THE EFFECT OF FOLIC ACID ON THE TOXICITY OF ITS ANALOGUE
4-AMINOPTEROYLGLUTAMIC ACID (AMINOPTERIN)

By GEORGE M. HIGGINS, Ph.D.

With the technical assistance of MURIEL STEMBER AND HARRY MONSEN

Ever since Woods described a competition between sulfanilamide and paraaminobenzoic acid for an enzyme system related to the use of this vitamin in metabolic processes, widespread interest has centered on the synthesis of analogues which are chemically related to a metabolite but which seriously interfere with its normal function. There are now available a large number of these metabolite antagonists which in very minute amounts seriously interfere with the functions of cell catalysts, such as vitamins and hormones.

Such antagonists or displacing agents have now been synthesized for every known member of the vitamin B-complex. Martin, Tolman and Moss prepared the first antagonist to pteroylglutamic acid. They produced methylfolic acid, which was shown to be an effective displacer of folic acid with a ratio of inhibitor to metabolite of 1:150. The growth-promoting action of pteroylglutamic acid for Streptococcus faecalis R was antagonized by this analogue.

Franklin, Stokstad, Belt and Jukes fed a crude antagonist, prepared by Hultquist and Smith, to weanling rats. This preparation accelerated and intensified the signs of pteroylglutamic acid deficiency in their rats, lowering hemoglobin levels and granulocyte counts and seriously impairing the maturation of cells in the bone marrow. These effects were all completely prevented, however, by the addition of suitable amounts of pteroylglutamic acid to the diet. Similar results were obtained by giving this same antagonist to mice and to chicks. Pteroylglutamic acid in appropriate amounts also prevented the appearance of this syndrome in these animals. Welch and colleagues provided the same crude antagonist to a pig which was fed a purified diet. They noted a retarded growth rate, alopecia, anorexia, profuse diarrhea and severe anemia. The interference with normal metabolism by the antagonist was removed by giving a crude source of extrinsic factor, essentially free of pteroylglutamic acid, together with normal human gastric juice.

More recently, another analogue of the vitamin, 4-aminopteroylglutamic acid, known as aminopterin, has been synthesized and experimentally tested on mice. This analogue was produced by the replacement of the hydroxy group of the pteridine ring by an amino group, resulting in a much more potent analogue than the 7-methylfolic acid produced by Martin and colleagues.

Since conjugates of folic acid—namely, pteroyltriglutamic acid and pteroyldiglutamic acid—have been shown to produce an acceleration of the leukemic process in children, it was concluded that the use of antagonists of pteroylglutamic acid in such cases was certainly indicated. Accordingly, Farber and col-
leagues reported their early results of the use of the more recently synthesized antagonist, 4-aminopteroylglutamic acid, in the treatment of a series of 16 children who had acute leukemia. Marked clinical improvements were noted, and influences were exerted on the immature cells of the peripheral blood, the spleen and lymph nodes. However, the toxic effects which accompanied the use of analogue were severe, including stomatitis and early ulceration.

Stickney, Hagedorn, Mills and Cooper reported that administration of this analogue produced remissions in the clinical course of certain patients who had acute leukemia. In some cases, however, improvement was not elicited. Toxic symptoms, including stomatitis, diarrhea, alopecia and deafness, were recorded.

Jacobson, Levin and Holt studied 10 patients with acute leukemia who received aminopterin or methopterin (10-methylpteroylglutamic acid). They concluded that methopterin was superior to aminopterin in the treatment of such leukemias in view of the fact that it was less toxic.

Pierce and Alt reported their results with aminopterin in a series of cases of acute leukemia. Remissions characterized by a severe marrow aplasia followed by rapid regeneration were obtained in 5 of the 11 cases. Berman, Axelrod, Vonder-Heide and Sharp reported their results with the use of aminopterin in 9 patients with chronic leukemia. Although they recognized definite hematologic effects, subjective improvement in any patient was not claimed. They, too, described the toxic effects which followed the administration of the drug.

Since all reports to date indicate that the severe toxic reactions which ensue on administration of this analogue must restrict any extended clinical use of it in spite of its therapeutic value, we undertook a study, in white rats, of some of the toxic manifestations of the analogue together with the modifications of those reactions which were induced when folic acid was given together with aminopterin.

METHODS

Fifty-six young healthy male white rats, weighing from 110 to 130 Gm., were selected from our Institute colony. These were arranged into seven groups of 8 animals each, so that the average weight of rats composing each group was essentially alike. All animals were maintained in metal cages, on open meshwork screens, thus greatly restricting coprophagy. Our standard laboratory ration was provided ad libitum, and water was available at all times in water bottles attached to the cages.

Six of the seven groups served as test groups and one as an uninjected control. Aminopterin was given intraperitoneally in amounts equivalent to 50 micrograms daily, and folic acid was given by stomach tube daily. The various test groups were arranged as follows: each animal in group A received aminopterin alone. Each animal in group B received the same amount of aminopterin plus 5 mg. of folic acid. Group C received aminopterin plus 10 mg. of folic acid. Group D received aminopterin plus 20 mg. of folic acid. Group E received aminopterin plus 30 mg. of folic acid. Group F received aminopterin plus 50 mg. of folic acid. The animals of Group G were given neither aminopterin nor folic acid and served as a normal control group.

At the end of the sixth day, the heart blood of all surviving animals was sampled and total erythrocyte and leucocyte tabulations and the differential distributions were recorded. Each animal was killed by etherization and the spleen, adrenals and thymus were removed and weighed on a precision balance.

* 4-aminopteroylglutamic acid was made available for our use by the Lederle Laboratories, Inc., to whom we are extremely grateful.
Bone marrow imprints were made of samples of marrow obtained from the distal third of the femur. These imprints were stained by the May-Grünwald-Giemsa technic.

**RESULTS**

1. **Body Weight Changes.** The changes in the weights of these test animals given aminopterin and the influences exerted by varying daily amounts of folic acid are shown in figure 1. For the first three days, increases of weight were recorded for all animals, but those receiving the analogue without the vitamin gained only 2 Gm. during that three-day period. Greatest gains were recorded by the animals receiving the analogue plus 5 mg. of folic acid, although all vitamin-supplemented rats gained more than the controls, which were fed the standard ration and given neither analogue nor vitamin. The data indicate, for the first three days, that the analogue was not seriously toxic and that even the smallest amounts of the vitamin counteracted its effects.

After the third day, however, the toxicity of the analogue was clearly indicated. A severe loss of weight occurred on the fourth day in all animals receiving the analogue alone (group A), and 5 mg. of folic acid was ineffective in preventing loss of weight. Gains of weight, however, were recorded for all animals receiving the larger amounts of vitamin. Ten milligrams of folic acid daily failed to inhibit the toxic effects of the analogue after the fourth day, and 20 mg. of the vitamin did not prevent the loss of weight induced after the fifth day. Thirty milligrams and 50 mg. of the vitamin when given with the analogue maintained
body weights during the fifth and six days, but increases of weight were not recorded.

2. Gross Appearance. The marked contrast in the appearance of animals receiving aminopterin alone and those receiving the analogue plus the vitamin is clearly portrayed in figure 2a and b. The rough coat, the stained hair, the encrusted eyelids and ears, and the extreme diarrhea were all prevented, during the six-day test period, by the administration of 30 mg. of folic acid daily by mouth together with the daily intraperitoneal administration of aminopterin.

3. Food Intake. Extreme anorexia was not evident until the third day of the test period, but the average intake of all animals taking the analogue without the vitamin was less than 1 Gm. a day (fig. 3). The addition of 5 mg. of the vitamin stimulated the appetite only slightly, although 10 mg. daily increased the food intake to more than three times that of animals taking the analogue alone and 30 mg. of folic acid, when given with the analogue, so stimulated the appetite as to maintain an average food intake in excess of 9.0 Gm. daily. However, in the amounts given, folic acid did not so nullify the effects of its analogue as to maintain appetites in any test animal equal to those of animals constituting the untreated control group.

4. The Weight of the Adrenal Glands. The adrenal glands invariably reflect unto-
ward reactions of an organism to toxic substances. Hyperplasia of the adrenal cortex, together with marked atrophy of the thymus, constitutes part of a syndrome embracing the reactions of an organism to unfavorable environments induced by a number of different factors. In this test, too, of the toxicity of aminopterin, the increased weights of the adrenal glands indicated an untoward reaction of the animals toward the drug.

The combined weights of the adrenal glands recorded at necropsy of all surviving animals of all seven groups on the sixth day are graphically portrayed (fig. 4). The average combined adrenal gland weight of all control animals was 20.6 mg. but in the group given aminopterin alone (A) the average combined weight of the adrenals was slightly in excess of 1.0 mg. The addition of only 5.0 mg. of folic acid daily reduced the average combined weight of the adrenal glands to 41.5 mg., and the administration of 30 mg. of folic acid daily to animals given the analogue resulted in restricting the weight of both adrenal glands to 18.0 mg. The administration of 30 mg. of the vitamin daily was less effective than that of 30 mg. in restricting hyperplasia.

5. The Weight of the Thymus. Atrophy of the thymus constitutes a part of the syndrome which ensues within an animal on the administration of toxic or harmful substances, so that atrophy of the thymus usually accompanies adrenal hyperplasia. The data obtained by weighing the thymus of all animals at necropsy are
Fig. 4.—Weights of the adrenal glands of animals receiving aminopterin and those receiving aminopterin plus varying amounts of folic acid, when the animals were killed on the sixth day.

Fig. 5.—Weights of the thymus of animals receiving aminopterin and those receiving aminopterin plus varying amounts of folic acid, when the animals were killed on the sixth day.

recorded in figure 5, and photographs of the glands of 2 animals receiving aminopterin and of 2 animals receiving aminopterin plus 30 mg. of folic acid daily are shown (fig. 6).
The average weight of the thymus of the 8 control animals (group G) was slightly less than 400 mg., while that of animals receiving aminopterin alone (group A) was slightly less than 150 mg. The range of thymus weight of animals within group A extended from 59.6 mg. to 296.8 mg. The administration of the various amounts of the vitamin (pteroylglutamic acid) had a considerable influence on restricting the extent of the atrophy, but the daily administration of 50 mg. of the vitamin resulted in maintaining an average thymus weight of 320.5 mg., a figure considerably less than that recorded for the control animals (group G).

6. The Weight of the Spleen. The size of the spleen is ordinarily not a reliable criterion for recording systemic reactions. A vascular organ, with capillaries and venous sinuses, the spleen is subject to rather rapid changes in size, correlated with the extent to which fluid or other blood constituents are sequestered within it. Splenic size varies considerably with the anesthetic agent used. Ether has a constricting effect on the organ, while pentobarbital sodium (nembutal) will ordinarily dilate the sinuses and enlarge it.

Since the data herein reported were obtained on animals correspondingly etherized, there is reason to believe that they constitute a reasonably accurate response of the spleen to the experimental restrictions imposed by the study. The data assembled on the weights of the spleens of all animals are given in figure 7. Since aminopterin inhibits blood cell formation, it is obvious that the spleen, a blood-forming organ, would be affected by this drug. Our data indicate wide variability in the size of the spleen in animals receiving aminopterin alone (group A). In some instances the spleen appeared as a narrow pale band of tissue, weighing as little as 91.8 mg.; in others it was more nearly normal, and in one animal it weighed 408.4 mg. The average weight of the spleen encountered for group A was 266.0 mg., which is considerably less than the average weight recorded for the control group G; namely, 600.0 mg. Photographs of the spleens of 2 animals receiving the drug and of those of 2 animals receiving the drug plus the vitamin are shown (fig. 8).

As in the data assembled on adrenals and thymus, so in those recorded for the weights of the spleens of the various groups, folic acid in the amounts given...
A = Aminopterin alone
B, C, D, E, F = Aminopterin plus folic acid
G = Controls

Fig. 7.—Weights of the spleens of animals receiving aminopterin and those receiving aminopterin plus varying amounts of folic acid, when the animals were killed on the sixth day.

Fig. 8.—Spleens of animals receiving aminopterin without folic acid (left), and of animals receiving aminopterin plus 30 mg. of folic acid daily (right).
maintained more nearly normal weights than in group A. Five milligrams of the vitamin exerted a considerable effect, but 30 mg. daily maintained in group E an average spleen weight (525.0 mg.) closely approaching the average weight in the untreated controls.

7. The Changes in Leukocytes in the Peripheral Blood. Aminopterin markedly restricted the total number of leukocytes in the peripheral blood of these young rats. In a series of 8 rats, selected from the same age group as those used to test the effects of this analogue, and known as a preinjection control group, the total leukocyte count was 14,000 cells per cubic millimeter of blood. Of these, approxi-

![Graph showing distribution of leukocytes](image-url)

**Fig. 9.—Distribution of leukocytes in the peripheral blood of normal animals, those receiving aminopterin, and those receiving aminopterin plus varying amounts of folic acid daily, on the sixth day of the experiment.**
tions began, it is obvious that aminopterin restricted both categories of white blood cells. The total number of lymphocytes dropped from a level of 11,000 to one of 3,300 per cubic millimeter, and the total number of neutrophilic leuko-

FIG. 10.—Imprints of the femoral bone marrow: a. Normal rat. b. Animal receiving aminopterin daily for six days. c. Animal receiving aminopterin daily for six days plus 5 mg. of folic acid daily. d. Animal receiving aminopterin daily for six days plus 30 mg. of folic acid daily. e. Animal receiving aminopterin daily for six days plus 50 mg. of folic acid daily.

cytes dropped from a level of 3,000 to one of 500 cells per cubic millimeter. The smallest amount of the vitamin administered (5 mg. daily) had no effect whatever on the total number of neutrophilic leukocytes but did considerably elevate
the total lymphocyte count. For reasons difficult to interpret, 10 mg. of the vitamin daily resulted in a considerable increase in the total numbers of neutrophilic leukocytes—up to 2,200 cells per cubic millimeter—and 5,800 lymphocytes for the same amount of blood, while those animals receiving the larger amounts of the vitamin—20, 30, and 50 mg. respectively—had lesser numbers of neutrophilic cells. Increased numbers of lymphocytes, however, were found to occur in animals receiving 50 milligrams of the vitamin daily (group F), nearly approximating the lymphocyte level which obtained in the postinjection control group (G').

8. The Bone Marrow. Imprints of bone marrow from the distal end of the femur were obtained at necropsy from representative animals of each group. These were stained by the May-Grünwald-Giemsa technic. Figure 10 rather well portrays the changes which ensued when the analogue was given and the modifications of these changes which followed the administration of the varying amounts of the vitamin together with the analogue.

Normal rat marrow contains cells of both the erythroid and the myeloid series in varying stages of maturation (fig. 10a). Attempts were not made in the present study to establish changes in the percentage distributions of the various cellular categories, but it was clearly obvious that marked inhibitory changes were incurred. A glance at the marrow preparations of animals receiving the analogue for the six days (fig. 10b) shows how completely the maturation of the myeloid cells had been suspended. These imprints show that the marrow, in the region examined, was composed of cells almost exclusively erythroid, although a very few early myeloid forms were present.

The administration of 5 mg. of folic acid to animals receiving the analogue incited a slight myeloid response (fig. 10c), although the smears indicated that the marrow was still largely erythroid. But there were larger numbers of myelocytes and metamyelocytes in the imprints stimulated by the added small amount of vitamin. The response of the bone marrow to the increasing amounts of folic acid was more nearly proportional to the amounts injected than in any other organ observed. As the amounts of the vitamin were increased, the percentages of myeloid cells in the marrow were correspondingly elevated. When 30 mg. of the vitamin were given, larger numbers of immature myeloid cells were identified in the imprints, and their maturation resulted in many fully mature granulocytes (fig. 10d). The oral administration of 50 mg. of the vitamin resulted in the retention of a marrow pattern which was entirely normal (fig. 10e). The numbers of mature granulocytes appeared even to exceed those accepted as a normal distribution.

Comment

This study, incomplete though it is with respect to all the many other toxic reactions induced by the analogue, was undertaken to determine the extent to which the vitamin, folic acid, would counteract the untoward effects of the antagonist, 4-aminofolic acid. The therapeutic effects of this folic acid antagonist are of sufficient value clinically to warrant studies directed toward a modification of the toxic symptoms which accompany its use. If the analogue could be so modified as to
restrict the extreme degrees of enteritis which develop on its administration and yet retain the marked inhibitory effect on the development of myeloid cells in the bone marrow, its effective clinical use would be assured.

By the administration of the vitamin together with the analogue we have demonstrated satisfactorily that, for a certain period, a given amount of vitamin will completely nullify a given amount of antagonist. The toxic reactions which were so severe in animals of group A, given aminopterin alone, included anorexia, atony of the entire gastro-intestinal tract with gastric and intestinal distention, marked diarrhea, adrenal hyperplasia and atrophy of the thymus, spleen and bone marrow, with resulting leukopenia.

Partial remission of these disorders was obtained by giving small amounts of the vitamin daily; but larger amounts, 30 mg. daily, essentially inhibited the destructive effectiveness of the analogue in all of the categories enumerated. To be sure, a complete return to normal gland weights and to normal blood levels was not attained in all animals receiving that amount of folic acid daily; yet the gross appearance of the animals, the character of the gastro-intestinal tract, and the restored appetite all certified to the general deduction that, for the six-day test period, it required 30,000 micrograms of folic acid daily to counteract the toxic effects of 50 micrograms of 4-aminofolic acid daily. This is a ratio of inhibitor to metabolite of 1:600.

The toxic effects of aminopterin are not immediately evident on its administration. For three days, animals showed no ill effects of its intraperitoneal administration. Then, extremely rapidly, even over night, all the foregoing toxic manifestations may present themselves. This delay in the onset of symptoms, we presume, is due to the presence of a reserve of folic acid in the organism at the outset of the experiment. It may be that as soon as the analogue had destroyed this reserve of the vitamin, the toxic symptoms appeared. And yet these symptoms cannot all be ascribed to a folic acid deficiency, for we are not aware that they ensue to this extent in animals fed diets deficient in folic acid. Nevertheless, they did not develop when large amounts of the vitamin were fed, for the six-day period, together with aminopterin.

Although this report covers a short-time study of the relationship of the vitamin, folic acid, to the toxicity exerted in rats by the antagonist, aminopterin, and shows unquestionably the inhibition exerted by the vitamin for a six-day period, yet we have other data which show that this inhibition was not effective indefinitely. Our results show that the characteristic syndrome incited by the amounts of aminopterin we administered, was not inhibited for periods longer than fourteen days by giving 30 mg. of folic acid daily. We have not extended our observations to include the results of giving 50 mg. of the vitamin for the longer period. Reasons for the failure of the vitamin to inhibit the antagonist for longer periods are not clear. It may be that further increase of the amounts of folic acid could well antagonize the analogue, so as to restrict permanently the onset of the toxic effects. There is need, therefore, for much more research on the functional interrelationships of this vitamin and its powerful antagonist, aminopterin.
EFFECT OF FOLIC ACID ON TOXICITY OF AMINOPTERIN

SUMMARY AND CONCLUSIONS

A study of some of the toxic reactions of 4-aminofolic acid together with the modifications of these reactions induced by giving varying amounts of folic acid daily to white rats is reported.

Seven groups of young male rats ranging in weight from 110 to 130 Gm. were arranged. Aminopterin (4-aminofolic acid) was given intraperitoneally, in amounts equivalent to 50 micrograms daily, to all rats of six of these seven groups. Folic acid was given by mouth to these animals in such amounts as to provide 5, 10, 20, 30 or 50 mg. daily to each animal respectively of five of the six groups receiving aminopterin. One group received the analogue without the vitamin. One group of 8 animals received neither the vitamin nor its analogue.

Observations continued for six days, when all surviving animals were killed and necropsy was performed. Data were assembled on the appetite, body weights, the weights of adrenals, thymus and spleen, the distribution of leukocytes in the peripheral blood and the changes in the bone marrow. The following conclusions seem warranted:

1. Aminopterin is extremely toxic and incites within six days anorexia, extreme diarrhea with atony of the entire gastro-intestinal tract, adrenal hyperplasia, atrophy of the thymus and spleen, and an inhibition to development of myeloid cells in the bone marrow.

2. Small amounts of folic acid are essentially without effect on the toxicity of that amount of the analogue we chose to administer.

3. Larger amounts of folic acid daily (30 mg.) proved effective in essentially inhibiting the development of the severe toxic reactions for the six-day period.

4. The severe toxicity of aminopterin does not manifest itself until the third or fourth day of its daily administration. The onset of these symptoms is thereafter extremely rapid.

5. Thirty milligrams of folic acid daily will not indefinitely counteract the toxic symptoms induced by 50 micrograms of aminopterin. In our experience, within twelve to fourteen days, the characteristic syndrome will appear in spite of the continuous administration of the vitamin.

REFERENCES


GEORGE M. HIGGINS

THE EFFECT OF FOLIC ACID ON THE TOXICITY OF ITS ANALOGUE
4-AMINOPTEROYLGLUTAMIC ACID (AMINOPTERIN)

GEORGE M. HIGGINS, MURIEL STEMBER and HARRY MONSEN