by *p*-aminobenzoic acid can be seen from the data in Table IV.[7] The values in this table were collected in the absence of dimethyldiaminobenzene, riboflavin, vitamin B_{12} and pteroylglutamic acid, but the same results were obtained in the presence of these metabolites.

TABLE III

RANGE OF REVERSAL BY *p*-AMINOBENZOIC ACID OF THE INHIBITION OF GROWTH OF *S. aureus* CAUSED BY COMPOUND 1

PAB γ per cc.	Compound 1 required for half-max. inhibition, γ per cc.	Compound 1 required to counteract the added PAB, γ per cc.	Inhibition index
0	7	..	...
.01	11	4	400
.03	20	13	433
.10	40	33	330
1.0	100	93	93

TABLE IV

EFFECT OF COMPOUND 1 ON THE GROWTH OF *S. aureus* IN THE PRESENCE OF EXCESS *p*-AMINOBENZOIC ACID

PAB γ per cc.	Compound 1 γ per cc.	Turbidity[a]	PAB γ per cc.	Compound 1 γ per cc.	Turbidity[a]
1.0	0	58	3.0	100	71
1.0	80	75	3.0	50	69
1.0	40	68	3.0	25	60
1.0	20	68			

[a] Expressed as per cent. of incident light transmitted by the culture as compared to the uninoculated medium.

Effects of Some of the Aggregate Analogs on Mice.—Because of the high potency of some of the aggregate analogs for species such as *S. aureus* and particularly because of the irreversible character of the toxicity, the determination of their toxicity to higher animals had chemotherapeutic importance. If the working hypothesis previously outlined[4] is valid, these compounds would be expected to be relatively harmless to living things such as mice which have a dietary need for riboflavin, vitamin B_{12} and pteroylglutamic acid. The following data show that this expectation was realized, in that toxicity for mice was low, and in some instances, not detected.

Acute toxicity was determined by injection of neutral, aqueous solutions or suspensions of each compound intraperitoneally into adult mice. Graded doses were thus administrated to groups of six animals or more for each dose level. These mice were fed stock rations, and observed for abnormal behavior and for survival. Chronic toxicity was determined in the same way, except that the test substance was injected daily for 5 days. The data are summarized in Table V.

TABLE V

TOXICITY FOR MICE OF AGGREGATE ANALOGS INJECTED INTRAPERITONEALLY

Compound number	Acute LD/50, mg. per kg.	Chronic LD/50,[a] mg. per kg. per day	Well tolerated dose,[b] mg. per kg. per day
1	>400	>400	400
2	>400	120	
3	120	80	
4	120	60	30
8	>400		[c]

[a] Based on response to daily intraperitoneal injection for 5 days. [b] Amount which caused no deaths among groups of 6 mice injected daily for at least one month; not necessarily the maximal well tolerated dose. [c] The compound neutralized and fed as 1% of the diet for one month was well tolerated.

Although compound 1 did not show appreciable toxicity as measured by survival, all animals which had been injected daily with large doses of it (10 mg. per day) for long periods (1–2 months) showed a characteristic rounding of the edges of the liver. This was invariably seen at autopsy. In addition, pronounced adhesions of the abdominal viscera were usually observed. Despite these manifestations, however, young mice treated with large amounts of this compound grew at a normal rate, and showed no discernible disease.

Compounds 3 and 4 were the most toxic of those examined, and with both, the signs of acute poisoning were identical. Shortly after injection of a lethal dose, mice so treated became hyperirritable and died in most profound rigor. The entire body was quite rigid, and this was true even of the tail. The tip of the tail was always bent at an angle to the main part.

Discussion

The results of this study show that by proper construction of an aggregate analog a biologically active substance can be obtained with 2 highly desirable properties: (a) high potency and (b) irreversible action. The aggregate analogs were produced by forming a molecule which contained within it 2 differing portions, each closely akin to one of two metabolites which function in living things in a closely connected series of vital metabolic processes. When these metabolites are chosen, as they were in the present investigation, with an eye to the formation of selectively toxic antimetabolites (*cf.* 4), then a further desirable property from the standpoint of chemotherapeutic use may be realized. Thus, the substances of high toxicity for *S. aureus* were relatively harmless to mice.

Some instructive relationships of chemical structure to biological activity can be discerned from the information at hand. The aggregates examined fall into 2 classes: those in which *p*-aminobenzoic acid and dimethyldiaminobenzene have been joined through the acidic group of PAB and the basic group of DMDA, with appropriate alterations of the structure to convert metabolites into antimetabolites (compounds 1–7); and those in which PAB and DMDA have been joined at the basic groups of both (compounds 8–11). The most active example of the first class is compound 4, or 1,2-dichloro-4-(*p*-nitrobenzenesulfonylamido)-5-nitrobenzene. Of the second class, the best was compound 8, or 1,2-dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene. In the first series (compounds 1–7) one sees

(7) Although in the preceding paper[2] compound 1 was assigned a potency less than that shown here, this was due to the presence of *p*-aminobenzoic acid in the test medium there employed.

that the coupling of the unchanged metabolite *p*-aminobenzoic acid with the antimetabolite dichlorodiaminobenzene gives rise to a substance of moderate potency (compound 7). The action of this compound is not reversed by the related metabolites. The activity is considerably greater than would be expected from the acylation of an amino group of dichlorodiaminobenzene with an indifferent acid radical.[2] When both metabolites in the aggregate are changed in a fashion expected to convert them into antimetabolites, as in compound 1, activity is increased, but the issue is obscured by the fact that the compound behaves to a considerable degree as just a powerful congener of sulfanilamide. Nevertheless, it is slightly more than that because *p*-aminobenzoic acid did not completely abolish its antibacterial action. Two more structural alterations bring us to compounds 3 and 4, which are both highly potent, and irreversibly active. Here, just as in other series studied earlier[2,8] the introduction of properly situated nitro groups resulted in the acquisition of high potency along with irreversible activity.

In the second class of aggregate analogs the conversion of DMDA to 1,2-dimethyl-4-amino-5-hydroxybenzene (a true antimetabolite of it[2]) and the union of this with unchanged PAB gave the most active compound. When the PAB portion was also changed into a sulfonamide, as in compound 11, all activity was lost. In fact, even the esterification of the carboxyl group of the PAB moiety, as in compound 9, or the elimination of the carboxyl group, as in compound 9 of the preceding paper,[2] abolished activity. These facts are of interest when one remembers that among the antibacterial agents derived from PAB alone, the effective ones have a free *p*-amino group while, on the other hand, many substitutions can be made for the acidic function, as in the host of congeners of sulfanilamide.

Some may contend that these aggregate analogs are not antimetabolites. It is not intended to debate this question in this paper. Rather, it is intended to show that by forming such aggregates, desirable properties may be built into pharmacological agents which formerly were good antimetabolites.

The concept of how these aggregates function biologically is essentially that of a multiple block in a series of connected and coördinated reactions in a vital process of the cell. Even though one half of the aggregate may form a reversible combination with the one active, enzymic center, it is still anchored by its other half to the second enzyme which is anatomically and spatially oriented with the first. The obstruction offered by the first block effectively relieves the cell of the ability to cope with the second. If concurrently a further difficulty is presented by introducing chemical groupings in the first moiety which cause difficulty in reversing the first block, as by introduction of halogen and nitro groups, then the efficiency of inhibition is even more enhanced. It is important to emphasize that this is a conceptional hypothesis of the mode of action which has not been definitely proved.

The idea of achieving increased potency by blocking sequential metabolic reactions is not new with this paper, or with the one preceding it.[2] Thus, to quote only some of the cases, Roblin, *et al.*,[9] made analogs of the purines and tried them in connection with sulfanilamide when it became known that not only PAB, but also adenine would reverse the antibacterial effect of sulfanilamide. More recently, Potter and Simonson[10] have used malonate and fluoroacetate together to inhibit 2 stages in the Krebs cycle. What is new in the present work, and that which was published earlier,[2] is the construction of a single molecule which contains within itself the properties to cause the multiple block with consequent emergence of desirable pharmacological properties.

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(10) V. R. Potter and H. Simonson, *Proc. Soc. Exp. Biol. Med.*, **76**, 41 (1951).