as high as 55 cc per minute in a 13 kg dog. This value is comparable to that reported by Landis and his co-workers.

The increases in filtration rate and renal plasma flow which follow such infusions as we have studied do not attain their maximal values until an hour or more after the termination of the infusion(1). Slow infusions do not increase the venous or arterial pressure, and the increments induced by rapid infusions have generally disappeared within 30 minutes after the end of the infusion. We conclude, therefore, that except for the early abrupt increase in filtration rate, which may in part be related to increased arterial pressure and hemodilution, the changes induced in renal function by such infusions are not related to changes in venous or arterial pressure, increased plasma or blood volume or protein dilution.

Summary. The intravenous infusion of 1100 to 1600 cc of a modified Locke's solution at rates of 140 to 220 cc/min. in dogs causes only a transient increase in venous and arterial pressure and pulse rate. These values usually return to control levels within half an hour. Plasma and blood volume as measured by changes in plasma protein concentration and hematocrit are increased markedly at first, but return to within 20% of control values 70 minutes after the infusion.

These systemic changes do not coincide in time with the increase in filtration rate and renal plasma flow induced by such infusions, and it is concluded that the changes in renal function cannot be attributed to them.

Received May 22, 1950. P.S.E.B.M., 1950, v74.

On the Mechanism of Action of Aminopterin.* (17922)

C. A. NICHOL AND A. D. WELCH

From the Department of Pharmacology, School of Medicine, Western Reserve University, Cleveland, O.

In studies on the metabolism of folic acid and its derivatives, it was observed that rat liver slices form from pteroylglutamic acid (PGA) a substance or substances utilized for growth by *Leuconostoc citrovorum* 8081(1). The existence of a factor of unknown chemical nature, referred to as citrovorum factor (CF), was first described by Sauberlich and Baumann(2) and its relation to folic acid was indicated by Sauberlich(3). Using the liver slice system, conditions influencing the synthesis of CF were studied(1), and it was found that the presence of certain reducing agents, particularly ascorbic acid, stimulated the formation of CF, both from synthetic PGA and from compounds present in normal rat liver. It was suggested that a reductive step may be involved in the enzymatic synthesis of CF. The 4-amino analogue of PGA, aminopterin, is one of the most potent inhibitors of the functions of PGA. However, in contrast to the reversible antagonism that often exists between metabolites and structural analogues that interfere with their biological action, aminopterin proved to be reversed very inefficiently by PGA. Thus, Franklin, *et al.*(4) reported that even high levels of PGA were unable to counteract the toxic effects in mice caused by feeding aminopterin at a level of 1 mg per kg of diet.

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

1. Nichol, C. A., and Welch, A. D., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 52.

2. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1948, v176, 165.

3. Sauberlich, H. E., *J. Biol. Chem.*, 1949, v181, 467.

4. Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 398.

TABLE I.
Aminopterin Inhibition of Conversion of PGA to Citrovorum Factor in Liver Slices; Comparison of Normal and PGA-deficient Rats.

		Additions to vessels containing liver slices* Citrovorum factor activity—units/gram of tissue					
					Ascorbic acid—10 mg		
	Aminopterin, μg/vessel		10 μg PGA	100 μg PGA		10 μg PGA	100 μg PGA
PGA-deficient rat liver (6)	0	70	1530	2700	180	8450	14000
	6.25		350	720		1190	4000
	12.5		200	880		880	3350
	25			430			1920
	50	50		200			1190
	50†			2320†			17200†
Normal rat liver (4)	0	1310	1770	2310	3990	5770	7250
	12.5		1200	1410		3220	3330
	25		1110	1690		5150	3480
	50	1240		1160			3510
	50†			2540†			6880†

* The contents of the vessels and their manner of treatment are described in the text. Each vessel contained about 1 g of liver slices from the number of animals indicated by the figure within the parentheses.

† These values represent the levels of CF found when the aminopterin was added at the *end* of the period of incubation. In comparison with the control values, they indicate that aminopterin had no appreciable effect on the response of the microorganism to the CF present.

Also, Higgins(5) observed that daily injection of 50 μg of aminopterin in rats caused signs of acute PGA-deficiency and death in spite of the simultaneous oral administration of PGA in doses as high as 30 mg per day. On the other hand, in the microorganism *Leuconostoc citrovorum*, Sauberlich(6) noted that inhibition of growth by aminopterin was counteracted effectively by concentrates of CF derived from liver or from urine. The possibility that aminopterin inhibits the functions of PGA by interfering with either the conversion of PGA to CF or the utilization of CF, or both, has been the subject of the present investigation.

Methods and results. Experiments in vitro. Weanling albino rats were depleted of PGA by feeding a purified casein-cerelose diet containing succinylsulfathiazole, as described previously(1). The normal rats were raised on a complete stock diet. Liver slice experiments were carried out in small beakers containing 4.5 cc of Krebs-Ringer phosphate solution (pH 7.4) to which appropriate additions of PGA, aminopterin, and ascorbic acid were made; the final volume of fluid was 5 cc in each case. Consecutive slices of liver were placed in the beakers in regular sequence in order to minimize variations in composition; the vessels were weighed before and after the addition of the slices. In all experiments, the incubations were carried out with shaking in a covered water bath at 37° for 2 hours. The ascorbic acid solution was freshly neutralized and was added quickly to the vessels as soon as they had reached the temperature of the water bath. After incubation, the vessels were steamed at 100° for 5 minutes, and the contents of each vessel were ground in a glass homogenizer. The mixture was adjusted to pH 6.5, transferred to a glass-stoppered test tube, diluted to 25 cc, steamed at 100° for 10 minutes, thoroughly mixed and filtered. The clear filtrates were assayed microbiologically with *Leuconostoc citrovorum* 8081, using the basal medium described by Sauberlich and Baumann(2). Turbidimetric readings were made after about 20 hours at 37° using the Klett-Summerson photoelectric colorimeter with a 660 mμ filter.† In all experiments conducted *in vitro* a comparison

5. Higgins, G. M., *Blood*, 1949, v4, 1142.

6. Sauberlich, H. E., *Arch. Biochem.*, 1949, v24, 224.

was made of the production of CF in the presence and absence of ascorbic acid, because of the previously demonstrated(1) favorable influence of reducing substances on the formation of the factor. Table I shows clearly that the CF-content of rat liver tissue, *in vitro,* was augmented by the addition of PGA. In the hepatic tissue of rats deficient in PGA, in contrast to liver slices from normal rats, very low values for CF were obtained. For this reason investigation of the inhibitory action of aminopterin on the synthesis of CF from PGA was facilitated by the use of PGA-deficient rats, rather than normal animals.

The data of Table I show that aminopterin markedly reduced the amount of CF formed from PGA by the liver slices of PGA-deficient rats. Since aminopterin is a potent inhibitor of the growth of *Leuconostoc citrovorum,* it was necessary to prove that the results observed actually were due to inhibition of hepatic synthesis of CF and were not to be accounted for by an antibacterial action on the microorganism subsequently used for assay. Absence of a significant effect of aminopterin, in the dilutions of the liver filtrates used for assay, is shown by the values for CF, designated in Table I by an asterisk, obtained when aminopterin was added to the slices at the end rather than at the beginning of the incubation period. These values agree closely with the corresponding values for CF found in the absence of aminopterin. The table shows also that the activity of aminopterin for the microorganism, unlike that of PGA, was not altered significantly by rat liver slices, since the CF activity found in control vessels containing no PGA did not differ appreciably from that of similar vessels in which 50 μg of aminopterin had been incubated with liver. Further, the inhibitory action of even higher concentrations of aminopterin on *Leuconostoc citrovorum,* grown in the presence of a limiting amount of CF, was not increased by a preliminary incubation of the inhibitor with liver slices.‡ The effect of ascorbic acid in augmenting the yield of CF, both in the absence and in the presence of PGA, is shown in Table I. The percentage inhibition of the production of CF caused by aminopterin, 50 μg per g of PGA-deficient liver tissue, was of the same order whether ascorbic acid was or was not present. In the amounts used, aminopterin, added to PGA-deficient slices, did not block completely the formation of CF from high concentrations of added PGA, although the yield was reduced by as much as 90%. On the other hand, addition of the antagonist (50 μg per g of tissue) to liver slices derived from normal rats abolished completely the extra CF formed from added synthetic PGA.

N^{10} - formyl - pteroylglutamate,§ "pteroyltriglutamate," and "pteroylheptaglutamate" were compared with pteroylglutamic acid, not only as precursors of CF, but also with regard to the effect of ascorbic acid and the inhibitory action of aminopterin on the derivation of CF from them. Each of these compounds yielded CF when incubated with liver slices from PGA-deficient rats and, in each case, the amount of CF synthesized in the tissue was increased in the presence of ascorbic acid. N^{10}-formyl-PGA was less active than PGA as a precursor of CF; however, the sample employed had not been subjected to rigid tests of purity and no particular significance is attached to the lower activity. "Pteroyltriglutamate" and "pteroylheptaglutamate" (crystalline vitamin B_c conjugate) were tested at concentrations approximately equimolar to PGA and showed about 50%

† A liver fraction is used in this laboratory as a constant reference standard; one unit of citrovorum factor is defined as that amount needed per 10 cc assay tube, to produce half-maximal turbidity in 20 hours at 37°.

‡ In the concentrations encountered, PGA does not influence the 20-hour growth of the organism. Studies of the stability of thymidine and of a naturally occurring material of unknown composition(7), under conditions which destroy CF, indicate that the accuracy of determination of the CF-values reported in this paper was not significantly influenced by these substances.

7. Nichol, C. A., and Welch, A. D., *Fed. Proc.,* 1950, v9, 367.

§ We are indebted to Dr. R. M. Anker who synthesized the N^{10}-formyl-pteroylglutamic acid in this laboratory according to the method of Gordon, *et al., J. Am. Chem. Soc.,* 1948, v70, 878.

TABLE II.
Inhibition by Aminopterin-Treatment, *in Vivo*, of the Conversion of Folic Acid to Citrovorum Factor by Liver Slices, *in Vitro*.

Additions to vessels containing liver slices*	Citrovorum factor activity—units/g of tissue Source of tissue	
	PGA-deficient rats	PGA-deficient, aminopterin-treated rats
None	80	40
PGA 10 μg	1610	60
PGA 100 μg	2600	110
Ascorbic acid, 10 mg	170	90
" " " " + PGA 10 μg	6960	170
" " " " + PGA 100 μg	11600	260

* The contents of the vessels and their manner of treatment are described in the text.

of the activity of PGA as precursors of CF, both in the presence and absence of ascorbic acid. Aminopterin inhibited the formation of CF from each of these forms of PGA. In the presence of 12.5 μg of aminopterin per g of liver slices, the yield of CF from PGA and from each of these derivatives of PGA was lowered to approximately 20% of that obtained without aminopterin. The effect of aminopterin on the ability of liver to convert PGA to CF also was studied by injecting aminopterin into PGA-deficient rats prior to the preparation of liver slices from these animals. Two groups of 8 rats each were selected from PGA-deficient animals which had not increased in body weight during the previous week. The rats of one group were given no additional treatment; each animal of the other group was injected intraperitoneally with 50 μg of aminopterin per day. The injected rats developed marked loss of body weight within 24 hours and severe diarrhea and secretion of porphyrin were observed on the second and third day of treatment. On the day following the third injection of aminopterin, 4 animals from each group were sacrificed for the comparison reported in Table II. Of the 4 remaining aminopterin-treated rats, 3 died on the third day and the last on the fourth day following the first injection. In the control group of PGA-deficient rats, body weight did not change appreciably and no deaths occurred during this time.

In contrast to the tissue from rats in the control group, liver slices from the injected rats were unable to form an appreciable amount of CF from PGA, even in the presence of ascorbic acid (Table II). Similarly, no CF was formed when formyl-PGA or the conjugates of PGA were incubated with liver slices from the aminopterin-treated rats, either in the presence or absence of ascorbic acid. Filtrates prepared from liver samples from rats which were injected with aminopterin did not inhibit growth of *Leuconostoc citrovorum* except at amounts per assay tube much greater than those required in the analyses. The prior treatment of the rats with aminopterin was more effective in reducing the yield of CF to be obtained from PGA added to the liver slices, than in those cases where the inhibitor was added *in vitro*.

Experiments in vivo. Sauberlich has shown that the urinary excretion of CF by rats is roughly proportional to the amount of PGA ingested(3). The inhibition by aminopterin of the conversion of PGA to CF observed in liver slice experiments, was tested *in vivo* by measuring the effect of aminopterin on the urinary excretion of CF by adult rats injected daily with PGA. Female rats (av wt, 150 g) were placed in individual metabolism cages equipped with paraffined-metal collecting funnels. These animals had been raised on a purified diet(1) containing 50 μg of PGA per 100 g and were fed this diet during the experimental period. Four groups were arranged, each containing three rats. Urine from each rat was collected under toluene during successive 48-hour periods and was kept refrigerated until assayed microbiologically with *Leuconostoc citrovorum* 8081. Urine from normal rats receiving 25 μg aminopterin per day was added to assay tubes

TABLE III.
Effect of Aminopterin on Urinary Excretion of Citrovorum Factor in Rats Receiving Pteroylglutamic Acid.

Group No.	Collection period, days	Treatment: PGA 2 mg/day	Treatment: Aminopterin, 25 μg/day	Treatment: Ascorbic acid, 50 μg/day	Citrovorum factor excreted per rat per 48 hr, units	Survival after initiation of aminopterin treatment, days
I	1-2	—	—	—	80	
(3 rats)	3-4	+	—	—	13800	
	5-6	+	+	—	4870	
	7-8	+	+	—	1770	
	9-10	+	+	—	1720	
	11-12	+	+	—	1420	
	13-14	+	+	—	1470	
	15-16	+	+	—	1660(2)*	12–14
II	1-2	—	—	—	80	
(3 rats)	3-4	+	—	+	52000	
	5-6	+	+	+	9840	
	7-8	+	+	+	6370	
	9-10	+	+	+	6250	
	11-12	+	+	+	2870	
	13-14	+	+	+	5720	
	15-16	+	+	+	2030(1)*	11–14
III	1-2	—	—	—	70	
(3 rats)	3-4	—	—	—	100	
	5-6	+	+	—	1030	
	7-8	+	+	—	900	
	9-10	+	+	—	1520	
	11-12	+	+	—	900	
	13-14	+	+	—	1210(2)*	8–11
IV	1-2	—	—	—	70	
(3 rats)	3-4	—	+	—	290	
	5-6	+	+	—	500	
	7-8	+	+	—	890	7–8

* Figures within parentheses represent the number of animals surviving the collection period indicated. CF values stated for all other periods represent the average of 3 urine samples.

containing limiting amounts of CF. It was found that the aminopterin excreted in the urine did not interfere appreciably with the growth of the organism at the dilutions used for assay. As indicated in Table III, daily doses of PGA, aminopterin, and freshly neutralized ascorbic acid were administered intraperitoneally. Group II duplicated the treatment of Group I, except that ascorbic acid was given along with PGA, since ascorbic acid has been found to increase the urinary excretion of CF by rats and human subjects receiving PGA(8). Groups I, III, and IV differed only in the times when administration of PGA and aminopterin were begun. Group I was given PGA during a 48-hour period prior to the initiation of treatment with aminopterin; Group IV received aminopterin during a 48-hour period prior to the initiation of treatment with PGA; in Group III PGA and aminopterin were administered simultaneously.

The data obtained from this experiment are presented in Table III. Normal rats fed a purified diet(1) containing succinylsulfathiazole (2%) excreted from 60 to 100 units of CF per 48 hours (all groups). Administration of PGA (2.0 mg per day) increased the CF content of the urine by about 150-fold (Group I). An even greater increase in the excretion of CF followed treatment with both PGA and ascorbic acid (Group II). When aminopterin was given simultaneously with PGA the amount of CF excreted was markedly reduced (1030 units, Group III,

8. Unpublished data from this laboratory and Welch, A. D., Trans. Assoc. Amer. Physicians, 1950, in press.

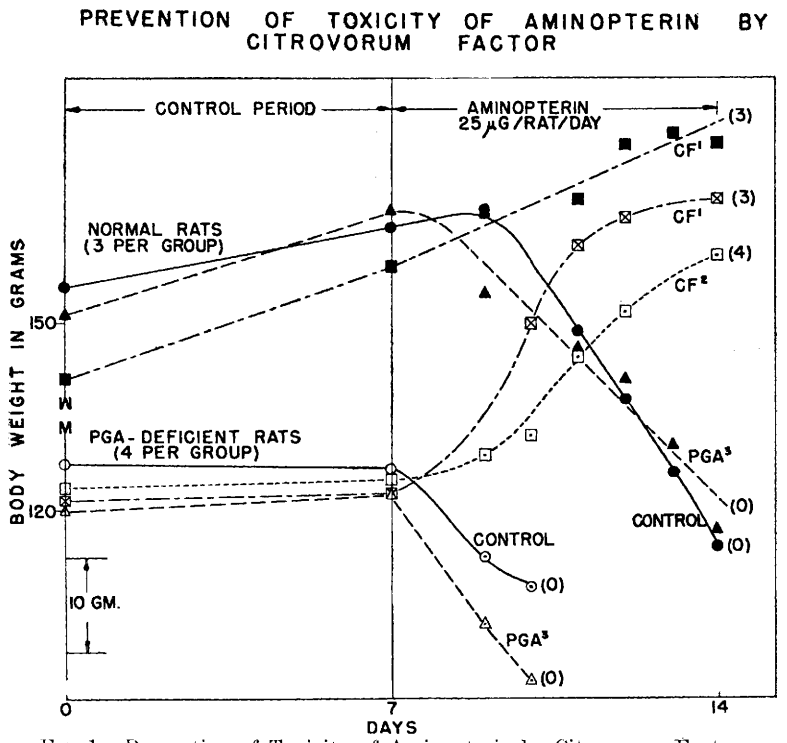

FIG. 1. Prevention of Toxicity of Aminopterin by Citrovorum Factor.

Figures enclosed in parentheses show the number of animals surviving on the day indicated.

1 500,000 units of CF (3 mg of concentrate) were inj. daily after 7th day.

2 250,000 units of CF (1.5 mg of concentrate) were inj. daily after 7th day.

3 5 mg of pteroylglutamic acid were inj. daily after the 7th day.

as compared with 13,800 units, Group I). Pre-treatment of rats with aminopterin for 2 days prior to initiation of PGA injections reduced further the urinary elimination of CF formed from PGA (Group IV). In the rats which at first were injected only with aminopterin (Group IV) there was a suggestion of a slight increase in the elimination of CF; this will be discussed subsequently. The large amounts of CF excreted by the rats first given PGA or PGA plus ascorbic acid decreased markedly when additional injections of aminopterin were begun. During continued treatment with PGA and aminopterin, some CF was formed, as evidenced by the excretion data. However, all rats died within 14 days following initiation of treatment with aminopterin. Administration of PGA prior to treatment with aminopterin increased the survival time. Since aminopterin inhibits the metabolic alteration of PGA, the product of this reaction might be capable of competing more effectively than PGA with this antagonist. Accordingly, the ability of concentrates of CF to prevent the toxicity of aminopterin was tested in PGA-deficient and in normal rats. In these experiments young female rats were given a purified diet(1) during a period of 6 weeks. To the diet of one group ("normal"), PGA was added at a level of 50 μg per 100 g. During a control period of one week, the PGA-deficient rats showed no significant change in body weight, while the "normal" rats continued to gain at a uniform rate, as is shown in Fig. 1. During the period of treatment, all rats were injected with aminopterin (25 μg per day) intraperitoneally. The control groups received no additional treatment. Other groups were given injections of PGA (5 mg per day) or of a concentrate of CF supplied by the Lederle Laboratories; this concentrate was

tested at two levels in the PGA-deficient rats (1.5 mg per day, providing 250,000 units, and 3.0 mg per day, providing 500,000 units of CF). In the "normal" rats the CF concentrate was administered intraperitoneally at a level of 500,000 units per day.

PGA failed to protect the rats from the lethal action of aminopterin. Although the "normal" rats were somewhat more resistant to the analogue than were the PGA-deficient animals, none survived the eighth day of injection with aminopterin. Comparable treatment of the PGA-deficient rats led to death by the fourth day of treatment. In contrast to the ineffectiveness of PGA, the concentrate of CF exerted a marked protective action against the toxic effects of aminopterin (see Fig. 1). "Normal" rats given the concentrate, together with aminopterin, maintained the same rate of growth that was observed during the control period. Of the PGA-deficient rats which were given the concentrate of CF, as well as aminopterin, only one animal died (on the third day of treatment). The other rats maintained a remarkable rate of growth. The number of animals used in this preliminary experiment was limited by the small amount of CF concentrate available. Consequently, no significance is attached to the difference in rate of growth between the groups receiving the two levels of CF concentrate. However, only those animals which received this concentrate survived and these rats remained healthy following the cessation of treatment (see Fig. 1).

Discussion. The data presented show clearly that aminopterin interferes, both *in vitro* and *in vivo*, with the conversion of pteroylglutamic acid to the factor(s) which promotes rapid growth of *Leuconostoc citrovorum* 8081. The product of the metabolic alteration of PGA, citrovorum factor, not only is capable of preventing the toxicity of aminopterin, but also is a biologically active derivative of PGA, as demonstrated by the growth response of PGA-deficient rats which received aminopterin and a concentrate of CF concurrently. Inhibition of the conversion of PGA to CF was more complete in liver slices from rats which were injected with aminopterin than when the antagonist was added directly to the tissue *in vitro*. Administration of aminopterin to the intact animal apparently allows greater opportunity for saturation of the enzymes responsible for the conversion of PGA to CF. Whether these enzymes are widely distributed throughout the tissues or are restricted to the liver has not yet been determined. The failure of massive doses of PGA to prevent the toxic effects of aminopterin has been demonstrated in several species(4,5,9,10,11). This antagonist appears to have much greater affinity than PGA for the enzymes which accomplish the conversion of PGA to CF. Sufficiently high concentrations of PGA in the tissues should limit the ability of the antagonist to combine with these enzymes, but in most cases this concentration apparently is not readily attainable. Thus, PGA at a level of 10 mg per 100 g of diet failed to counteract the lethal effect of aminopterin (100 μg per 100 g in mice)(4); recently, however, Ershoff, Hoffstadt and McWilliams(12) reported positive protection in this species when a ratio of PGA to aminopterin of 500:1 was used. Also, Hertz and Tullner(13) observed that inhibition by aminopterin of the estrogen-induced growth in the female genital tract of estradiol-treated ovariectomized rats and stilbesterol-treated chicks could be counteracted at PGA to inhibitor ratios of 300:1 or 400:1.

The action of aminopterin cannot be explained solely on the basis of an interference with the metabolic alteration of PGA. Studies of the effect of this antagonist on certain bacterial species, particularly *Leuconostoc citrovorum* 8081, indicate that aminopterin interferes with the utilization of CF(6,14,15). Data presented in this paper support this

9. Goldsmith, E. D., Schreiber, S. S., and Nigrelli, R. F., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 299.

10. Karnofsky, D. A., Patterson, P. A., and Ridgway, L. P., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 447.

11. Farber, S., *Blood,* 1949, v4, 160.

12. Ershoff, B. H., Hoffstadt, J. P., and McWilliams, H. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 501.

13. Hertz, R., and Tullner, W. W., *Endocrinology,* 1949, v44, 278.

conclusion. Rats given injections of both PGA and aminopterin excreted from 1000 to 2000 units of CF in the period immediately preceding death (Table III). This amount of CF far exceeds that excreted by "normal" rats fed a diet adequate in PGA (50 μg per 100 g), yet this amount of CF excreted in the urine reflects tissue concentrations incapable of reversing the toxic effects of aminopterin. However, larger amounts of CF effectively counteracted the antagonist (Fig. 1). Presumably aminopterin competes with CF for combination with an apoenzyme or enzyme with which CF normally reacts. Preliminary evidence suggests that the activity of 1 μg of pure CF may lie between 1000(16) and 10,000 units(15). Since 250,000 units of CF protected rats completely against the toxic effects of 25 μg of aminopterin, it may be suggested that, on a weight basis, the ratio of CF to aminopterin required to prevent the effects of the antagonist is less than 1:1 or 10:1. Recent studies by Broquist, *et al.*(15), using mice, also have shown that concentrates of CF can prevent the toxic effects of aminopterin. Whether CF can reverse the toxicity of aminopterin when treatment is delayed until the toxic effects of the drug are strongly manifested is under investigation.

The survival time of rats injected with aminopterin was increased by prior treatment with PGA (Table III). This effect may be due to saturation of the tissues with CF so that depletion of CF and inhibition of its functions by aminopterin require a longer time. Also, CF may be formed from certain precursors in the tissue by reactions which are not inhibited by aminopterin. This is indicated by data in Table I which show that, in normal liver slices, aminopterin did not influence significantly the amount of CF which was derived from tissue-precursors. Also, the additional CF formed from tissue-precursors in the presence of ascorbic acid was not blocked by aminopterin. However, under the same conditions, the conversion of added PGA to CF, which also is increased by ascorbic acid, apparently was inhibited completely by the analogue. Conceivably, these findings might signify that, in the conversion of PGA to materials with high activity for *Leuconostoc citrovorum,* at least two steps are involved. Only the first of these may be blocked by aminopterin, while the second step could involve a reduction facilitated by ascorbic acid but not interfered with by aminopterin. According to this view, there may be present in normal rat liver a compound with a structure intermediate between those of PGA and CF, and on which the effect of ascorbic acid is exerted.

Swendseid, *et al.*(17), observed a small increase in the urinary elimination of CF following administration of aminopterin to human subjects. A similar observation was made in each rat injected with aminopterin alone (Table III). This may reflect a displacement of CF from tissue complexes by the antagonist.

Information derived from these studies indicates that PGA is converted metabolically into a biologically more effective compound and provides an explanation for the biochemical mechanism of action of a very potent poison and for the failure of PGA effectively to counteract its toxicity. Further, and in confirmation of the work of Broquist, *et al.* (15) in mice, a means is afforded of counteracting the easily developed toxic action of aminopterin, a compound of considerable utility in the therapy of certain types of acute leukemia in children.

Summary. Rat liver slices form from synthetic folic acid (PGA) a factor (CF) utilized for growth by *Leuconostoc citrovorum;* this conversion is inhibited by aminopterin. In rats given PGA, a similar inhibition of the synthesis of CF is shown by a marked decrease in the urinary excretion of CF following the administration of aminopterin. The antagonist not only prevents the metabolic altera-

14. Franklin, A. L., Stokstad, E. L. R., Hoffmann, C. E., Belt, M., and Jukes, T. H., *J. Am. Chem. Soc.*, 1949, v71, 3549.

15. Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H., *Fed. Proc.*, 1950, v9, 156.

16. Anker, R. M., Boehne, J. W., and Welch, A. D., *Fed. Proc.*, 1950, v9, 351.

17. Swendseid, M. E., Swanson, A. L., Miller, S., and Bethell, F. H., *Fed. Proc.*, 1950, v9, 372.

tion of PGA but also competes with the product (CF) derived from PGA. The lethal action in rats of aminopterin, 25 μg daily, is not prevented by PGA, but the daily administration of 250,000 units of CF completely counteracts the toxic effects of the antagonist. That CF is a biologically active derivative of PGA is shown by the fact that rats in which growth is arrested by PGA-deficiency, and which due to aminopterin are refractory to PGA, grow remarkably when given concentrates of the citrovorum factor.

We are especially appreciative of the invaluable technical assistance given by Marilyn F. Wilson and Caroline A. McKalen.

Pteroylglutamic acid, "pteroyltriglutamate" and some of the concentrates of citrovorum factor were furnished by the Lederle Laboratories Division of America Cyanamid Co., through the courtesy of T. H. Jukes; other concentrates of the citrovorum factor were prepared by R. M. Anker and J. W. Boehne, III, of this laboratory. Crystalline vitamin B_c conjugate was a gift from J. J. Pfiffner of Parke, Davis & Co., and succinylsulfathiazole was provided by Sharp and Dohme, Inc., through the courtesy of W. A. Feirer.

Received May 2, 1950. P.S.E.B.M., 1950, v74.

Tocopherol Requirements of Rats by Means of the Hemolysis Test.* (17923)

CATHARINE S. ROSE AND PAUL GYÖRGY

From the Nutritional Service of the Department of Pediatrics and the Gastrointestinal Section of the Medical Clinic, School of Medicine, University of Pennsylvania.

It has been found that in rats deficient in vitamin E, injection of alloxan or its reduction products, alloxatin and dialuric acid, is followed within a few minutes by hemolysis so severe that in some cases the cell volume is reduced from the normal value of over 50% to 10% or less(1,2). The same reaction may be produced in washed red blood cells with dialuric acid(2,3). Alloxan itself is not hemolytic *in vitro* so it may be presumed that its action *in vivo* depends on reduction to dialuric acid by sulfhydril or other reducing compounds. *In vivo* or *in vitro* the blood cells of animals receiving adequate amounts of tocopherol are completely protected against the hemolyzing effect of dialuric acid. The *in vitro* hemolysis test requires only a few drops of blood and has proved to be a convenient method of studying some aspects of the requirement and utilization of tocopherol in the rat.

Experimental. The rats used in these studies were females of the Sprague-Dawley strain weighing about 70 g when they were received. Depletion studies were not begun until the rats weighed about 110 g. Until this time they were given the Steenbock stock diet(4) supplemented with milk and cabbage, and the animals referred to as "stock" or "normal" received this ration throughout the whole experimental period. The vitamin E-deficient diet contained casein (General Biochemicals, vitamin test), 20; lard, 38; cod liver oil, 2; salt mixture (U.S.P. No. 2), 4; sugar, 36; and in some instances yeast, 5 (replacing an equal weight of sugar). Each animal received a daily supplement of 20γ thiamine chloride, 100γ calcium pantothenate, 20γ pyridoxine, and 25γ riboflavin. In most of the experiments the vitamin E supplement was given in the form of mixed natural tocopherols (Distillation Products) dissolved in cottonseed oil (when massive doses were given) or in peanut oil. In one study a synthetic DL-alpha tocopherol (Merck) was used.

For the hemolysis test 8 or 10 drops of blood

* Supported in part by a grant of Hoffmann-La Roche, Inc., Nutley, N. J.

1. Gyorgy, P., and Rose, C. S., *Science,* 1948, v108, 716.

2. Gyorgy, P., and Rose, C. S., *Trans. N. Y. Acad. Sci.,* 1949, v52, 231.

3. Rose, C. S., and Gyorgy, P. In press.

4. Steenbock, H., *Science,* 1923, v58, 449.