SELECTIVE TOXICITY OF 1,2-DICHLORO-4,5-DIAMINOBENZENE: ITS RELATION TO REQUIREMENTS FOR RIBOFLAVIN AND VITAMIN B_{12}*

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Recent investigations have indicated that one way in which to achieve selectively toxic antimetabolites is to find a biological *precursor* of a vitamin, and then to construct a suitable analog of it. Such an analog may then harm those species which synthesize this vitamin, and should not affect those which have a nutritional requirement for it. Thus, the selective toxicity of sulfanilamide and its congeners for certain bacteria in contrast to higher animals has been postulated to reside in the difference in requirement for folic acid (1–3). This postulate has received support in the finding that the harmful action of a pimelic acid analog could be foretold with fair precision from a knowledge of nutritional requirements for biotin (4). The vitamin which arises from the precursor by a series of biosynthetic reactions (*e.g.*, biotin) sometimes may be able completely and non-competitively to erase the effects of the analog.

The present investigation was undertaken with two questions in mind. (*a*) Could additional substances of predictable selective toxicity be realized by construction of structural analogs of a precursor of another vitamin? (*b*) Could a means be found to foretell antimetabolites of the precursor which would not be counteracted by the vitamin?

With regard to the first question, the correlation of the selective toxicity of sulfanilamide and of the pimelic acid analog with nutritional requirements for the vitamins, folic acid or biotin, was just cited. In the present study a single precursor of both riboflavin and vitamin B_{12} was sought, an analog of this precursor was prepared, and it has been shown to retard the growth of all species examined except those with a nutritional need for these two vitamins. This demonstration added evidence in favor of the postulate that selectively toxic substances can be achieved in this general way.

The bearing of the second question on some of the problems of chemotherapy and of pharmacology can be seen from consideration of the action of sulfanilamide, in contrast to that of the pimelic acid analog mentioned above. This

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latter antimetabolite retarded the growth of biotin-synthesizing organisms, and this action was overcome competitively by pimelic acid (4). Such organisms have been shown probably to carry out the formation of biotin by a series of reactions starting with pimelic acid (5, 6). The growth retardation is therefore probably due to an interference with this synthesis. In accord with this opinion it was found that if biotin was present in the culture fluid, the pimelic acid analog was no longer toxic. Similarly, those species which lacked the ability to synthesize biotin, and depended on their food for it, were not susceptible to the toxicity of the analog.

Nevertheless, the ability to counteract the analog with the vitamin related to it further up the biosynthetic pathway probably accounts in part for the failure of such compounds as therapeutic agents in infectious diseases. In such chemotherapy it is necessary to retard one species selectively when it is growing in mixed culture with another (*i.e.*, the host animal). This is a considerably more exacting task than is the selective inhibition of isolated species. In terms of the example with the pimelic acid analog, biotin-independent forms can be affected, and biotin-dependent ones are not when tested singly. However, when tested in mixed culture, the biotin needed to secure any growth of the latter forms negates the action of the analog on the former ones. As a result, neither is harmed. This has proved to be true experimentally. Thus, although mice were resistant to this pimelic acid analog, and tubercle bacilli were quite susceptible, it was found incapable of saving mice from mortal infection with tubercle bacilli (7). The biotin within the animals probably nullified the antibacterial power of the compound. Of course, this explanation is not the only one which may apply, but it seems the most plausible one in view of existing knowledge.

The chemotherapeutic action of sulfanilamide indicates the probable point of difference of a successful drug from one which is not so. This analog of *p*-aminobenzoic acid would be expected to be counteracted by folic acid, the product somewhat further up the biosynthetic path. However, in practically all cases this is not found to be the case. Only in the case of a few species of enteric streptococci will folic acid nullify the antibacterial action of the sulfonamide drugs (2, 8). These organisms are among those which are resistant to sulfonamide therapy when they invade animal tissues. Perhaps the folic acid in these tissues accounts for the failure of the drugs to act. When grown in isolated culture in the absence of excess folic acid, the enteric streptococci are susceptible to sulfanilamide (2). The chemotherapeutic success of sulfonamide drugs thus may depend on an inability of folic acid to erase its antibacterial powers, just as the chemotherapeutic failure of the pimelic acid analog may be related to the ability of biotin to do so. The problem then is to understand the basis of this difference, or failing that, to comprehend enough of it to provide conditions favorable to the formation of similarly irreversible agents which will act in mixed cultures.

The working hypothesis which has been explored in this study is that selectively acting compounds which are not nullified by vitamins further along the biosynthetic path, can be made by choice of a metabolic precursor of *two or more* vitamins and production of a suitable analog of this precursor. The desirable type of action of sulfanilamide is believed to reside in such a situation. It is an analog of *p*-aminobenzoic acid, which is a precursor of folic acid, and possibly of some other essential metabolite as well. These other essential metabolites are not considered to be the purines, serine, or valine (9), because, although the latter influence the action of sulfanilamide, the bulk of evidence points to the conclusion that they are products arising from reactions of folic acid, and hence only secondarily from *p*-aminobenzoic acid. (See for example references 10–12.) It is not possible to give any well documented reason why an analog of a precursor of two or more vitamins should act in the desired irreversible fashion. One might expect a mixture of these two or more metabolites to erase the effect just as biotin does for the pimelic acid analog. Although to support the postulate an argument can be constructed which is based on competing rates of reaction for the precursor and on differences in the way the two vitamins are metabolized, sufficient data are not available to make it definitive.

The working hypothesis can be tested in the following way. A precursor of two or more vitamins should be sought, and an antimetabolite of it should be made. This antimetabolite ought then to prove harmful to species which do not have a nutritional need for both of these vitamins, and harmless to those which require them. Furthermore, both vitamins should be unable to nullify the toxic effect of the analog, while the precursor to which it is structurally related should do so competitively.

1,2-Dimethyl-4,5-diaminobenzene was recognized as a probable precursor of riboflavin and of vitamin B_{12}, and some evidence for this opinion was found. This diamine occurs in riboflavin fused to a pyrimidine ring and a ribityl side chain. It has also been identified in vitamin B_{12} as part of a dimethylbenzimidazole riboside which occurs in acid hydrolysates of the vitamin (13). An antimetabolite of it, namely 1,2-dichloro-4,5-diaminobenzene showed those biological properties expected of such an analog.

EXPERIMENTAL

1,2-Dichloro-4,5-diaminobenzene.—This substance seems to have been obtained previously only as an impure oil of unestablished constitution (14). It can be prepared readily as a pure substance in either of the following ways.

Eighteen gm. of 1,2-dichloro-4,5-dinitrobenzene (15) was suspended in 120 cc. of concentrated HCl, the mixture was heated on a steam bath, and 30 gm. of mossy tin was added slowly. The mixture was shaken vigorously during the addition of the tin, and the flask was equipped with an air condenser during the operation. When the order of addition was changed, the product was black, and difficult to purify. The mixture was heated for half an hour on the steam bath after all the tin had been introduced, and then 200 cc. of water and about 3 gm.

of norit were added. The mixture was filtered while it was still hot, and the colorless filtrate was concentrated under reduced pressure until extensive crystallization had occurred (to about 100 cc.). The mixture was cooled and saturated with HCl gas and the crystals were collected, washed with concentrated HCl, and recrystallized from 50 cc. of water by saturating the solution with HCl. The yield was 13.4 gm. of white needles. When heated on a microscope stage the shape of the crystals changed at about 155° from needles to plates, and these melted with decomposition at 193°. These values were unchanged by recrystallization.

$C_6H_8N_2Cl_4$. Calculated, N 11.2; found, N 11.3

A substance with the same characteristics was formed when 1,2-dichloro-4-amino-5-nitrobenzene was similarly reduced with tin and HCl. The starting material for both routes was 1,2-dichloro-4-nitrobenzene. The direct nitration required in the first method limited the scale of the operations, but the over-all yield was better than by the second procedure.

The free base was obtained by solution of the dihydrochloride in water, addition of an excess of 1 N NaOH, and extraction with ether. Evaporation of the ether from the extract left white or pink crystals which were purified by recrystallization from hot water. These crystals began to shrink at about 130° and slowly changed as the temperature was raised, until they had disappeared at about 160°.

$C_6H_6N_2Cl_2$. Calculated, C 40.7, H 3.4, N 15.8
Found, C 40.9, H 3.4, N 16.0

Sources of the Organisms Used.—Cultures of *Lactobacillus leichmanni* 313 were obtained from Dr. V. du Vigneaud and from Dr. L. D. Wright, who also supplied *L. lactis* (Dorner) and *L. bifidis*. *Euglena gracilis* was from Dr. S. H. Hutner. *Chlorella vulgaris* and the 6 plant pathogens, *Pseudomonas tabaci*, *P. angulata*, *Corynebacterium michiganense*, *C. fascians*, *Agrobacterium tumefaciens*, and *Xanthomonas pelargoni* were provided by Dr. A. C. Braun. *L. brevis* was from Dr. M. S. Dunn, and *Ophiostoma multiannulatum* from Dr. N. Fries. *Shigella sonnei* was supplied by Dr. W. F. Goebel, *Salmonella typhimurium* was from Dr. H. A. Schneider and *Proteus* and *Bacillus tenuis* were from Dr. R. J. Dubos. The assistance of these donors is gratefully acknowledged. All other species were those used in this laboratory for several years.

Manner of Testing Effect of Analog on Growth of Microorganisms.—A culture medium adequate for the growth of the species under observation was distributed in 10 cc. portions in test tubes, and sterilized at 120°. Neutralized, aqueous solutions of the dichlorodiaminobenzene dihydrochloride were sterilized separately, and added aseptically just before inoculation. Sufficient water was omitted from the tubes in a series to allow for this addition. The heating of any phenylenediamine derivative in the basal media was avoided. The cells from a young broth culture of the organism were washed once, and diluted to 1/100 of their original concentration, and 1 drop was used to inoculate each tube. Incubation at the temperature best suited to each species was continued until maximal growth was observed in the unsupplemented basal medium. Prolonged incubation invariably made the diamine appear less toxic. The amount of growth was determined by measurement of turbidity in an Evelyn colorimeter, and in addition, where this could be done, by titration of the acid produced. Dose-response curves were constructed, and from them the amount of substance which allowed half-maximal growth was estimated.

The basal medium employed for nearly all species was that of Landy and Dicken (16) in which folic acid was supplied as pteroyl glutamic acid (0.01 γ per cc.), and in which riboflavin was present in 0.1 γ per cc. An effort was made to use the same basal medium for all species in order to eliminate the chance of differences in toxicity arising from dissimilarities of the culture fluid. However, for a few of the species special media were necessary to secure growth. For *O. multiannulatum* the one described by Fries *et al.* (17) was used, and for *Saccharomyces cerevisiae*, one developed earlier (18) was employed. Because *P. tabaci* and *P. angulata* failed to grow in the enriched medium of Landy and Dicken, tests with these organisms were done

in the mixture of glucose and salts proposed by Hook *et al.* (19). For *L. lactis* (Dorner) and *L. leichmanni* a tryptic digest of casein (0.5 mg. per cc.) and crystalline vitamin B_{12} (0.003 γ per cc.)[1] were added to the Landy-Dicken medium as recommended by Hoffmann *et al.* (20). This was done in preference to use of the solution proposed by these authors, because the concentration of heavy metal salts which they employed was great enough to cause darkening and precipitation of the dichlorodiaminobenzene. In order to induce *L. bifidis* to grow in this medium, the increased vitamin levels used by Skeggs *et al.* (21) were employed. The effects of such additions to the medium of Landy and Dicken, on the toxicity of the analog for *Staphylococcus aureus*, and for *L. casei* and for *E. coli* were found to be negligible in comparative trials. Therefore, these alterations did not seem to be of great importance for the values observed. Similarly, vitamin B_{12} did not influence the toxicity for any species except the two to be discussed below. Those organisms which did not require the presence of riboflavin were tested with the analog in the absence of this vitamin as well as with 0.1 γ of it per cc., but the results were comparable. *E. gracilis* and *C. vulgaris* were grown in the medium described by Hutner *et al.* (22). The requirements of each species for riboflavin and for vitamin B_{12} were determined in these media by omission of one or the other and observation of growth response in comparison to a supplemented control in the usual fashion.

TABLE I

Growth-Inhibitory Effect of 1,2-Dichloro-4,5-diaminobenzene Dihydrochloride on Staphylococcus aureus

Analog	Light transmission by the culture
γ per cc.	*per cent*
0	65
3	70
10	96

Inhibition of Growth of Staphylococcus aureus by 1,2-Dichloro-4,5-Diaminobenzene.—The data in Table I illustrate the effect of the analog on the growth of *S. aureus* when the test was conducted in the manner just indicated. Half-maximal inhibition was achieved with 6 γ per cc. In a series of similar trials, the value varied from 5 to 10 γ depending somewhat on the time of incubation.

Selective Toxicity of 1,2-Dichloro-4,5-diaminobenzene Correlated with Nutritional Requirements for Riboflavin and Vitamin B_{12}.—When a number of microbial species were examined in the manner just indicated, the toxicity of the analog for each was found to be as shown in Table II. The requirements for riboflavin and for vitamin B_{12} were also observed to be as indicated there, and these were in close agreement with existing knowledge about such needs. The maximal concentration of the dichlorodiaminobenzene dihydrochloride which could be tested was 300 γ per cc., because with larger amounts a precipitate formed during the incubation period.

Those species which required both vitamins were insusceptible to the growth-

[1] Crystalline vitamin B_{12} was very generously supplied by Drs. E. L. R. Stokstad and T. H. Jukes of Lederle Laboratories; and by E. R. Squibb and Sons, Inc.

inhibiting action of the analog, while those which needed neither vitamin were usually quite susceptible. The Gram-negative enteric bacilli (*E. coli*, *S. typhimurium*, *S. sonnei*, and *Proteus*) were considerably more resistant than other forms which showed no nutritional need for riboflavin and vitamin B_{12}. This was noteworthy in view of a somewhat similar situation with respect to the comparative resistance of such species to sulfanilamide. The organisms

TABLE II

Toxicity of 1,2-Dichloro-4,5-diaminobenzene Correlated with Nutritional Requirements for Riboflavin and Vitamin B_{12}

Toxicity is expressed as amount of the dihydrochloride which caused half-maximal inhibition of growth in a synthetic medium containing all known nutrients.

Organism	Temperature of incubation	Time of incubation	Amount of analog	Riboflavin requirement	Vitamin B_{12} requirement
	°C.	*days*	*γ per cc.*		
Lactobacillus brevis	30	2	6	None	None
Lactobacillus arabinosus	30	1	10	"	"
Saccharomyces cereviseae	30	1	6	"	"
Staphylococcus aureus	37	1	6	"	"
Streptococcus fecalis R	30	1	40	"	"
Bacillus tenuis	37	1	20	"	"
Chlorella vulgaris	25	10	10	"	"
Ophiostoma multiannulatum mutant strain 1671	30	5	20	"	"
Leuconostoc mesenteroides	30	3	4	"	"
Xanthomonas pelargoni	25	3	1	"	"
Corynebacterium fascians	25	7	1	"	"
Corynebacterium michiganense	25	9	0.6	"	"
Pseudomonas angulata	25	5	100	"	"
Pseudomonas tabaci	25	3	30	"	"
Agrobacterium tumefaciens	25	4	60	"	"
Escherichia coli	37	1	200	"	"
Proteus strain 4	37	1	100	"	"
Shigella sonnei	37	1	200	"	"
Salmonella typhimurium	37	1	100	"	"
Lactobacillus casei	37	2	140	Required	"
Hemolytic streptococcus H69D	37	1	180	"	"
Euglena gracilis	25	7	100	None	Required
Lactobacillus leichmanni strain 313	37	2	No effect at 300*	Required	"
Lactobacillus bifidis strain 4963	37	2	" "	"	"
Lactobacillus lactis (Dorner) strain 8000	37	2	" "	"	"

* This was the largest concentration which could be dissolved in test media.

which required only one of the two vitamins preformed in the medium tended to be more resistant than closely related species which required neither, but they were still affected by the analog when present in sufficient concentration. Thus *L. casei* was somewhat more resistant than *L. arabinosus*, and *E. gracilis* was more so than *C. vulgaris*. However, the number of such species was not large enough to warrant a sound conclusion on this point.

The correlation of susceptibility and nutritional need for riboflavin and vitamin B_{12} appeared to best advantage when the different lactobacilli were compared. Among this group of physiologically and morphologically related species those which showed the nutritional requirements were quite resistant, while those which did not were among the most susceptible.

Tolerance of Mice for 1,2-Dichloro-4,5-diaminobenzene.—This compound was relatively non-toxic to mice. For these tests Swiss strain albino animals ranging in age from 4 to 12 weeks were usually employed, but other strains were also tested with entirely similar results. All animals were fed a stock ration (purina fox chow). Eleven individuals injected daily with an aqueous solution of 10 mg. of the dihydrochloride for 10 days showed no detectable sign of injury. Sixteen animals responded similarly when the dihydrochloride was made to constitute 0.5 per cent of the food, and in addition, were injected daily intraperitoneally with 6 mg. of the free base dissolved in olive oil. This combined treatment was continued for 1 month, during which time the animals gained weight.

Competitive Antagonism of the Action of the Dichlorodiaminobenzene with 1,2-Dimethyl-4,5-diaminobenzene in Bacteria.—The growth-inhibiting properties of the dichloro compound were antagonized, and in a competitive fashion, by 1,2-dimethyl-4,5-diaminobenzene,[2] the postulated metabolic precursor to which the halogenated analog was structurally related. The data in Table III illustrate this point in the case of *S. aureus*. A similar situation was found with each one of the susceptible species, except with the Gram-negative enteric bacilli, which were not tested in this way.

The concentration of dimethyldiaminobenzene required to antagonize the action of the dichloro analog was somewhat greater than that which might be expected if the former were a true metabolic precursor of the 2 vitamins under consideration. Thus, to antagonize a minimally inhibitory amount of the dichloro compound in *S. aureus* about 1 γ of the dimethyldiaminobenzene per cc. was demanded. By analogy with the sulfanilamide-*p*-aminobenzoic acid case, one would expect considerably less to be effective. Furthermore the dimethyldiaminobenzene was itself toxic in all species tested when sufficiently high concentrations were employed. Thus, 200 γ per cc. caused half-maximal inhibition of the growth of *S. aureus*. This toxicity may be reminiscent of the antimicrobial effect exerted by high concentrations of *p*-aminobenzoic acid.

[2] A sample of this compound was generously supplied by Dr. R. B. Pringle of these laboratories.

Antagonism of the Action of 1,2-Dichloro-4,5-diaminobenzene with o-Phenylenediamine.—In the case of *S. aureus*, the growth-inhibiting action of the dichloro compound was overcome by *o*-phenylenediamine. The latter compound was considerably less active than was 1,2-dimethyl-4,5-diaminobenzene,

TABLE III

Competitive Antagonism of 1,2-Dimethyl-4,5-diaminobenzene Dihydrochloride and 1,2-Dichloro-4,5-diaminobenzene Dihydrochloride to the Growth of Staphylococcus aureus

Dimethyldiaminobenzene added	Dichlorodiaminobenzene needed for half-maximal inhibition	Additional dichloro analog needed*
γ per cc.	*γ per cc.*	*γ per cc.*
0	8	—
1	11	3
3	20	12
9	30	22
27	69	61

* This is the amount necessary to antagonize the dimethyldiamine added to the medium and is column 2 minus the amount of dichloro analog found necessary with no added dimethyl compound.

TABLE IV

Antagonism between o-Phenylenediamine and 1,2-Dichloro-4,5-diaminobenzene Dihydrochloride in the Growth of Staphylococcus aureus

o-Phenylenediamine	Dichlorodiaminobenzene	Light transmission by the culture
γ per cc.	*γ per cc.*	*per cent*
0	0	69
0	10	95
0	3	80
100	0	100
30	0	82
20	0	71
20	10	74
10	10	81
10	3	73

but 20 γ of it per cc. overcame almost completely the effect of 10 γ of the dichloro compound per cc. Data to illustrate this antagonism are contained in Table IV. Because *o*-phenylenediamine was itself toxic above 20 γ per cc. no more extensive study of its apparently competitive behavior could be made.

Activity of 1,2-Dimethyl-4,5-diaminobenzene in Replacement of Riboflavin and Vitamin B_{12}.—The dimethyldiaminobenzene exhibited some slight activity as riboflavin when tested with *L. casei*, and some potency as vitamin

B_{12} for *L. leichmanni*, and for *E. gracilis*. The assays for riboflavin activity were performed in the basal medium described earlier in this paper, from which riboflavin was omitted. The methods of Hutner *et al.* (22) and of Hoffmann *et al.* (20) were employed for vitamin B_{12}. The diamines to be tested were added after the media had been sterilized. 1,2-Dimethyl-4,5-diaminobenzene was 1/100,000 as active as riboflavin for *L. casei*, and 1/1,000,000 as active as vitamin B_{12} for *L. leichmanni*. For *E. gracilis* it was between 1/500,000 and 1/1,000,000 as potent as vitamin B_{12}. In all assays, full growth could not be attained with the compound because its limited solubility restricted the amount which could be added. The comparisons of activity with the two vitamins were therefore made only on the lower portion of the standard dose-response curves.

1,2-Dimethyl-4-amino-5-ribitylaminobenzene[3] was similarly tested. It proved to have no activity for vitamin B_{12}, but was 1/10,000 as effective as riboflavin for *L. casei*. This compound is one stage nearer to riboflavin than is the dimethyldiaminobenzene, but is somewhat more removed from vitamin B_{12}. Riboflavin activity for it has been observed previously (23).

Effect of Riboflavin Plus Vitamin B_{12} on the Toxicity of 1,2-Dichloro-4,5-diaminobenzene for Microorganisms.—The effects of adding vitamin B_{12} (from 0.001 to 0.1 γ per cc.) plus riboflavin (from 0.1 to 10 γ per cc.) on the toxicity of a minimally effective dose of 1,2-dichloro-4,5-diaminobenzene were studied for most of the species which were susceptible to this analog. No diminution in harmful action was found except with *L. casei* and hemolytic streptococcus H69D. With both of these riboflavin-requiring forms the mixture of the 2 vitamins rendered the dichlorodiaminobenzene slightly less inhibitory of growth. The effect was observable only at levels of the latter which still permitted fair growth in the absence of the additional riboflavin and the B_{12}. An amount of the dichloro compound which was able to suppress growth completely could not thus be rendered less harmful. The data in Table V illustrate these facts. The magnitude of this antagonism was such as to raise doubts about its existence but the effect was observed regularly.

Excessive amounts of riboflavin (10 γ per cc. of culture medium) inhibited the growth of some of the species, *e.g.*, *S. aureus*. These harmful concentrations of the vitamin enhanced rather than diminished the toxicity of the dichlorodiaminobenzene. This was found to be the case whether vitamin B_{12} was present or absent.

Effect of Replacement of Vitamin B_{12} by Desoxyribosides on the Toxicity of 1,2-Dichloro-4,5-diaminobenzene for L. bifidis.—Because the nutritional requirement for vitamin B_{12} can be met by additions of desoxyribosides in the case of *L. bifidis* (21), an effort was made to find whether the replacement of

[3] Samples of this substance were very kindly supplied by Dr. J. A. Aeschlimann of Hoffmann-La Roche, Inc.

vitamin B_{12} in the medium in this way would render this species susceptible to the dichlorodiaminobenzene. Vitamin B_{12} was therefore omitted, and desoxyguanosine (10 γ per cc.) was added to the basal medium. Under these conditions 300 γ of the analog per cc. did not retard growth. The result was the same when desoxyinosine was used in place of desoxyguanosine.

The experiments were repeated with *L. leichmanni* strain 313. However, in contrast to reports in the literature (24), neither desoxyguanosine nor desoxyinosine would support growth in the absence of vitamin B_{12}. Therefore a trial with the dichlorodiamine was not possible.

TABLE V

Effect of Riboflavin Plus Vitamin B_{12} on the Growth-Retarding Action of 1,2-Dichloro-4,5-diaminobenzene Dihydrochloride for Lactobacillus casei

Riboflavin	Vitamin B_{12}	Dichlorodiaminobenzene dihydrochloride	Light transmission
γ per cc.	*γ per cc.*	*γ per cc.*	*per cent*
0.1	0	0	35
0.1	0.05	0	32
1.1	0	0	32
0.1	0	100	48
0.1	0	30	35
1.1	0.05	100	40
1.1	0.05	30	34
1.1	0	100	47

DISCUSSION

The success in predicting what species would be susceptible to 1,2-dichloro-4,5-diaminobenzene from a consideration of nutritional requirements adds more support to the postulate that selectively toxic substances can be realized in the same way. Similarly, the results harmonize with the idea that agents may be formed which are not counteracted by substances further up the biosynthetic metabolic path if a metabolite is chosen which is the precursor of two or more of them. This latter point is still quite tenuous, and requires much further exploration. Several parts of the argument lack sufficient experimental support. In the first place, no convincing evidence was found that 1,2-dimethyl-4,5-diaminobenzene actually is the metabolic precursor of riboflavin and vitamin B_{12}. Points in favor of its being such are (*a*) its undeniable occurrence in the two vitamins, (*b*) its slight activity in replacement of each of these two vitamins, and (*c*) its ability to counteract in a competitive fashion the toxicity of 1,2-dichloro-4,5-diaminobenzene. Data difficult to reconcile with such a view are (*a*) that it is not more active in counteracting the harmful effects of the dichloro analog, and (*b*) that *o*-phenylenediamine will overcome the toxicity of the dichlorodiaminobenzene.

If the concept outlined in this paper is correct, then clearly some discrimination must be exercised in its application to other cases. The choice of the metabolic precursor may be important, because present knowledge indicates that just any one will not do, even though it may be the starting point for the formation of two or more important constituents of living cells. Thus, glutamic acid is most probably used in the synthesis of both glutamine and glutathione, and possibly of several more metabolites as well. And yet, structural analogs of glutamic acid have not proven to be selectively harmful to those species which perform the syntheses of these compounds. Indiscriminate application of the present concept would require them to do so.

The bases for a discriminating choice do not seem clear. Perhaps, if one wishes to inhibit cell division, one should select a precursor of some of those substances which are directly concerned in cell division, rather than in some other processes such as energy transport or the building of anatomical structures. In any event, metabolic precursors do not appear to be of equal aptitude for this purpose.

It is quite conceivable that the toxicity of dichlorodiaminobenzene has no relationship to the biosynthesis of riboflavin and of vitamin B_{12}. The antagonism of its effect with the dimethyldiaminobenzene could then be viewed as follows: Cells possess a mechanism for metabolizing aromatic amines, which are poisonous for unknown reasons. The dimethyldiamine is intrinsically less toxic than the dichlorodiamine. (This has actually proved to be the case.) The less toxic dimethyl compound may thus be able to exclude the more harmful dichloro compound and thus protect the cell. While such a view is tenable, it seems less likely than the other one proposed, especially in light of the correlations of toxicity with nutritional needs for two vitamins which possess the dimethyldiaminobenzene structure within them.

SUMMARY

In a series of 26 species selected from widely differing classes, 1,2-dichloro-4,5-diaminobenzene was toxic to those which did not exhibit a nutritional need for riboflavin plus vitamin B_{12}. It failed to retard the growth of those which needed both of these vitamins. The compound was conceived as an antimetabolite of 1,2-dimethyl-4,5-diaminobenzene. This latter, which is contained within the structures of the two vitamins, was pictured as a metabolic precursor of them. It was found to have very slight activity as either riboflavin or as vitamin B_{12} for lactic acid bacteria and algae. The growth-inhibiting action of the dichlorodiaminobenzene was overcome competitively by the dimethyldiaminobenzene, and also, to a lesser extent, by *o*-phenylenediamine. The toxicity was not influenced by additions of riboflavin plus vitamin B_{12}, except in the cases of two species, where the influence was slight. These facts were considered to support the idea that properly constructed analogs of a precursor of two or more essential participants in cell division may be able to

circumvent the counteraction which the vitamin has been found to exert on an antimetabolite of its precursor. Alternate explanations of the observed data were likewise considered.

BIBLIOGRAPHY

1. Woolley, D. W., *Ann. Rev. Biochem.*, 1947, **16,** 359.
2. Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, 1946, **164,** 485.
3. Woods, D. D., and Nimmo-Smith, R. H., *Symposia Soc. Exp. Biol.*, No. 3, Growth, 1949, 12.
4. Woolley, D. W., *J. Biol. Chem.*, 1950, **183,** 495.
5. du Vigneaud, V., Dittmer, K., Hague, E., and Long, B., *Science*, 1942, **96,** 186.
6. Tatum, E. L., *J. Biol. Chem.*, 1945, **160,** 455.
7. Pierce, C., Dubos, R. J., and Woolley, D. W., unpublished data.
8. Auhagen, E., *Z. physiol. Chem.*, 1948, **283,** 195.
9. Winkler, K. C., and Haan, P. G., *Arch. Biochem.*, 1948, **18,** 97.
10. Woods, D. D., *Bull. Soc. chim. biol.*, 1948, **30,** 730.
11. Woolley, D. W., and Pringle, R. B., *J. Am. Chem. Soc.*, 1950, **72,** 634.
12. Plaut, G. W. E., Betheil, J. J., and Lardy, H. A., *J. Biol. Chem.*, 1950, **184,** 795.
13. Brink, N. G., Holly, F. W., Shunk, C. H., Peel, E. W., Cahill, J. J., and Folkers, K., *J. Am. Chem. Soc.*, 1950, **72,** 1866.
14. Hartley, P., and Cohen, J. B., *J. Chem. Soc.*, 1904, **85,** 865.
15. Kuhn, R., Weygand, F., and Moller, E. F., *Ber. chem. Ges.*, 1943, **76,** 1044.
16. Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, 1942, **27,** 1086.
17. Fries, N., Bergström, S., and Rottenberg, M., *Physiol. Plant.*, 1949, **2,** 210.
18. Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, 1943, **78,** 489.
19. Hook, A. E., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Biol. Chem.*, 1946, **165,** 241.
20. Hoffmann, C. E., Stokstad, E. L. R., Franklin, A. L., and Jukes, T. H., *J. Biol. Chem.*, 1948, **176,** 1465.
21. Skeggs, H. R., Spizizen, J., and Wright, L. D., *J. Am. Chem. Soc.*, 1950, **72,** 811.
22. Hutner, S. H., Provasoli, L., Schatz, A., and Haskins, C. P., *Proc. Am. Phil. Soc.*, 1950, **94,** 152.
23. Pringle, R. B., private communication.
24. Kitay, E., McNutt, W. S., and Snell, E. E., *J. Biol. Chem.*, 1949, **177,** 993.