A Naturally Occurring Antimetabolite of Methionine *in the Causation of a Disease*

By D. W. Woolley, PH.D.

It is becoming increasingly clear that antimetabolites are not merely the creation of organic chemical laboratories. On the contrary, it emerges that Nature knows about the phenomenon of antimetabolites, and many of her creatures make them and use them in normal physiological processes. This concept arose more than ten years ago, but gained acceptance so slowly that even now it is not believed in many quarters. This has been partly because the numbers of naturally occurring and recognizable antimetabolites are not nearly so large as the synthetic ones. Then, too, earlier notions about the modes of action of some of the natural members have tended to persist and to distract attention from the fact that these substances really are antimetabolites.

One of the first cases of a naturally occurring antimetabolite was discussed in a paper at the first meeting of this society about ten years ago here in New York. This was the case of the pellagragenic agent of corn which is an antagonist of nicotinic acid. However, this case leaves much to be desired because the exact structure of the antagonist is not yet established.

Today, I would like to tell you about a more recently discovered and clearer example of a naturally occurring antimetabolite. This is the toxin of *Pseudomonas tabaci,* which is an antimetabolite of methionine.

Basic Principles

First, let us briefly review what is meant by an antimetabolite.[1] Such a compound is a structural analogue of some vitally essential component of living things. However, the structural analogy does not, of itself, make a compound an antimetabolite. In addition, the analogue must be able to antagonize the action of the metabolite. In other words, it must be able to exclude its related essential metabolite from the specific cellular reactions for which the latter is designed. A deficiency of this essential metabolite is thereby created. The antimetabolite competes with the metabolite when the latter is serving as a substrate in an enzymic or semi-enzymic reaction. Because it preferentially attaches to the enzyme, it is able to prevent the formation of the normal product of the reaction. In this way the deficiency is produced. The structural analogy of the antimetabolite to the metabolite is presumably what allows it to make this attachment. Being so nearly like the metabolite, the antimetabolite fits into the specific receptor of the enzyme, much as the metabolite itself does.

Now let us consider the toxin of *Pseudomonas tabaci.* There is a disease of tobacco, and of certain other crops, which is caused by a pathogenic bacterium. This organism, *Pseudomonas tabaci,* enters the leaves of the plants, grows there, and produces lesions which consist of necrotic spots surrounded by yellow halos. The disease can spread so rapidly through a field that it is called wildfire disease. The lesions are caused by an extracellular toxin which is formed by the pathogen. Thus, if the organism is grown in the laboratory, and a filtrate of the culture is made, this filtrate will, when it is applied as a drop to a tobacco leaf, reproduce the signs of the disease (see Figure 1). The amount of toxin in a drop can be measured by determination of the diameter of the halo. Within certain limits, the larger the spot, the more toxin was present. This has been the basis for quantitative assay of the toxin. This method has

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been used by Dr. A. C. Braun who has done much of the biological investigation of this disease, and with whom we have had the good fortune to collaborate.[2]

Dr. Braun found that a unicellular plant, viz., *Chlorella vulgaris*, was also susceptible to this toxin. In this alga, he found that the growth-inhibiting property of the *Pseudomonas* toxin could be reversed by methionine. This action was quite specific in that no other amino acid was effective. Furthermore, the antagonism between methionine and the toxin was of the reversible and competitive kind. This character of the antagonism is always compelling evidence, although we must not suppose, as some do,[3] that it is the *sine qua non* of antimetabolite action. In fact, although the toxin was a competitive antagonist of methionine in *Chlorella*, in tobacco plants this amino acid was not able to protect against the toxin. Much experimentation has gone into the understanding of this failure, and it now appears likely that extraneous causes of penetrability and toxicity of methionine in the tobacco tissue beclouded the picture.

Because of the clear-cut results with *Chlorella*, the working hypothesis was formed that the toxin was an antimetabolite of methionine and that it owed its pathogenic action to the induction of a specific deficiency of this amino acid. To test this hypothesis it was necessary to isolate the toxin in pure form, and to establish its chemical structure. This has been done, and the idea that it would be analogous to methionine has been verified.

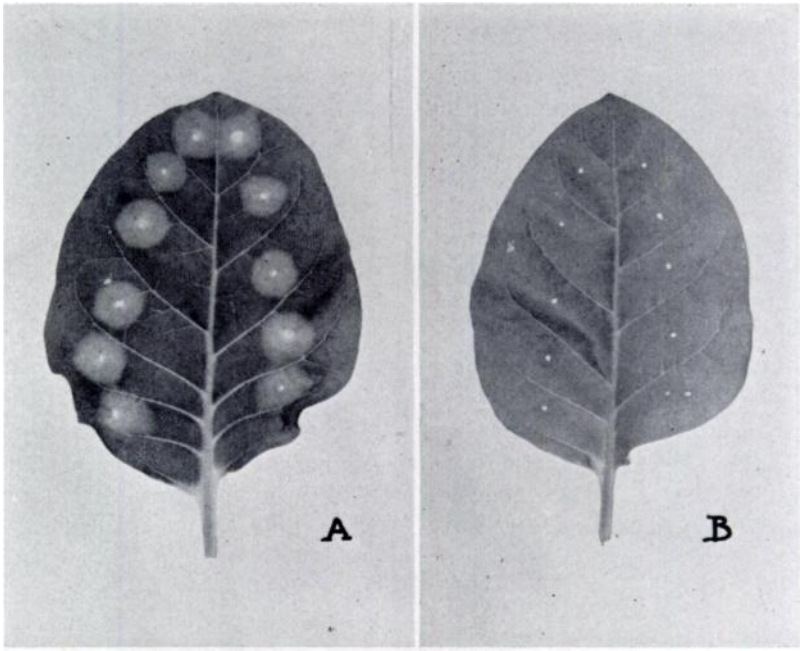

Fig. 1. Tobacco leaves inoculated with (*A*) sterile wildfire toxin; (*B*) control media.

Before beginning these chemical studies, we had a further indication of the soundness of the working hypothesis. This was that a synthetic antimetabolite of methionine would reproduce the disease in tobacco plants. This synthetic compound was methionine sulfoximine, which, at that time, had just been shown to be the causative agent of "running fits" in dogs. You will recall that this antimetabolite arises in wheat flour when the latter is treated with nitrogen trichloride. It is clearly a structural analogue of methionine, and many of its toxic effects can be overcome by this amino acid. When methionine sulfoximine was applied to tobacco leaves, it produced the typical lesions of wildfire disease. It was of much interest, however, to find that methionine would not prevent these lesions any more than it would prevent those called forth by the

Pseudomonas toxin. If *Chlorella* instead of tobacco was the test plant, then, just as with the toxin, the action of methionine sulfoximine could be overcome. The structures of methionine and of methionine sulfoximine are shown in Figure 2.

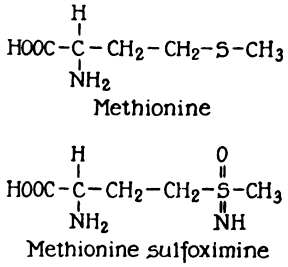

Figure 2

Isolation

The isolation of the *Pseudomonas* toxin proved to be very difficult. This was because of its instability, especially to very weak alkali, and also because of the fact that it was a water-soluble substance quite insoluble in almost all organic solvents. Furthermore, it was present in very small amounts in the culture filtrates. A method of isolation was finally devised[4] which is summarized in Figure 3. It consisted of adsorption of the toxin to alumina under acidic conditions in methanolic solution, and elution with acidified water. The material so obtained was then separated on large columns of powdered cellulose. It had been found that the toxic activity was associated with a substance which gave a ninhydrin test, and which had an R_F on paper chromatograms of 0.26 in a mixture of propanol and water. By use of these properties, tests were devised for locating the position of the toxin in effluents of the columns. Results so obtained were always verified by assays on tobacco leaves.

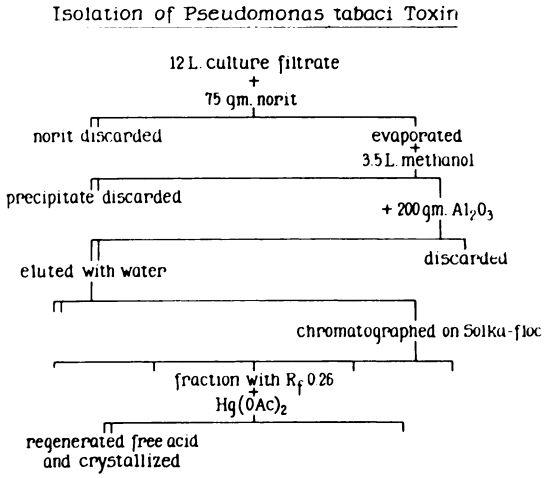

Figure 3

The active fraction from the chromatographic columns was contaminated with an unknown organic acid. In order to bring about final purification of the toxin, it was precipitated as the mercury salt, and the free toxin was then regenerated and caused to crystallize. The yield was about 4 mg per liter of culture.

The isolated toxin was very active in causing the wildfire lesion of tobacco leaves. Thus, it was detectable at about 0.005 μg.

Chemical Structure

The chemical structure of the pure toxin was established by suitable analyses and degradations to known compounds.[5,6] The main features of the degradations are shown in Figures 4 and 5. The proposed structure of the toxin is also shown in Figure 5. The elementary analyses revealed that the empirical formula was most probably $C_{10}H_{16}O_6N_2$, and that the toxin was α-amino acid. One of the two nitrogen atoms occurred as a free α-amino group, and the other one was an acylated amino group.

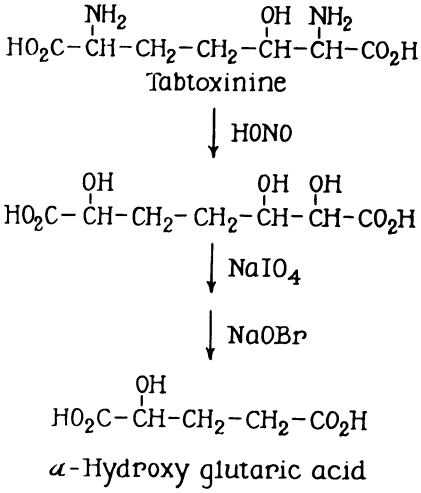

Figure 4

Complete hydrolysis with acid gave rise to lactic acid plus a new amino acid which had previously not been known. This new compound contained both of the nitrogen atoms of the original toxin and seven of the ten carbon atoms. It was identified as α,ε-diamino-β-hydroxypimelic acid by suitable degradations to glutamic acid with periodate, and to α-hydroxyglutaric acid with nitrous acid, hypobromite and periodate (see Figure 4). This new amino acid was named tabtoxinine.

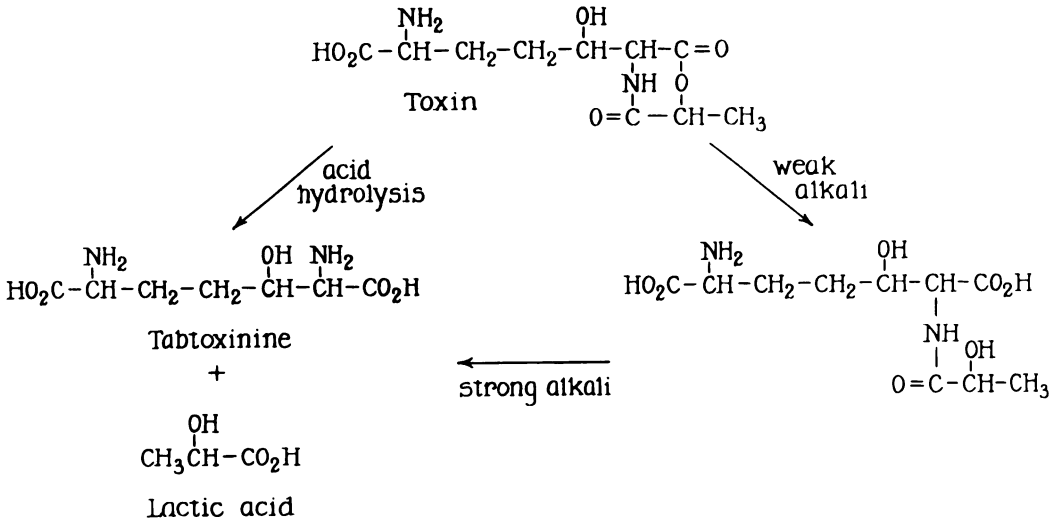

Figure 5

With these two products of complete hydrolysis, viz., lactic acid and tabtoxinine, all of the carbon and nitrogen atoms were accounted for, and the problem resolved itself into the question of how they were united in the toxin. Very mild alkali destroyed the biological activity of the toxin, and gave rise to a new compound which was isolated and identified as most probably α-lactyltabtoxinine. In other words, the lactic acid was the acylating group which bound one of the nitrogen atoms. The toxin itself was the lactone formed by ring closure of the α-carboxyl and the hydroxyl of the lactyl residue.

Although the formula shown in the figure seemed to be the most probable one for the toxin, there was still some little doubt about it. Thus, small amounts of formic acid were also recognized as a product of acid hydrolysis. At one time[7] it was felt that the acyl group was formyl rather than lactyl. However, a considerable amount of work all pointed to the conclusion that the formic acid probably arose from more profound decomposition of the recognized constituents. The proposed structure for the toxins seemed adequate to account for its chemical properties, but we must carry with us this slight reservation about its absolute correctness.

I fear that I may have bored many of you with this brief description of the chemical work. These days, many are content to buy a chemical compound from the supply houses, or to write to someone and request a gift. If these two easy procedures fail, the general reaction seems to be not to investigate the substance. Nevertheless, I feel that a knowledge of organic chemistry is at the very heart of the study of antimetabolites. From the very beginning, it has been necessary to synthesize all sorts of strange new compounds, for without them the whole field of antimetabolites would have been almost barren. If we rely on only the compounds we can buy or beg, I fear that our understanding of this whole subject will be considerably stunted. It is partly for this reason that I have ventured into a discussion of the organic chemistry of the *Pseudomonas* toxin. You will recognize, too, that the determination of its chemical formula was crucial to the concept outlined.

Relationships

Let us now ask how close the resemblance in structure is between methionine and the *Pseudomonas* toxin. The formulations of Figure 6 will illustrate this point. Consider first the relationship of methionine to the recog-

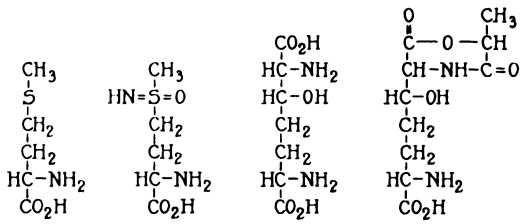

Figure 6

nized antimetabolite methionine sulfoximine. In passing from the metabolite to the antimetabolite the change has been to add an oxygen and an imino group to the sulfur. The analogy is clear. You will also recall that methionine sulfoximine is able to reproduce the signs of wildfire disease in tobacco leaves, although from a quantitative standpoint it is considerably less potent than is the natural toxin. The analogy of methionine sulfoximine to tabtoxinine is also rather close. The sulfur atom has been replaced by two carbon atoms (a well-known method of making antimetabolites[1]). The oxygen atom has gone with one of these carbons, and the nitrogen atom with the other. The double bonds have been reduced so that we have hydroxyl and amino rather than ketone and imino, and the methyl group has been correspondingly oxidized up to a carboxyl group. These changes bring us to tabtoxinine, the hydrolytic product of the toxin. The entire toxin results from the acylation of the amino group and closure of the lactone ring.

It is attractive to think that these last changes in structure are just a device for assurance of ease of permeability for the toxin. Thus, tabtoxinine has a highly ionized group at both ends of the molecule, and presumably, such highly polar substances find difficulty in penetrating cells. Methionine, on the other hand, is ionized at only one end. It would seem possible that the lactyl group on the amino group, and the lactone linkage of the carboxyl of the toxin are maneuvers to cancel out the charge at one end of the tabtoxinine molecule, and thus to allow the toxin to penetrate to its site of action in the plant. We plan to test this idea directly by synthesis of the appropriate derivative of tabtoxinine. Both tabtoxinine and the alkali-inactivated toxin have no toxic effects on tobacco leaves, and as you can see, these both have ionizable groups at both ends of the molecules.

Of course, one must not forget that the lactone grouping may play a role in anchoring the toxin to the methionine site in the tobacco cell. Such a chemically reactive grouping as this lactone would present possibilities for strongly binding the toxin by a covalent bond to this site in the victim. This may help to explain the very high potency of the toxin in contrast to methionine sulfoximine. It has been recognized previously[1] that the possession of chemically reactive groupings in addition to structural analogy to the metabolite, frequently makes the antimetabolite both highly potent and irreversible in its effects, and for quite evident reasons.

The fact that this toxin is an amino acid, while the bacterial toxins of microbial diseases of animals are usually proteins, is noteworthy. The plants do not have a circulatory system in any way comparable with the blood stream of animals. Consequently, large molecules travel slowly in plants. An invading parasite should find it advantageous to elaborate small, readily diffusible toxins rather than proteins. The case of the *Pseudomonas* toxin is not the first example to show that phytopathogens do this. The preceding case of the toxin of *Fusarium lycopersici*, which causes disease in tomato plants, was shown by work both in Switzerland and in our laboratory to be not a protein, but a modified tripeptide.[8,9] This tripeptide was shown to have a structural analogy, and an antagonistic action toward peptides which possessed strepogenin activity.

The *Pseudomonas* toxin now emerges as a second phytopathogenic substance which is a modified amino acid, and which is an antimetabolite of another essential metabolite,

viz., methionine. Some day it may be possible to show that the proteins which are the toxins of pathogenic bacteria in higher animals have a structural analogy to some specific and important proteins of the host, and that the toxins owe part of their poisonous effects to an antagonism to these constituents of the host.

Summary

To come back now from the realm of hypothesis, let us remember that a compound of chemical structure related to methionine occurs naturally as the toxin of the plant pathogen *Pseudomonas tabaci*. This toxin, which has been isolated in pure form and identified, reproduces the lesions of the disease. The same lesions can be caused by a synthetic antimetabolite of methionine. In the unicellular plant *Chlorella vulgaris*, the poisonous action of the *Pseudomonas* toxin can be overcome in a competitive fashion by methionine, and by no other known substance. For these reasons it seems rather clear that the *Pseudomonas* toxin probably owes much of its disease-producing properties to the fact that it is a naturally occurring antimetabolite of methionine.

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