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Distribution of Amethopterin in Normal Mouse Tissues Following Intravenous Injection.* (19818)

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The antifolic activity of amethopterin (4-amino-N^{10}-methyl pteroyl-glutamic acid) has been well established, bacteriologically(1) and by animal experiments(2-3). It has been shown to be effective in prolonging the survival time of leukemic mice(4) presumably by an induced relative deficiency of pteroylglutamic acid (PGA) and citrovorum factor (CF) which retards the development of the leukemic process(5). In a similar manner it causes remissions in a significant percentage of acute leukemias in childhood(6-9).

Probably as a result of the lack of a satisfactory simple method for assaying amethopterin directly, little has been reported on the fate of this compound following its administration either to human subjects or to the experimental animal. Swendseid(10) using a back titration modification of the standard method for microbiological assay of PGA, has reported on the excretion of aminopterin (4-amino-pteroylglutamic acid) during treatment of leukemic patients. The recent development(11) of a simple disc method for determining the concentration of amethopterin in blood and urine led us to broaden the scope of this technic to assist in the study of the metabolism of this compound in the normal mouse. As a preliminary to these studies, the distribution of amethopterin in the tissues of the normal mouse following intravenous injection was determined.

Materials and Method. Young mice of the inbred Akm stock, weighing approximately 20 g each, were used. All the mice were maintained on a standard diet of Purina Laboratory Chow prior to and during the experiment. Water was allowed *ad libitum* throughout the experiment. Each mouse was given a single injection of amethopterin at a dose of 0.5 mg/kg by the intravenous route. This was considered an optimum dose, producing readily assayable amounts in the tissues and yet it was well below the maximum tolerated daily dose, and approximately 1/200th of the acute LD_{50} dose(2). Mice were sacrificed at 15, 30, 60, 120, 180, and 240 minutes after injection. Under ether anesthesia, blood was collected in a heparinized syringe by severing the axillary vessels, the animal being finally killed by decapitation. In those mice injected 1, 2, and 4 hours previously the liver, spleen, kidneys and lungs were then removed

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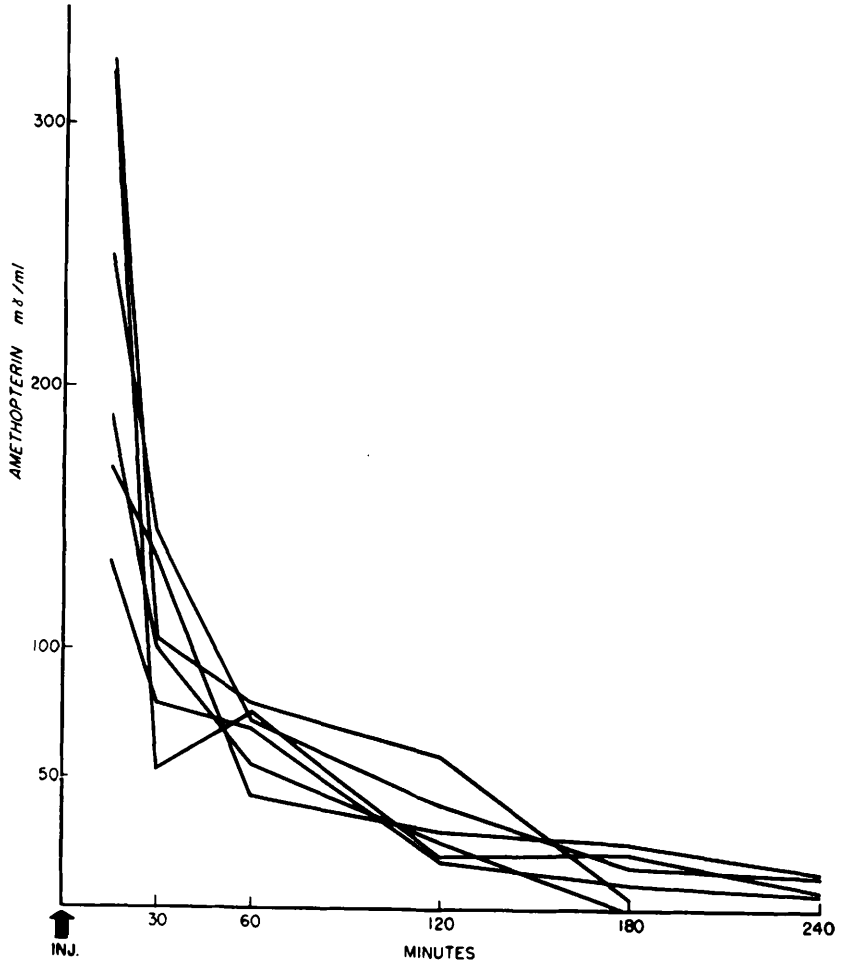

FIG. 1. Serum levels of amethopterin in normal mice after i.v. injection at .5 mg/kg.

and a sample of muscle taken from the hind leg. Each specimen was weighed and homogenized in distilled water immediately after removal.

Assay of Homogenate for Amethopterin Content. The samples were divided into 2 groups: (A) Unboiled, and (B) Homogenate boiled immediately for 5 minutes to destroy autolytic enzymes. The samples from group (B) were centrifuged and the supernatant assayed. In Group (A) the whole homogenate was assayed. Suitable dilution was carried out in both groups, following which 0.09 ml was delivered on to paper discs (Schleicher & Schuell penicillin assay discs, 12.7 mm diameter) placed on a pourplate of Difco folic acid assay medium containing 1 mγ/ml of PGA inoculated with *Streptococcus faecalis* (ATCC 8043). Following incubation over night the zones of inhibition were measured and calculated against standard zones of known concentration of amethopterin(11).

Results. Serum Levels. Fig. 1 shows the levels of amethopterin in the serum in 6 series of mice. It will be seen that after the first 30 minutes following injection the levels are closely related. In all cases the serum concentration has either reached or approximated zero in 4 hours.

Tissue Concentration. Table I shows the concentration of amethopterin in the tissues. It will be observed that much higher concentrations of amethopterin were assayable in the boiled specimens (B) in the case of the liver

TABLE I. Average Concentration of Amethopterin in Tissues of the Mouse Following i.v. Injection at .5 mg/kg Expressed in mγ.

Time following inj.	1 hr			2 hr			4 hr			24 hr	
	A	B		A	B		A	B		B	
		Content, mγ	mγ/g		Content, mγ	mγ/g		Content, mγ	mγ/g	Content, mγ	mγ/g
Liver (1–1.3 g)	424(6)	1400(6)	1215	0(3) 369(3)	868(6)	734	0(6)	628(6)	594	449(4)	410
Kidneys (.25–.35 g)	0(4)	227(6)	712	0(4)	162(6)	562	0(4)	120(6)	412	127(4)	384
Spleen (.07–.1 g)	0(3) 33(1)	20(6)	225	0(3) 22(1)	26(6)	273	0(3) 25(1)	20(6)	200	20(1) 0(3)	210 0
Lung (.15–.2 g)	0(3) 90(1)	0(3) 16(3)	0 96	0(4)	0(5) 15(1)	0 72	0(4)	0(5) 18(1)	0 84	0(4)	0
Muscle (.2–.25 g)	—	0(3) 5(1)	0 25	—	0(4)	0	—	0(3) 10(1)	0 40	0(4)	0

A—Unboiled specimens (mγ/organ). B—Boiled specimens (total content in mγ/organ and conc. in mγ/g).

Figures in parentheses denote No. of mice tested.

and kidneys and to a lesser extent in the spleen. This was readily explained by the fact that the enzymatic freeing of folic acid and CF from their conjugates was prevented by boiling. In the unboiled specimens (A) on the other hand, incubation resulted in freeing of folic acid and CF and hence reversal of some or all of the amethopterin activity. This was significant as it was an indication as to the folic acid and CF content of the tissue and suggested that the highest concentration was present in the liver and kidneys. Owing to the small quantity of free folic acid and CF present in tissues(12) it was presumed that the boiled specimens gave an accurate estimate of the amount of amethopterin present in the tissues. It can therefore be observed that the majority of the compound was present in the liver, followed in sequence by the kidney and spleen, while no significant quantities were found to be present in the lung after one hour and at no time in the muscle.

The retention of relatively large quantities of amethopterin in the liver and kidneys after 4 hours, at which time it had approximated zero in the serum, prompted us to determine the amount of the compound present after 24 hours. As may be seen, readily assayable quantities, little different from those found after 4 hours, remained.

Discussion. It was shown by this experiment that following the administration of amethopterin, it became concentrated in folic acid and CF-containing tissues. In addition it was observed that the highest concentration of amethopterin was found to be present in the liver followed in order by the kidney and spleen. This is of interest in view of the fact that a similar distribution of folic acid and CF has been demonstrated in the rat, guinea pig and chick tissues by direct microbiological assay(12). This tissue selectivity of amethopterin is not unexpected in view of the known properties of this compound, and supports the view that the antimetabolite replaces the metabolite (in this case amethopterin, and folic acid and CF) in the enzyme systems in which it is associated. A similar experiment has since been carried out in mice with advanced leukemia (Ak4). The results to date suggest a more widespread distribution and higher concentration of amethopterin in the tissues, which may be an indication of the affinity of the leukemic cell for amethopterin. In view of a correspondingly high serum level, however, the possibility of these findings being a consequence of renal dysfunction secondary to the leukemic process cannot be excluded.

Another finding of interest was the presence of amethopterin in significant quantities (ap-

proximately 6% of the injected dose) in the liver and kidney 24 hours after injection. Its presence was even more interesting since the serum level had almost reached zero within 4 hours of administration.

Previous studies(11) have shown that following oral or intramuscular administration to normal adult human subjects of amethopterin at a dose of 5 mg, 40-57% was excreted in 24 hours, the majority within 4-6 hours. Applying the knowledge obtained from the above experiment (Table I) it appears safe to presume that the remainder of the drug is metabolized or retained in the tissues. It is suggested from this experiment that following an injection of amethopterin most of the compound is rapidly excreted or metabolized within 4 hours, the remainder being concentrated in folic acid and CF-containing tissues. The continued presence of amethopterin activity in these tissues after 24 hours or more indicates a failure to complete the metabolism of amethopterin.

It will be noticed that we have referred to the substance in the tissues with growth-inhibiting properties for *Streptococcus faecalis* as amethopterin. Preliminary chromatographic studies suggest that this substance may indeed be amethopterin. However, the possibility that it may be the deaminated derivative, N^{10}-methyl-PGA, cannot be excluded at present as both have identical Rf values in the aqueous Butanol and acetic acid system and both are inhibitory to *S. faecalis*. In an attempt to reach a more definite conclusion as to its nature, however, further chromatographic studies are in progress.

Summary. 1. The concentration of amethopterin in the serum, liver, kidney, spleen, lungs and muscle of normal mice at intervals following intravenous injection of 0.5 mg/kg has been ascertained. 2. The relative concentrations of amethopterin in the tissue appeared to parallel the folic acid and CF content *viz.* liver, kidney, and spleen in descending order. No significant quantity of amethopterin was found in the lungs or muscle. 3. Amethopterin-like activity was found to be still present in the liver and kidney in relatively large quantities 24 hours after injection although the serum level had reached or approximated zero in 4 hours.

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