2. Dewan, J. G., *Biochem. J.*, 1938, v32, 1378.
3. von Euler, H., Adler, C., Gunther, G., and Das, N. B., *Z. physiol. Chem.*, 1938, v61, 1938.
4. Adler, C., Gunther, G., and Everett, J. E., *Z. physiol. Chem.*, 1938, v14, 255.
5. von Euler, H., Adler, C., and Steenhoff-Eriksen, T., *Z. physiol. Chem.*, 1937, v248, 247.
6. Copenhaver, Jr., J. H., McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, 1950, v183, 73.
7. Potter, V. R., Pardee, A. B., and Lyle, G. G., *J. Biol. Chem.*, 1948, v176, 1075.
8. Landua, A. J., and Awapara, J., *Science*, 1949, v109, 385.
9. Needham, D. M., *Biochem. J.*, 1930, v24, 208.
10. Nakada, H. I., and Weinhouse, S., *J. Biol. Chem.*, 1950, v187, 663.
11. Awapara, J., *J. Biol. Chem.*, 1952, v197, 695.

Received May 8, 1953. P.S.E.B.M., 1953, v83.

Persistence of Amethopterin in Normal Mouse Tissues.* (20362)

JAMES R. FOUNTAIN,† DORRIS J. HUTCHISON,‡ GEORGIA B. WARING, AND JOSEPH H. BURCHENAL.

From the Division of Experimental Chemotherapy, Sloan-Kettering Institute, the Chemotherapy Service, Memorial Center for Cancer and Allied Diseases, and the Sloan-Kettering Division of Cornell University Medical College, New York City.

In a previous report(1) it was shown that following the intravenous administration of amethopterin (4-amino-N^{10}-methyl-pteroylglutamic acid) to normal mice in a single dose of 0.5 mg/kg, the greater part of the compound was rapidly excreted or metabolized within approximately 4 hours. The remainder was found to be concentrated mainly in those tissues (liver and kidneys) which have been reported(2) as containing relatively high concentrations of folic acid and citrovorum factor.

As a consequence of this observation it was of interest to determine the persistence of the antimetabolite in various tissues after the administration of amethopterin, by various assay schemes, and furthermore to establish the identity of the anti-metabolite.

Materials and methods. The mice used in this experiment were of the inbred AkR stock, weighing approximately 20 g. All were young mice of approximately the same age. Groups of mice of different sexes were used. Previous to and throughout the experiment the mice were fed the standard Purina Laboratory Chow diet. For convenience of description the mice used in this study are divided into 6 groups based on the administration of amethopterin. *Group I* (31 mice) received amethopterin intraperitoneally at a dose of 3 mg/kg on alternate days for a total of 3 injections. *Group II* (18 mice) received 1 mg/kg on the above schedule. *Group III* (10 mice) received a single intraperitoneal injection of 3 mg/kg, and *Group IV* (12 mice) was given 1 mg/kg intravenously on alternate days for 3 injections. *Group V* (18 mice) was given amethopterin as for Group I. Seventy-two hours after the last injection 2 mice were sacrificed, and thereafter at daily intervals all the remaining mice were given citrovorum factor (CF)§ intraperitoneally at doses ranging from 0.25 to 30.0 mg/kg. Mice were sacrificed daily 24 hours after last injection of CF. The procedures employed in obtaining and preparing tissues for assay were those previously reported(1) and the assay procedure was that of Burchenal *et al.*(3).

Group VI. For purposes of identification

* This investigation was supported by a research grant from the National Cancer Institute, of the National Institutes of Health, Public Health Service, by an institutional grant from the American Cancer Society, and by funds from the Damon Runyon Memorial Fund for Cancer Research, the Lasker Foundation and the Grant Foundation.

† Visiting Research Fellow of the British Empire Cancer Campaign and the American Cancer Society.

‡ Research Fellow of the National Cancer Institute.

§ Leucovorin (Lederle) supplied through the kindness of Dr. James Ruegsegger, Lederle Laboratory Division American Cyanamid Co., Pearl River, N. Y.

TABLE I. Concentration of Amethopterin (mγ/g) in the Liver, Kidneys, Spleen and Lungs of Mice over a 3 Week Period.

		Days after last inj.									
	Group	1	2	3	4	7	10	12	14	17	21
Liver (mγ/g)	I	338(3)	345(4)	410(3)	305(3)	384(2)	—	482(2)	427(2)	268(2)	426(3)
	II	294(4)	—	512(2)	—	408(2)	275(2)	432(1)	410(3)	—	375(2)
	III	332(2)	315(2)	—	309(1)	330(1)	368(1)	—	315(1)	—	284(2)
	IV	280(2)	—	412(2)	—	—	340(1)	350(1)	387(1)	261(1)	320(1)
Kidneys (mγ/g)	I	430(1)	453(2)	405(2)	430(1)	279(2)	—	327(2)	233(2)	218(2)	238(3)
	II	380(2)	320(2)	—	—	331(2)	260(1)	—	212(2)	—	101(2)
	III	—	—	—	290(1)	219(1)	231(1)	—	156(1)	—	126(2)
	IV	—	—	—	—	—	—	—	285(2)	380(1)	226(1)
Spleen (mγ/g)	I	0(1)	219(2)	140(1) 0(1)	0(1)	0(2)	—	0(2)	0(2)	0(2)	420(1) 0(2)
	II	0(2)	—	0(2)	—	0(2)	0(1)	—	0(3)	—	0(2)
	III	—	—	—	0(1)	0(1)	0(1)	—	0(1)	—	0(1)
	IV	—	—	—	—	—	—	—	0(2)	630(1)	0(1)
Lungs (mγ/g)	I	240(1)	80(2)	0(2)	0(1)	0(2)	—	0(2)	0(2)	0(2)	150(1) 0(2)
	II	150(1) 0(1)	—	0(2)	—	0(2)	0(1)	—	0(3)	—	0(2)
	III	—	—	—	0(1)	0(1)	0(1)	—	0(1)	—	0(1)
	IV	—	—	—	—	—	—	—	0(2)	475(1)	0(1)

Group I. Amethopterin 3 mg/kg × 3 i.p. alternate days. Group II. 1 mg/kg × 3 i.p. alternate days. Group III. 3 mg/kg i.p. single inj. Group IV. 1 mg/kg × 3 intrav. alternate days.

of the inhibitory substance in the liver, a series of mice was injected intraperitoneally with amethopterin at a dose of 3 mg/kg. These animals were sacrificed at ½ hour intervals up to 3 hours, then at 4, 5, 7, 17, 24, 48, 70 hours, 4, 8, and 14 days. Other mice not in this series were tested up to 8 months after injection. The liver was removed from each animal and treated as previously mentioned, after which the supernatant was concentrated *in vacuo* to approximately one ml. As controls for this experiment homogenates of 4 normal livers were prepared. Amethopterin (0.5 γ/g) was added to the first preparation, methopterin, N^{10}-methyl-PGA, (2 γ/g) to the second, aminopterin, 4-amino-PGA (1 γ/g) to the third, and to the fourth control was added a single preparation containing the 3 folic acid antagonists in the concentrations given above. These controls were boiled, clarified, and concentrated like the test samples. 10 μl of the liver concentrates were placed on Eaton-Dikeman No. 613 paper strips; these strips were developed for 6-68 hours in 3 different solvent systems.‖ After the strips were removed and air dried they were placed on large pyrex plates which contained Difco Folic Acid Assay agar plus PGA at 0.5 mγ/ml seeded with *S. faecalis* (ATCC 8043). The bioautographs of the inhibition zones were observed after an incubation of 16 hours.

Results. Table I shows the concentration of amethopterin in mγ/g of wet tissue in the organs of mice in Groups I-IV. Throughout the 21-day period mice in all groups showed the presence of amethopterin in the kidneys and liver. The levels remained remarkably constant, and the size of the dose bore no relationship to the amethopterin concentration. Similar results have been observed previously (1) following a single intravenous injection of 0.5 mg/kg and also in these experiments with a single dose of 20 mg/kg.

The serum was of interest in that amethopterin was detectable in all groups throughout the time of the experiments. The serum levels of Group I mice are shown in Fig. 1A, and similar levels were detected in Groups II-IV.

The serum level of amethopterin rose to significantly higher levels following the ad-

‖ 1% K_2HPO_4 aqueous(4)
5% NaCitrate and iso-amyl alcohol(4)
70% Iso-propanol, 10% NH_4OH, 20% distilled water. The paper strips were also buffered with 0.08 M Na_2HPO_4.

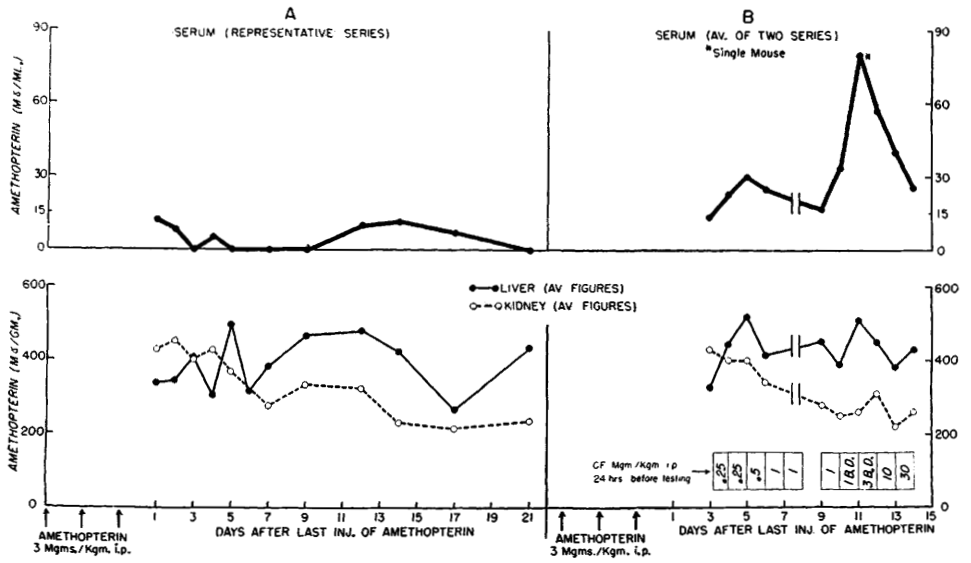

FIG. 1. Concentration of amethopterin in the serum, liver and kidneys of two groups of mice. (A) Amethopterin 3 mg/kg × 3 i.p., alternate days. (B) As for (A), followed by daily i.p. inj. of citrovorum factor.

ministration of CF (Fig. 1B). In order to be assured that the serum was relatively free of CF, it was not tested for amethopterin in the majority of instances until 24 hours following the injection of CF. The actual amount of CF administered did not appear to bear any relationship to the height of the amethopterin serum level.

It has been possible to identify the antimetabolite which persisted in the mouse liver and follow its course in a series of mice (Group VI). A bioautograph of a series of liver samples and of the clinical amethopterin (parenteral preparation) is presented in Fig. 2. Strip No. 1 shows the separation of aminopterin, methopterin and amethopterin, Strip No. 2 represents the particular sample of clinical amethopterin¶ that was injected into this series of mice. In the liver concentrates aminopterin was not detected after one hour and methopterin was present at 7 hours but not at 17 hours. It is possible that these 2 antifolics were excreted, but it is also possible that they cannot be detected because folic acid and CF have approximately the same Rf values as aminopterin and methopterin respectively. Amethopterin was detectable after 14 days, and in another series it could be identified by chromatography and bioautography after 8 months.

Identification of amethopterin in a liver taken 24 hours after injection is demonstrated in Fig. 3. Strip 1 shows the 3 spots representing aminopterin, methopterin, and amethopterin seen when these compounds were added to normal liver. Strip 2 shows the single spot found in liver of a mouse 24 hours after injection of amethopterin. When amethopterin was added to this liver preparation, only one spot was noted (Strip 3) but when methopterin (Strip 4) or aminopterin (Strip 5) was added a second spot appeared in addition to the original one representing amethopterin. Similar results have been obtained in the iso-amyl alcohol and K_2HPO_4 systems.

Discussion. The finding of 3 components in the amethopterin preparations complicates any metabolic study, but we have attempted to consider only the amethopterin content of tissues since neither of the other 2 components was detectable after 17 hours.

It has been noted that in normal volunteers (3) and in leukemic patients(5,6) variable

¶ Lederle Lot No. 7-8528.

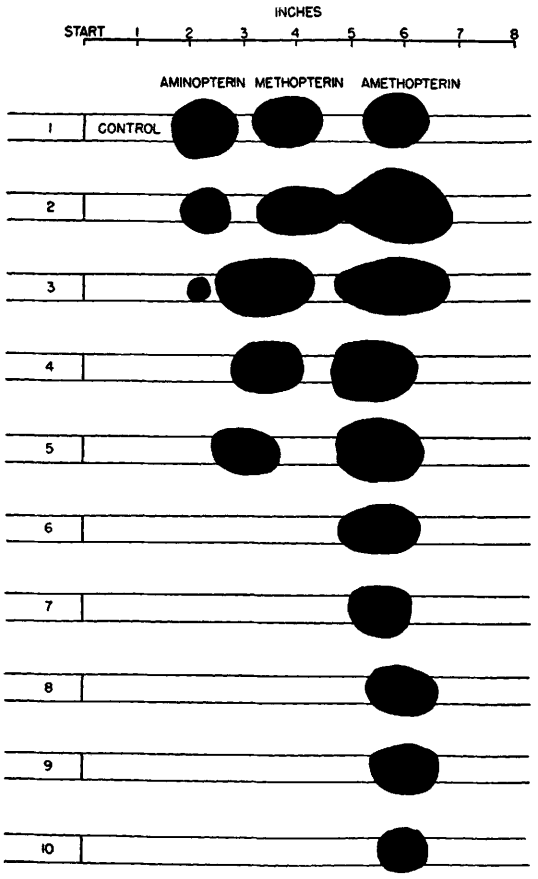

FIG. 2. Bioautographs of strips from alkaline iso-propanol system on *S. faecalis* (8043).

Strip 1. 10 μl of control liver concentrate which contained amethopterin, methopterin, and aminopterin (see text).

Strip 2. 100 mγ of amethopterin, Lederle Lot No. 7-8528.

10 μl of liver concentrates following administration of amethopterin (3 mg/kg).

Strip 3. After 1 hr
4. 2
5. 7
6. 48
7. 4 days
8. 8
9. 14
10. 8 mo

amounts of amethopterin or aminopterin cannot be accounted for by renal excretion alone.

It was evident from the experiments reported here that a relatively high concentration of amethopterin persists in tissues of the normal mouse for a period of 3 weeks and in one instance as long as 8 months in the liver. Moreover, it appeared that within the dosage range used in these experiments the mouse could rapidly eliminate within 24 hours all the amethopterin in excess of a threshold amount which is retained in the liver and kidneys. The effects of very large doses of amethopterin on tissue retention were not studied, as it was considered desirable to keep the dosage below the range which produces toxic manifestations(7) and within the chemotherapeutic range(8).

Although small quantities of amethopterin were found to be frequently present in the serum, there was little indication that any was being metabolized or excreted after the first 48 hours. Urine samples taken on several occasions at the time of sacrificing failed to show the presence of amethopterin.

In keeping with the hypothesis that antimetabolites function by excluding a structur-

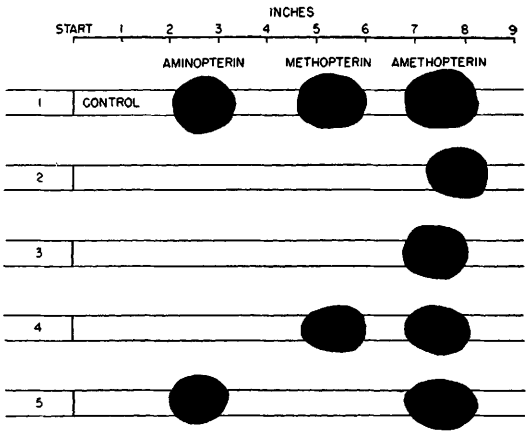

FIG. 3. Bioautographs of strips from alkaline iso-propanol system on *S. faecalis* (8043).

Strip 1. 10 μl n.l.h.* + 0.5 γ/g amethopterin (control).
15 μl n.l.h. + 2 γ/g methopterin (control).
10 μl n.l.h. + 1 γ/g aminopterin (control).

Strip 2. 10 μl l.c.* 24 hr after inj. of amethopterin (3 mg/kg).

Strip 3. 10 μl l.c. 24 hr after inj. of amethopterin (3 mg/kg) + 10 μl amethopterin control.

Strip 4. 10 μl l.c. 24 hr after inj. of amethopterin (3 mg/kg) + 15 μl methopterin control.

Strip 5. 10 μl l.c. 24 hr after inj. of amethopterin (3 mg/kg) + 10 μl aminopterin control.

* n.l.h. = normal liver homogenate; l.c. = liver concentrate.

ally related metabolite from combination with its enzyme(9) and thus preventing the formation of an enzyme substrate complex, it is probable that the amethopterin retained for such long periods of time is incorporated up to a relatively constant concentration in enzymes ordinarily associated with folic acid and CF, and any excess is rapidly excreted.

Summary. 1. Amethopterin given parenterally in various doses to normal mice resulted in the retention of a more or less constant amount of amethopterin in the liver for a period of at least 3 weeks. A decrease in the kidney concentration of amethopterin was noted. 2. CF at doses ranging from 0.5 to 30 mg/kg intraperitoneally failed to have any detectable effect 24 hours later on the concentration of amethopterin in the liver and kidneys, but caused a significant rise in the serum level of amethopterin. 3. The inhibitory substance present in the mouse liver 24 hours to 14 days after the administration of amethopterin has the same Rf value as amethopterin in 3 different solvent systems, and is therefore considered to be amethopterin.

We wish to acknowledge the technical assistance of Mrs. Elizabeth Robinson Hollands and the valuable assistance of Mr. V. M. Doctor in the chromatographic differentiation of amethopterin and methopterin.

1. Fountain, J. R., Waring, G. B., Hutchison, D. J., and Burchenal, J. H., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 193.

2. Dietrich, L. S., Monson, W. J., Gwoh, H., and Elvehjem, C. A., *J. Biol. Chem.*, 1952, v194, 549.

3. Burchenal, J. H., Waring, G. B., Ellison, R. R., and Reilly, H. C., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 603.

4. Wieland, O. P., Hutchings, B. L., and Williams, J. H., *Arch. Biochem. and Biophys.*, 1952, v40, 205.

5. Swendseid, M. E., Swanson, A. L., Miller, S., and Bethell, F. H., *Blood,* 1952, v7, 302.

6. Ellison, R. R., Murphy, M. L., Hutchison, D. J., and Burchenal, J. H., Unpublished observations.

7. Ferguson, F. C., Jr., Thiersch, J. B., and Philips, F. S., *J. Pharm. and Exp. Ther.*, 1950, v98, 293.

8. Burchenal, J. H., Burchenal, J. R., Kushida, M. N., Jonhston, S. F., and Williams, B. S., *Cancer,* 1949, v2, 113.

9. Woolley, D. W., *A Study of Antimetabolites,* John Wiley and Sons, Inc., New York, 1952.

Received May 11, 1953. P.S.E.B.M., 1953, v83.

A Colorimetric Method for Hyaluronic Acid Estimation.* (20363)

W. A. PIERCE, JR., R. H. STEELE, AND A. G. C. WHITE.

From the Departments of Microbiology and Biochemistry, Tulane University School of Medicine, New Orleans, La.

Several methods are available for the quantitative measurement of hyaluronic acid. The material can be precipitated, purified, dried and weighed. The viscosity of its aqueous solutions can be measured, although this property varies, depending upon the amount of depolymerization that occurs during isolation and purification(1). The observation that hyaluronic acid forms an insoluble complex with protein at or near pH 4.0 led to the development of the turbidimetric test for hyaluronic acid(2). Although this technique has been widely employed and is currently used (with or without modification) for the measurement of hyaluronidase activity(3-6), the necessity for measuring the optical density of a suspension in which turbidity is increasing with time is a limitation. Under test conditions ordinarily employed turbidity formation proceeds rapidly at first and then at a decreasing rate for 10 to 20 minutes. The time elapsing between the addition of the reagents and the measurement of optical density must therefore be carefully controlled. Furthermore, when large amounts of polysaccharide are present flocculation may occur. The optical density then increases at first

* This work was supported in part by a grant from the U. S. Public Health Service.