Effects of 4-Amino-N^{10}-Methyl-Pteroylglutamic Acid on the Extracellular Fluids of Rats.* (18743)

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(With the technical assistance of Patricia Theodore.)

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During studies of the actions of folic acid antagonists in mice, rats, and dogs(1-3), it was noted that animals receiving fatal doses reduced their food and water intake, developed severe diarrhea, and appeared dehydrated at death. Such observations suggested that lethal effects might be related to alterations in body fluids. Accordingly, this report presents measurements of the changes produced in rat extracellular fluid by 4-amino-N^{10}-methyl-pteroylglutamic acid (aminomethyl-PGA).

Methods. The rats (Wistar) were equally divided by sex and weighed an average of 201 g. Those used for study of fluid volumes varied in weight from 148 to 259 g, a range in which plasma volume and "thiocyanate space" have been shown to be linear functions of body weight(4). Non-sterile injections of aminomethylPGA were given intraperitoneally in a dose of 100 mg/kg (fatal to most rats in 3 to 7 days)(3). Three or 4 days later, animals manifesting diarrhea, dehydration and weight loss of about 15% were sacrificed. Of the injected animals, 54% were sacrificed, 15% died and 31% survived. Heart blood was obtained under ether anesthesia and heparin-coated syringes were used when whole blood or plasma samples were required. Colorimetric procedures were employed for determination of calcium (method of Roe and Kahn), phosphorus (Fiske and SubbaRow), sugar (Folin-Wu), and creatinine (alkaline picrate method). Sodium and potassium were measured with the flame photometer(5). Nitrogen values were obtained by micro-Kjeldahl digestion and distillation. Total CO_2 was measured by the method of van Slyke and Neill. A modified iodometric method was used for chloride measurements (6). Plasma and circulating red cell volumes and "thiocyanate space" were determined simultaneously with sodium thiocyanate (20 mg/cc) and Evans Blue (1.2 mg/cc) given in a volume of 0.25 cc/100 g weight into the exposed saphenous vein of etherized animals. After a 5-minute interval, considered adequate for complete mixing(7), blood was drawn from the heart for determination of hematocrit and plasma concentrations of thiocyanate and Evans Blue(7) and calculation of circulating red cell volume. For balance studies, rats were maintained over wire screens in funnel bottom cages. To prevent scattering, ground food pellets (GLF Lab. Chow) were mixed with 10% by weight of peanut oil. Rats were maintained several days under these conditions before tests were begun.

Results. Table I summarizes results of the various analyses performed with rats intoxicated with aminomethylPGA. Plasma volume and "thiocyanate space" were reduced 18% and 17% respectively. Inasmuch as measures of "thiocyanate space" provide a useful estimate of extracellular fluid volume(8), these results indicate that intoxicated animals suffered significant losses of extracellular water.

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TABLE I. Effects of AminomethylPGA and of Starvation on Extracellular Fluid of Rats.

Factor	Units	Control rats Mean	Control rats No. of tests	Control rats Stand. dev.	Test rats Mean	Test rats No. of tests	Test rats Stand. dev.	Difference of means % change	Difference of means Statistically significant‡
Effects of aminomethylPGA, 100 mg/kg, I.P.									
Thiocyanate space	cc/100 g†	28.	10	1.8	23.	13	1.8	−17	Yes
Plasma vol.	"	4.4	19	.6	3.6	22	.6	−18	"
Red cell "	"	5.3	19	.8	5.6	22	1.4	+6	
Sodium (serum)	m Eq./l	144	6	1.2	144	5*	2.1		
Potassium (serum)	"	4.7	6	.8	4.5	5*	.4	−4	
Calcium (plasma)	mg/100 cc	5.5	6*	.6	3.7	7*	.5	−33	Yes
Phosphorus "	"	4.1	6	.3	3.1	6	.3	−24	"
Chloride "	m Eq./l	92	6*	8.7	96	6*	11	+4	
Total CO_2 "	"	32	6*	2.9	30	6*	1.3	−6	
Protein (plasma)	g/100 cc	5.5	6*	.3	4.5	8*	.3	−18	Yes
N.P.N. "	mg/100 cc	38	6*	3.7	36	8*	6.5	−5	
Creatinine "	"	0	6		.6	6	.5		Yes
Sugar (blood)	"	89	6	4.5	94	6	45	+6	
Effects of complete starvation									
Thiocyanate space	cc/100 g†	27	12	3.7	24	15	2.1	−11	Yes
Plasma vol.	"	4.7	12	.6	4.1	15	.6	−13	"
Calcium (plasma)	mg/100 cc	4.2	6	.3	3.9	6	.2	−7	
Phosphorus "	"	4	6	.4	3.6	6	.3	−10	
Protein "	g/100 cc	6.3	6	.6	5.9	6*	1	−6	
Creatinine "	mg/100 cc	0	6		0	6			

* Tests done on pooled samples.
† Expressed in g body wt at time of sacrifice.
‡ $P \leq .05$ (Fisher "t" test).

The full extent of the reduction in "thiocyanate space" may have been obscured by the coincident decrease in plasma protein concentration, since plasma proteins are known to bind thiocyanate ion(8,9). The loss of extracellular water was in keeping with the appearance of dehydration. Total circulating red cell volume did not change significantly in intoxicated rats, either when calculated in terms of the final weight (as in Table I) or original weight.

Among the blood electrolytes, plasma calcium and phosphorus values were respectively 33% and 24% lower in intoxicated rats than in controls. Serum sodium and potassium, as well as plasma chloride and total CO_2 concentrations were unaltered by aminomethylPGA. It should be noted that this maintenance of normal concentrations in a reduced volume represented a loss of these electrolytes. As shown in Table I, no detectable amounts of creatinine were found in control plasma samples (the method used did not give colorimetric readings at concentrations less than 0.06 mg %). However, the plasma of all but one rat intoxicated with aminomethylPGA contained measurable amounts. The mean value for whole blood sugar was essentially the same in both groups of rats, although poisoned animals showed extreme variations. Non-protein nitrogen remained unchanged.

Further experiments were performed to elucidate the mechanism by which the losses of protein and water occurred. Table II shows the effects of aminomethylPGA (100 mg/kg I.P.) on the weight, food consumption, water intake, urine volume, and urinary excretion of nitrogen and chloride of 12 rats. Two died on the fourth day, 8 others died by the seventh day, and 2 survived. It is apparent that increased excretion of water and nitrogen did not occur. However, the food consumption was almost completely eliminated by the fourth day, even in those rats which survived the effects of aminomethylPGA. The water intake, urine volume, and urine nitrogen and chloride excretion were somewhat reduced after administration of aminomethylPGA. In a second series of rats, the voluntary food consumption was observed on 2 control days

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TABLE II. Effects of AminomethylPGA and Starvation on Metabolism of Rats.

	Control (mean of 2 days)	Test days 1	2	3	4
Effects of aminomethylPGA (100 mg/kg I.P.) on 12 rats*					
Food consumed (g/rat/day)	11.3	7.4	5.5	1.8	.8
Wt (g)	213	213	210	196	191
Water intake (cc/rat/day)	19.7	13.2	12.2	10.9	11.8
Urine vol. ''	2.3	2.1	1.6	3	1.9
'' N (mg/rat/day)	78	71	53	57	53
'' chloride (m Eq/rat/day)	.039	.059	.022	.017	.015
Effects of progressive food restriction on 12 rats*					
Food supplied (g/rat/day)	11	7.2	5.4	1.8	.8
Wt (g)	204	205	199	192	185
Water intake (cc/rat/day)	20	18.6	14.6	11.2	9.4
Urine vol. ''	2.3	2.1	1.9	2.1	1.9

* Rats studied in pairs.

and thereafter restricted to amounts proportional to the food intake *ad libitum* exhibited by the intoxicated rats. These rats manifested essentially the same effects on weight, water intake and urine volume as the rats given aminomethylPGA (Table II).

Inasmuch as anorexia was a predominant feature of aminomethylPGA-intoxication, it seemed necessary to assess the role of starvation in the production of the biochemical changes observed in poisoned animals. For this purpose rats were completely deprived of food, though permitted free access to water, for a 3-day period, sufficient to reduce their weight by 15%. This weight reduction, although comparable to that found in intoxicated animals at the time of sacrifice, was not accompanied by overt signs of dehydration. Those factors which had been altered by administration of aminomethylPGA were determined in these starved animals (Table I). The "thiocyanate space," plasma volume, and plasma protein, calcium and phosphorus were all reduced by starvation but only in the case of the fluid volumes were the reductions statistically significant. Plasma volume was reduced 13% and "thiocyanate space" 11%, compared to the reductions of 18% and 17% respectively, which followed administration of aminomethylPGA. Plasma creatinine was not elevated by starvation. An incidental observation in these starved rats was an elevation of total red cell volume, a finding not seen in poisoned animals and for which no explanation was apparent.

An attempt was made to alter the toxicity of aminomethylPGA by changes in the state of hydration of animals. Mice were used, since the lethal response of rats to single doses of aminomethylPGA was previously shown to be erratic(3). Daily intraperitoneal administration of 2 cc of isotonic saline solution (approximately half the daily water intake of control mice) failed to lower the mortality of an LD_{50} of aminomethylPGA. Similarly, abstinence from water for 26 hours prior to injection of aminomethylPGA did not increase the toxicity of an approximate LD_{10}.

Discussion. Rats poisoned with aminomethylPGA manifested weight loss, reduced consumption of food and water, and reduced urinary output. Of these effects, only the reduced food consumption seemed due primarily to actions of the drug. The restriction in food intake was noted in previous studies(3) but its extent not fully appreciated. Even rats with little other evidence of intoxication refused food almost completely. This effect may be related to the intestinal lesions produced by this compound(3).

The loss of plasma protein and at least part of the loss of body water may also be ascribed to the intestinal lesion. As previously described(1-3), anti-folic acid agents cause desquamation of the intestinal mucosa and production of an ulcerative enteritis. In many animals extravasation of plasma into the intestinal lumen was observed microscopically and significant amounts of protein and water were undoubtedly lost by this means. Certainly urinary excretion cannot account for the losses of water and protein. Although starva-

tion was productive of a mild dehydration, the results stated herein, together with the reports of others(10,11) indicate that the restricted food consumption associated with intoxication by aminomethylPGA cannot account for the observed protein and water losses.

The mechanism by which aminomethylPGA elevated plasma creatinine and depressed plasma calcium and phosphorus concentrations is obscure. Starvation did not duplicate these effects. Probably part of the lost calcium was that combined with the plasma protein extruded into the intestine.

It is difficult to evaluate the significance of the observed biochemical changes. Both the starvation and the dehydration induced by administration of aminomethylPGA seem compatible with life. The failure of forced hydration to prevent death in intoxicated mice further minimizes the significance of the dehydration. The loss of plasma protein may be of greater importance. It may be calculated from the observed changes in body weight, plasma volume and plasma protein concentration that approximately 40% of the initial plasma protein was lost during the period of intoxication with this drug.

It is of interest to compare the effects of the present folic acid antagonist with those of the nitrogen mustards. The actions of both groups of compounds are characterized by anorexia, weight loss, bone marrow damage, diarrhea associated with intestinal lesions, and delayed death. Previous investigators(12,13), studying the effects of the mustards in dogs, found that fatally poisoned animals lost significant amounts of body water and protein, principally from the intestinal tract. However, as concluded in the present study with aminomethylPGA, these changes were not considered sufficient to account for the lethal actions of nitrogen mustards.

Summary. Administration of 4-amino-N^{10}-methyl-pteroylglutamic acid to rats caused reduction of food consumption, extracellular fluid volume, plasma volume and plasma concentrations of protein, calcium, and phosphorus. Plasma creatinine concentration was elevated. Starvation alone did not duplicate the effects of this compound. Increased urinary excretion of nitrogen and water did not occur. The loss of body water and plasma protein appears to be the consequence of a previously described intestinal lesion produced by anti-folic acid agents. The relation between these biochemical changes and the mechanism of lethal action remains obscure.

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A Spontaneous Epizootic of Mouse Encephalomyelitis.* (18744)

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Two main groups of mouse encephalomyelitis viruses were described by Theiler(1,2). The TO type virus produces flaccid paralysis,

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