Production of Fatty Livers by 4-Aminopyrazolo-(3,4-d)-Pyrimidine. Toxicological and Pathological Studies.* (22769)

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Recently interest has been aroused in the possible use of the pyrazolopyrimidines as potential chemotherapeutic agents for the treatment of human neoplasms. Robins(1) synthesized a series of such compounds with the hope that the isomers of the naturally-occurring purines might be active. Subsequently the adenine isomer, 4-aminopyrazolo-(3,4-d)-pyrimidine (4-APP), was shown by Skipper (2) to be the most active of the series in inhibiting the mouse mammary adenocarcinoma 755 and in increasing the life span of mice with L1210 or L5178 leukemia. The results indicated that 4-APP might be worthy of clinical trial and accordingly pharmacological studies were undertaken in laboratory animals.

Procedure. Mice, rats, cats, and dogs were employed. The mice in all cases were male Swiss albino (Millerton Research Farms). Male CFW (Carworth Farms Wistar) rats were used for the toxicological studies while the pathological studies were carried out with male CFN rats (Carworth Farms Nelson) (3,4). The latter are Wistar rats which have been bred free of chronic murine pneumonia. Most of the toxicological, hematological, biochemical, and pathological methods employed herein have been described(5,6). As the 4-APP was found to be hepatotoxic, alkaline phosphatase in plasma was added to other previously described tests of liver function in dogs. The procedure of Bodansky with the modification of Gomori(7,8) was employed for this purpose. For administration of animals the agent was solubilized in H_2O by the addition of molar equivalent amounts of HCl.

TABLE I. Toxicity of 4-APP.

Species	Route of admin.	No. of successive daily doses	LD_{50}	19/20 confidence limits*	S*
			⌐mg/kg/day¬		
Mice	Intraper.	1	290	260–320	1.11
	"	5	25	18– 64	1.8
	Oral	1	180	116–279	2.4
	"	5	12.5	11– 15	1.20
Rats	Intraper.	1	228	127–410	1.54
	"	5	17.5	10– 32	2.21
	Oral	1	141	108–185	1.42
	"	5	18	15– 20	1.10
Cats	Intrav.	1	50–100	—	—
Dogs	Intrav.	10†	1.25–2.5	—	—
	Oral	10†	1.25–2.5	—	—

* Calculated according to Litchfield and Wilcoxon(9). Animals were observed for at least 2 wk after end of treatment.

† Maximum No. of doses given daily excepting weekends.

Toxicity in rodents. In Table I are presented LD_{50} data in mice and rats. 4-APP was shown to be of the same order of toxicity in both species and to be as toxic, or possibly more toxic, when given by the oral route than when injected intraperitoneally. The most prominent finding in the rodents given high single doses either by mouth or intraperitoneally was the appearance within two hours of blepharostenosis, blepharospasm and hypersensitivity. Of those given 400 mg/kg intraperitoneally all of the rats and most of the mice died unobserved overnight; the remaining mice lost weight progressively and died 12 to 13 days later. Single lethal doses given orally to mice (equal to or less than 400 mg/kg) resulted in delayed deaths between 3 and 14 days after intubation. In rats oral doses of similar magnitude caused death between 1 and 4 days. Weight loss was the only consistent finding associated with the delayed

* This work was supported by grants (C-415) from National Cancer Institute of N.I.H., U.S.P.H.S. and the Elsa Pardee Foundation.

The 4-aminopyrazolo-(3,4-d)-pyrimidine was kindly supplied by Dr. R. K. Robins, New Mexico Highlands University.

fatalities; an occasional animal appeared unkempt or showed diarrhea. Rats and mice which had received 4-APP on each of 5 successive days by either route of administration showed progressive weight loss beginning 24 hours after the first dose. Such animals became unkempt and occasionally the rats had "bloody" exudates about the nares. Most of the deaths occurred between the fifth and seventh days after initiation of treatment.

Pathological changes in rats. The lesions associated with intoxication in rats were studied in the CFN strain. For this purpose an initial group of 8 animals was injected intraperitoneally with 4 successive daily doses of 25 mg/kg/day. At 96 hours after initiation of this treatment the animals showed only a moderate weight loss and appeared less intoxicated than desired for the pathological studies. Accordingly they were not sacrificed and all eventually regained their lost weight and survived. (It is interesting to note that in the CFW strain this dose had proved to be greater than the LD_{50}. The lesser susceptibility of the CFN strain in this instance is in keeping with our recent experience comparing the toxicity of various agents in both kinds of Wistar rat.) A second group of CFN rats was then injected with 50 mg/kg/day for 4 days. Six of these were sacrificed at 96 hours at which time all showed severe weight loss and an untidy, unthrifty appearance. The remaining 2 rats were dead by the seventh day. The *gross findings* in all the 6 sacrificed animals were: an average weight loss of 20%, pale livers, small spleens and pallor of the renal cortex. Two showed dark purple adrenals. On microscopic examination a fat nephrosis was found in the kidneys as evidenced by slight vacuolization in the epithelium of proximal convoluted tubules. The vacuoles were primarily subnuclear and were shown to contain fine drops of fat in frozen sections stained with Sudan IV. A fine, diffuse cytoplasmic vacuolization was also seen in hepatic cells and shown to be fat by the Sudan IV stain. Except for the fatty change the livers appeared normal; there was no evidence of necrosis, inflammation or bile duct involvement. The sternal bone marrow showed an 85-90% loss of nucleated elements. In 2 animals recent cortical hemorrhages were present in the adrenals and in one of this pair the medulla was similarly involved. The adrenals of a third animal showed only medullary hemorrhages. In all 6 animals the red pulp of the spleen was reduced in amount and, though the thymus was involuted, the other lymphoid tissues showed no significant microscopic changes. No alterations were observed in the intestines or in the other viscera studied.

Toxicity in cats. Three respective pairs were given single intravenous doses of 100, 50, and 25 mg/kg. The highest dose caused immediate urination, salivation, hyperpnea and panting, a sluggish pupillary reflex, and relaxation of the nictitating membrane. At 5 minutes one of the pair given this dose had slight convulsive movements and was unable to stand. Both cats vomited several hours after injection and at 6 hours, though hyperpnea and salivation had ceased, the animals appeared moderately depressed. Thereafter depression and ataxia were evident until the animals died, one during the second day and the other during the third day. Except for retching and vomiting at 3 to 4 hours after 50 mg/kg the two lower doses produced no significant signs of intoxication and all 4 recipients survived the 3-week period of observation.

Toxicity in dogs. Each of the following lethal doses were tested in a pair of animals: 10, 5, and 2.5 mg/kg/day intravenously and 5 and 2.5 mg/kg/day by mouth. The 2 given 10 mg/kg/day died at 5 and 6 days after initiation of treatment; 7 of the remaining 8 died between 7 and 16 days; the 1 survivor had received 5 mg/kg/day by mouth. During the course of intoxication weight loss, anorexia, and vomiting appeared in all animals between the second and fifth days after initiation of treatment. Small ulcerations of the buccal mucosa were noted in 2 of the 10 dogs while 2 others showed patches of slight congestion in this area. Increases in fluid intake and in urine output with a concomitant fall in the specific gravity of urine (to 1.010 or less) were noted in 8. Nine developed loose stools by the third day and 4 of these showed tarry stools with evidence of blood by the

end of the first week. Four others had tarry stools on the day of death. Jaundice was present at 7 to 10 days (as evidenced by a yellow cast in the sclera and buccal mucosa) in 2 of the animals given 2.5 or 5 mg/kg/day intravenously. There was no jaundice seen in any of the dogs treated orally with 4-APP. Body temperatures remained normal throughout the course of fatal intoxication except for hypothermia 24 hours before death in the 2 given 10 mg/kg/day intravenously. Pairs of animals were also given doses of 1.25 mg/kg/day and 0.63 mg/kg day, either orally or intravenously; all of these dogs survived and showed no untoward signs.

Pathological changes in dogs. Table II presents hematological and biochemical findings in a group of 4 dogs treated with intravenous doses of 4-APP. Dogs 1 and 2 received 5 mg/kg/day for 4 consecutive days while dogs 3 and 4 were given 9 successive daily injections of 2.5 mg/kg/day excepting weekends. All 4 animals were sacrificed 24 hours after the last injection. The course of intoxication in each was like that described above.

All developed reticulocytopenia and a depression of the nucleated cell count in bone marrow as seen in samples aspirated from the iliac crest. In 3 of the animals hemoconcentration was present at the time of sacrifice; in dog 4 an increased hematocrit was seen at 7 days. Significant lymphocytopenia was noted in all dogs; conversely 3 showed a neutrophilic granulocytosis. Dogs 3 and 4, which had been subjected to the more prolonged course of intoxication, had decreased numbers of circulating platelets by the eleventh day. Both dogs receiving the higher dose (5 mg/kg/day) had an obvious defect in blood coagulation as evidenced by the marked prolongations of clotting times (Table II). This disturbance was found to be associated with deficiencies of 2 of the components of the blood clotting mechanism. In Table II it will be seen that prothrombin times became greatly elevated and simultaneously the fibrinogen contents of plasma decreased significantly. Dogs 3 and 4 which had received 2.5 mg/kg/day shown no significant changes in clotting time but did develop increased pro-

TABLE II. Hematological and Biochemical Findings in Dogs Given 4-APP.

Dog	Day	Wt (kg)	Herit. (ml/100 ml)	Retic. (%)	PMN-N	Leyte	Pltlt.	BMNC	Clot time (min.)	Fbgen	Blrbn	BSP	Prot. time (sec.)	Alk. ptase (mg %)
					———	(10^3 cells/mm³)		———			(mg %)	———		
1	0	12.3	57	.1	7.8	1.4	134	170	5.8	216	.3	.2	13.6	.3
	4†	10.2	77	0	22.3	.2	192	24	>22.0	68	1.2	2.8	52.1	4.7
2	0	10.7	47	.1	5.2	1.1	190	126	3.8	305	.2	.3	12.4	2.5
	4	9.2	56	0	21.6	0	164	16	22.0	*	3.9	—	>120	7.8
3	0	9.8	52	.2	14.6	1.1	432	211	4.5	—	(1)	.1	15.4	(2)
	7	7.9	61	0	10.5	.2	262	24	6.8	—	—	—	16.3	—
	11†	7.1	59	0	13.6	2.1	106	—	8.5	—	.5	1.3	35.2	2.1
4	0	15.9	47	.8	8.8	2.3	206	224	5.0	—	(1)	.2	12.6	(2)
	7	13.7	53	0	9.6	3.8	218	61	5.3	—	—	.3	12.1	—
	11	13.5	47	0	16.1	.7	34	41	6.8	—	.4	.8	18.1	3.6

Abbreviations: Alk. ptase, plasma alkaline phosphatase; Blrbn, total plasma bilirubin; BMNC, bone marrow nucleated cells; BSP, bromosulfalein retention in blood; Clot., clotting; Fbgen, plasma fibrinogen; Herit, hematocrit; Leyte, lymphocytes; Pltlt, platelets; PMN-N, polymorphonuclear neutrophils; Prot., one-stage prothrombin; Retic., reticulocytes.

* The oxalated plasma failed to clot upon addition of $CaCl_2$ and thrombin. † Dogs 1 and 3 were moribund at sacrifice.

(1) Avg control value for 49 dogs = .2 ± .1; (2) avg control value for 30 dogs = 1.3 ± .9.

thrombin times. No plasma fibrinogen determinations were done in these dogs.

Dogs 1 and 2 had significant increases in plasma bilirubin. In dogs 1, 3 and 4 in which measurements were made on the day of sacrifice bromosulfalein (BSP) retention was found to be elevated. The extent of this elevation in dogs 1 and 3 may have been exaggerated by the concomitant hemoconcentration and moribund state of these animals. Changes in plasma alkaline phosphatase were not impressive.

In additional biochemical analyses, which are not recorded in Table II, plasma protein and blood glucose remained relatively constant. Non-protein nitrogen in plasma rose from initial values of 15-25 mg % to 32-59 on the day of sacrifice. Dogs 2 and 3 which had developed hemoconcentration also showed significant decreases in plasma chloride (from 116 and 113 to 100 and 105 mEq/l respectively; unfortunately this analysis was not carried out in plasma from dog 1.

On *microscopic examination* the most consistent findings were in the livers of all 4 dogs. In dogs 1 and 2 which had been given the higher daily doses of 4-APP a fine diffuse cytoplasmic vacuolization was present in the hepatic cells; in frozen sections Sudan IV stain revealed this to be fat. In addition both livers showed plugging of the bile canaliculi; one had a slight cholangiolitic proliferation. In neither animal was there evidence of any necrotic or inflammatory changes in the liver. Other significant findings in dogs 1 and 2 were recent hemorrhages in the adrenal cortices of both and in the medulla of one. One of this pair also had hemorrhages in the myocardium, lungs, and small and large intestines. The bone marrows of both were congested but the nucleated elements appeared normal. Dogs 3 and 4 which had received the lower daily doses also had fatty livers but to a lesser extent than did 1 and 2. Again neither necrotic nor inflammatory cells were present; in this pair abnormalities were not noted in the bile ducts and canaliculi. As in dogs 1 and 2 sections of vertebral and sternal bone marrow revealed marked congestion but again the number of nucleated elements was within normal limits. No pertinent changes were seen in the kidney, intestine, spleen, or any of the other viscera studied.

Discussion. Of the purine and purine analogs which have been studied toxicologically (10) 4-APP, an isomer of adenine, is the only one known to cause fatty livers in rats and dogs although 2,6-diaminopurine has been found to induce fatty metamorphosis in pigs (11). The present study has shown that in dogs the fatty change may be accompanied by stasis of bile in the canaliculi and by evidence for hepatic insufficiency, that is, elevations in the one-stage prothrombin time and decreases in the fibrinogen content of plasma (12,13). Such changes appear in conjunction with and probably account for the blood coagulation defect. Jaundice and increased plasma bilirubin may also be seen; the hyperbilirubinemia may be related to the intrahepatic biliary stasis.

The hepatic changes produced by 4-APP can be distinguished from those caused by a close analog, 6-mercaptopurine(5,10). The latter induces hepatic necrosis in mice and rats without fatty alteration. In dogs the same agent produces stasis of bile in canaliculi similar to, but more extensive than, that caused by 4-APP; there is no evidence for fatty metamorphosis. Interestingly, the changes in tests of hepatic function in dogs intoxicated with 6-mercaptopurine are those expected to result from biliary obstruction: hyperbilirubinemia, increased retention of BSP, and marked rise in plasma alkaline phosphatase. Prothrombin time, however, remains unaltered(10).† In contrast the prominent sign of hepatic insufficiency resulting from 4-APP is the disturbance of the prothrombin complex.

Microscopic study revealed no significant change in the kidneys of dogs though fat nephrosis was observed in lethally intoxicated rats. These observations are in contrast to those previously reported with adenine, 2-chloroadenine and purine(10). Intoxication of rats with the last-mentioned purines is characterized by the deposition of occlusive

† Unpublished.

crystals in the renal tubules.

Rats given 4-APP also showed a significant decrease in the content of nucleated cells in the bone marrow. In intoxicated dogs, though bone marrow aspirated from the iliac crest contained decreased numbers of nucleated elements, microscopic study of decalcified sections revealed marked congestion in the marrow capillaries but neither decreases nor abnormalities in hematopoietic elements. It is possible that the aspirated marrow appeared depleted due to dilution with blood from the congested capillaries.

Adrenal cortical hemorrhages were found in several of the rats and dogs which had been sacrificed for pathological study. The significance of these lesions is obscure; but their presence is interesting in light of the finding that 4-APP also induces hypochloremia and polyuria in dogs.

Summary and conclusions. The toxic effects of 4-aminopyrazolo-(3,4-d)-pyrimidine (4-APP) have been studied in mice, rats, cats, and dogs. The agent was found to produce a fatty change in liver which was associated neither with necrosis nor with inflammation. Hepatic insufficiency was evidenced by hyperbilirubinemia, increases in one-stage prothrombin time and decreases in plasma fibrinogen. In rats fat nephrosis and depletion of hematopoietic elements in bone marrow were also seen. Hepatic insufficiency should be anticipated as a possible hazard in clinical trials with 4-APP.

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Received July 30, 1956. P.S.E.B.M., 1956, v93.

Demonstration of a Marked Bence-Jones Proteinemia.* (22770)

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Previous reports of Bence-Jones proteinemia have been founded upon a variety of observations: precipitate formation in multiple myeloma sera when heated to 55-60°C(1-3); aberrant serum protein solubility characteristics on salt fractionation(4); undemonstrated but postulated identity of a crystalline substance from a myeloma serum with crystals in the corresponding Bence-Jones protein containing urine(5); appearance of a new or elevated serum peak in electrophoretic pattern which closely corresponds in mobility to that of urinary Bence-Jones protein(4); immunological cross reaction with and blocking of antibody to Bence-Jones protein by the myeloma serum(6); and demonstration of slow sedimenting serum components which are electrophoretically in the γ- and β-globulin areas(6).

Most of these criteria can not be considered

* This work was supported in part by a research grant (C-1786(C)) of the N.I.H., U.S.P.H.S.