This is in marked contrast to the inability of PGA to protect against much smaller doses of A-methopterin(9).

Thus, from these studies and those of others it would appear that aminopterin and A-methopterin are true antagonists of citrovorum factor rather than of folic acid.

Summary. 1. The administration of citrovorum factor 15 minutes prior to seven to thirty times as large a dose of A-methopterin, prevented the toxicity of the antimetabolite at levels ranging from 2 to 100 times the chronic LD_{50}. 2. No significant protective effect was noted when similar dosages of citrovorum factor were given four or more hours after the administration of A-methopterin. 3. A-methopterin appears to be a true antagonist of citrovorum factor rather than of pteroylglutamic acid.

Received January 18, 1951. P.S.E.B.M., 1951, v76.

Role of Aureomycin and Citrovorum Factor in "Folic Acid" Deficiencies.* (18498)

HARRY A. WAISMAN, M. GREEN, J. CRAVIOTO MUNOZ, A. RAMENCHIK, AND J. B. RICHMOND.

From the Department of Pediatrics, College of Medicine, University of Illinois, Chicago.

The use of folic acid antagonists in the treatment of acute leukemia in children has demonstrated that aminopterin and a-methopterin prolong life and give satisfactory clinical improvement in many cases. It has never been adequately explained why some cases similarly managed appear to be entirely unaffected by antagonist therapy. In several laboratory animals the antagonist is toxic to normal bone marrow and produces a leukopenia, growth retardation and hemorrhagic phenomena. No single metabolic action has as yet been suggested to account for these multiple findings. The consideration that more than pteroylglutamic acid (PGA) is antagonized by aminopterin finds some support in the inability of relatively large amounts of this vitamin to overcome small dosages of aminopterin. Our experiments to elucidate the mechanism of action of antifolic acid compounds were designed to test the possibility that more than PGA alone is affected by the antagonists. To obtain information on other factors that may influence the production of a PGA deficiency, it seemed necessary to compare the deficiencies induced by different means. At least three experimental approaches are available to obtain "folic acid" deficiencies: (1) The omission of PGA from a complete diet, (2) The use of PGA antagonists, and (3) The addition of relatively insoluble sulfonamide drugs to the diet. Since the deficiency produced by mere omission of the vitamin from the diet is time consuming and variable, this method was not used. The experiments described below provide evidence that the latter 2 methods may induce other deficiencies in addition to the now recognized PGA deficiency. Accordingly, the term "folic acid" deficiency will be used in the body of the paper to denote the condition observed on the sulfa diet or on the diet containing the antagonist. Since these diets probably produce a multiple rather than a single deficiency, reports by previous workers should be re-evaluated.

Experimental. Sprague-Dawley rats, 24 days of age, were given a synthetic diet consisting of sucrose, 73 parts; vitamin-free casein, 18 parts; salt mixture,† 4 parts; thiamine, 0.5 mg; riboflavin, 0.5 mg; niacin, 1 mg; calcium pantothenate, 2 mg; pyridoxine, 0.5 mg; inositol, 100 mg; biotin, 0.002 mg; and choline, 300 mg; vitamin B_{12}, .010 mg. Two drops of oleum percomorphum was given by mouth twice weekly to each rat as a

* Supported in part by a grant from the United States Public Health Service.

† Salts No. 2, Nutritional Biochemicals, Chagrin Falls, Ohio.

source of vitamins A and D. When 2% sulfaguanidine or 2% sulfaphthalidine was included in the synthetic basal diet, the sucrose was proportionately reduced.

Results. Aminopterin-induced "folic acid" deficiency. It had already been established by others(1) that 50-100 μg of aminopterin per 100 grams of diet produces poor weight gain, leukopenia, hemorrhage and death in a period of 3 to 4 weeks. In our experiments, however, despite the apparent uniformity of animals from a commercial source, the variability of response by the individual rats to the antagonist made adequate controls imperative. Data were obtained in several series to confirm the fact that aureomycin gives better growth than a diet without the antibiotic. The effect of the antagonist was tested, therefore, in a diet which included aureomycin. Adult rats previously fed the basal diet continued to thrive when given 75 μg of aminopterin plus 5 mg of aureomycin per 100 g of diet. Other adult rats given both 5 μg aminopterin and 5 mg of aureomycin as a single daily intraperitoneal injection for 14 days showed little change in weight, again demonstrating surprising protection by aureomycin against toxic levels of aminopterin. Because Weintraub *et al.*(2) and Goldin *et al.*(3) found that male mice are more susceptible than female mice to toxic levels of aminopterin, it seemed desirable to substantiate our findings under these more rigorous experimental circumstances by using only male weanling rats. A typical series

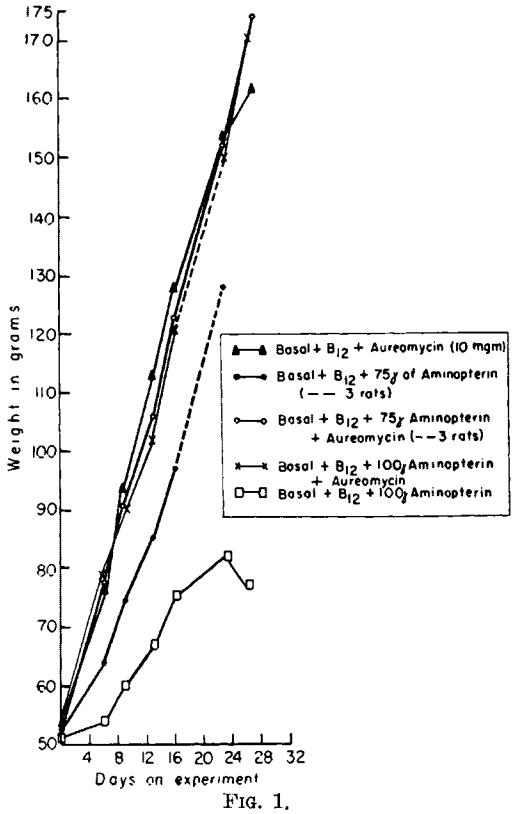

FIG. 1.

Effectiveness of aureomycin in overcoming the aminopterin induced "folic acid" deficiency.

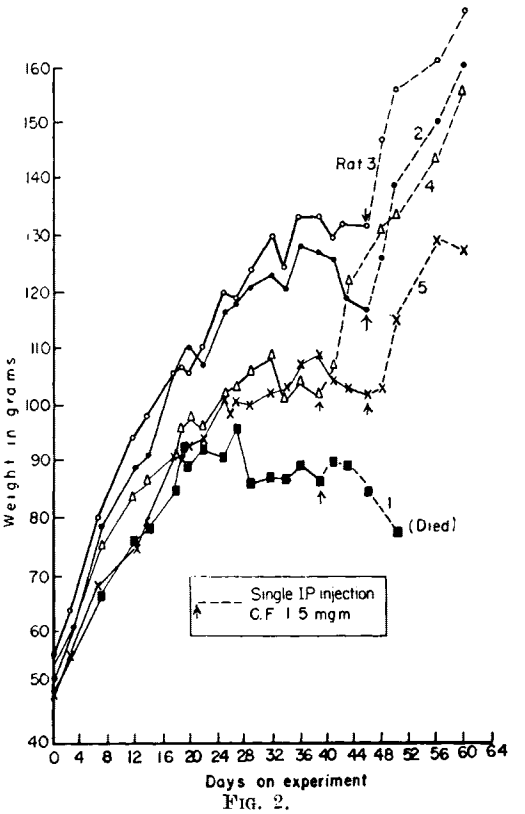

FIG. 2.

Typical growth response following a single intraperitoneal injection of citrovorum factor concentrate to individual rats fed the sulfaphthalidine diet.

1. Oleson, J. J., Hutchings, B. L., and SubBarrow, Y., *J. Biol. Chem.*, 1948, v175, 359.

2. Weintraub, S., Krause, S. D., and Wright, L. T., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 609.

3. Goldin, A., Greenspan, E. M., Goldberg, B., and Schoenbach, E. B., *Cancer*, 1950, v3, 849.

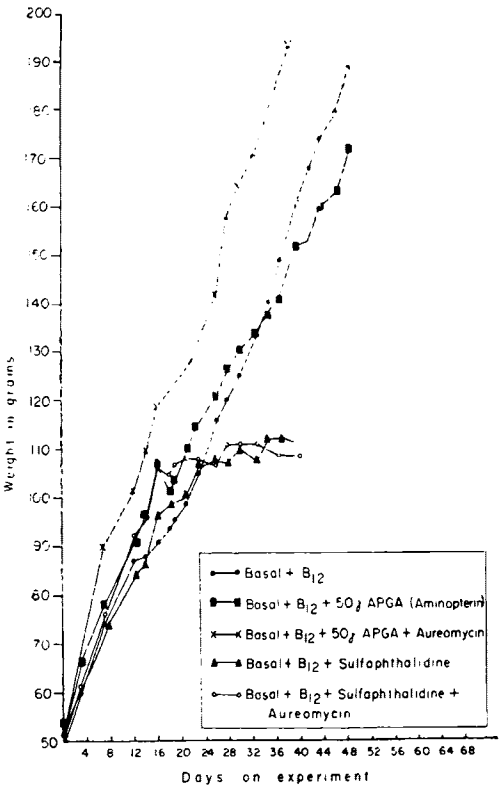

FIG. 3.

Ineffectiveness of aureomycin in group receiving sulfa diet in contrast to good growth response in group receiving minimal level of aminopterin.

contained groups in which 5 or 10 rats were given 50, 75 or 100 μg of aminopterin per 100 g of diet. Growth on 50 μg of aminopterin was slightly below that of controls not receiving the antagonist. The increasing toxicity of the 75 and 100 μg level in the diet was evidenced by the fact that 2 rats succumbed on the 75 μg diet, and all died on the 100 μg level. Fig. 1, 3 and 4 give graphic evidence that the beneficial effect of aureomycin occurs with 50 μg, 75 μg or 100 μg of aminopterin in the diet. It will be seen that while aminopterin control rats fail before 4 weeks (Fig. 1), the aureomycin plus aminopterin groups give as good growth as the control diet containing aureomycin but without aminopterin. Two rats which did not eat the aminopterin plus aureomycin diet well had poor weight gain; it was of interest, therefore, that these animals showed significant improvement in weight when 5 mg of aureomycin was given intraperitoneally. We have used 65 rats on aureomycin diets and twice this number on suitable control diets to confirm these observations.

The question whether aureomycin is a growth factor *per se* or whether its growth promoting properties are the result of antibiotic action cannot be completely answered at this time. It is known that autoclaving aureomycin at an alkaline pH destroys the antibiotic activity. Fig. 4 not only shows additional data on the ability of aureomycin to counteract aminopterin toxicity, but also provides evidence that alkaline autoclaving destroys the beneficial effect of the antibiotic on growth.

Sulfa-induced "folic acid" deficiency. It has always been presumed that PGA formed by intestinal bacteria is subsequently absorbed for purposes of growth in the host and that these bacteria are inhibited by an in-

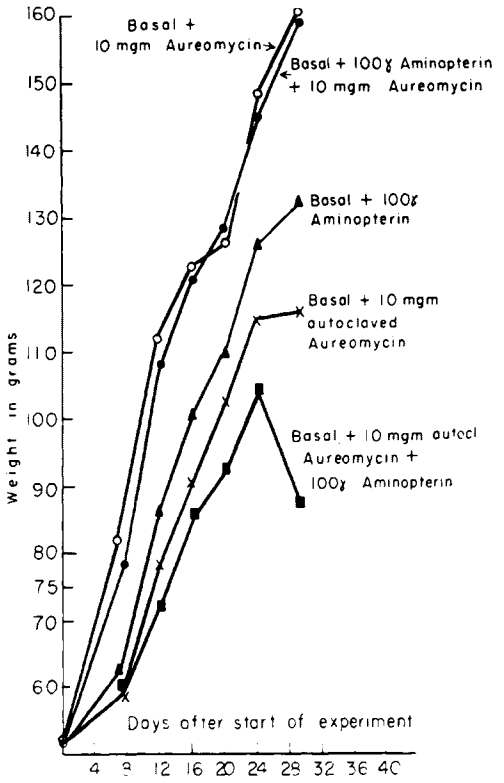

FIG. 4.

Ineffectiveness of autoclaved aureomycin in promoting growth or counteracting aminopterin toxicity.

soluble sulfonamide. The addition of PGA alone, however, does not provide optimum growth in rats on diets containing such sulfa drugs, and inhibition of intestinal flora may limit not only PGA but other growth factors required by the rat. Our data clearly show the effectiveness of concentrates of citrovorum factor‡ in correcting the "folic acid" deficiency. Fig. 2 shows the typical gain in growth that results when 10 x 10^6 citrovorum factor units§ are given as a single injection to individual rats fed the sulfaphthalidine diets. These responses were seen in more than 25 animals after they were maintained for 36-48 days on the sulfaphthalidine diet. In several instances (not illustrated) injection of the citrovorum concentrate into deficient animals was followed by a latent period before a manifest response. It is also of interest that in several rats citrovorum factor was ineffective once the rats had received single intraperitoneal injections of 200 μg of PGA.

The role of aureomycin as a growth stimulus has not been previously examined in rats receiving 2% sulfa diets. When 2.5 or 5.0 mg of aureomycin was either given intraperitoneally to the deficient animals or was added to 100 g of diet, increases in weight were not significantly higher than in the controls. A similar result was obtained when 10 mg of aureomycin was added to 100 g of diet containing 2% sulfaphthalidine from the start of the experiment (Fig. 3). This finding is important, especially since we were able to show that aureomycin could counteract the deleterious effect obtained on a diet which contained aminopterin.

Discussion. The present experiments have been primarily directed at elucidating the mode of action of the PGA antagonists. Although PGA is part of an important enzyme system necessary for growth or for cellular metabolic processes, recent work has indicated that citrovorum factor is perhaps the more active portion of that enzyme system. It was therefore not unexpected that citrovorum factor concentrates were more effective than PGA in correcting the "folic acid" deficiency produced either by aminopterin or sulfa drugs. It might be more exact, perhaps, to describe aminopterin as a citrovorum factor antagonist as well as a PGA antagonist. However, the fact that PGA is a precursor in the formation of citrovorum factor may indeed explain the effectiveness of aminopterin as an antagonist.

The most significant finding of the present report is the effectiveness of aureomycin in overcoming the toxic effects of aminopterin. The inability of toxic levels of aminopterin to exert their effect in the presence of aureomycin was apparent whether the aureomycin was given from the start or after the onset of the deficiency.

It is of more than passing interest that autoclaved aureomycin was not able to provide the growth response obtained with untreated antibiotic. The assumption can be made that since the antibiotic activity was destroyed, the mode of action of the compound is through its effect on intestinal flora. This may not be completely tenable, since if an additional growth factor exists in crystalline aureomycin, it too may be destroyed by the autoclaving. Further work on this point is necessary, but several possibilities may be entertained, none of which are as yet documented by adequate data: (a) It may be that the role of aureomycin is merely that of an antibiotic depressing the growth of those intestinal microorganisms that utilize the PGA produced by favorable bacteria, thus making the vitamin unavailable to the host. (b) Aureomycin may actually be a growth factor, and the findings here are unrelated to its antibiotic activity as such. All or part of the aureomycin molecule may function as a prosthetic group for an essential coenzyme. (c) Still another possibility is that aureomycin may interfere with those natural inhibitors of folic acid conversion to citrovorum factor. (d) A last possibility is that aureomycin has a structure essentially similar to that of PGA or citrovorum factor and can replace either in the diet. Experiments are in progress to determine whether any or all of these hypotheses have any credence.

‡ Kindly supplied by Dr. H. Broquist, Lederle Laboratories, Inc.

§ 10,000,000 units are 0.75 mg (Dr. H. Broquist).

Summary. 1. "Folic acid" deficiencies induced by aminopterin and by sulfa drugs in rats have been compared. 2. Aureomycin can counteract the toxic effects of 50, 75 and 100 μg of aminopterin/100 g of diet. The antibiotic is equally effective whether fed in the diet or given intraperitoneally. 3. Aureomycin is without effect on growth in rats made "folic acid deficient" on a sulfa diet. 4. Citrovorum factor concentrates are able to completely overcome the deficiency produced on either type of diet.

Received January 9, 1951. P.S.E.B.M., 1951, v76.

Complement-Fixing Murine Typhus Antibodies in Vitamin Deficiency States.* (18499)

KENNETH WERTMAN AND JOSEPH L. SARANDRIA

From the Department of Biological Sciences, University of Pittsburgh.

The effect of malnutrition in epidemic typhus fever has been recognized for some time. Efforts have been made to find an explanation for the observation that increased mortality occurs in malnourished populations exposed to typhus fever. FitzPatrick(1) injected weanling male white rats, subjected to various vitamin and protein deficiencies, with viable rickettsiae (*Rickettsia typhi*) of murine typhus fever. It was demonstrated that the animals on a reduced vitamin B complex intake of 1 10th the optimal level were more susceptible than those receiving ample B vitamins. Thiamin, riboflavin, and pantothenic acid deficiencies studied separately, caused increasing susceptibility. It was also reported that animals on a natural diet were more resistant than those on a complete synthetic ration. The purpose of this investigation was to extend these observations. It was felt that the increase in susceptibility might in some manner be correlated with the inability of the animals to rapidly produce circulating antibodies when antigenic material was introduced.

The production of circulating antibodies in vitamin deficient states has been reported by a number of investigators. The antigenic materials employed for these studies have been of a variety of types. Axelrod, Carter, McCoy and Geisinger(2) demonstrated that pyridoxin, pantothenic acid deficiencies effected the antibody production by the rat in response to the injection of human red blood cells as antigenic stimulus. Carter and Axelrod(3) reported that the content of circulating antibodies in the thiamin and biotin deficient rats was less than that of the control rats. Ludovici, Axelrod, and Carter(4) observed a low production of circulating antibodies in the pantothenic acid deficiency state when rats were injected with human red blood cells. Stoerk, Eisen, and John(5) reported that pantothenic acid deficiency did not affect the level of circulating antibodies when the rats were immunized with sheep erythrocytes. In the present study 5 different diets were utilized. In the initial investigation (Table I), 5 rats were placed on an adequate diet and 5 were placed on a diet containing only one-tenth of the amount of vit. B complex considered to be the optimum. The second experiment employed 4 groups of animals; these were total vit. B deficiency,

* This investigation was supported by a research grant from The National Institutes of Health, Public Health Service.

1. FitzPatrick, F., *Am. J. Pub. Health,* 1948, v38, 676.

2. Axelrod, A. E., Carter, B. B., McCoy, R. H., and Geisinger, R., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 137.

3. Carter, B. B., Axelrod, A. E., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 416.

4. Ludovici, P. P., Axelrod, A. E., and Carter, B. B., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 81.

5. Stoerk, H. C., Eisen, H. N., and John, H. M., *J. Exp. Med.,* 1947, v85, 365.