group attached to such a substance, whose effects are minimized by dilution. One point in Fischer's data might lend support to the idea of such a toxic factor. Fischer reports that either egg albumin or insulin, when added to dialyzed plasma, will protect cells placed thereon from histolytic disintegration. He attributes this effect to the nutritive properties of the labile S-S and S-H bonds in these materials, but both egg albumin and insulin are known to be highly active adsorbents capable of detoxifying various media. This appears to be the effect of serum albumin when added to media for the cultivation of bacteria[10] and may have been a factor in Spratt's experiments[11] with explanted early chick blastoderms (not tissue cultures). It seems quite possible that the beneficial effect of egg albumin or insulin when added to dialyzed plasma may be of similar origin. On the other hand, the defect might be due to an imbalance of nutritive materials. The plasma and embryo juice were dialyzed against a Ringer's solution containing 0.1% dextrose but lacking phosphate. The diluent used in preparing media (Tyrode's solution) contained no dextrose but did contain phosphate. The diluent was thus *not* in equilibrium with the dialyzed materials, and would both extract dextrose therefrom and add phosphate thereto. If 0.1% dextrose was too high a concentration for fibroblast growth, the defect would be corrected by dilution. Since phosphate is required for growth, the defect might be a simple deficiency as postulated by Fischer, but not necessarily a deficiency of organic materials. However, Fischer's evidence, that a suitable amino-acid mixture prepared with physiological salt solution rather than Tyrode's solution does, in fact, partially correct the defect of dialyzed plasma-embryo juice, shows that phosphate was not the critical factor. It shows also that the low molecular weight materials removed by dialysis, such as the amino acids, *are* of great and at this level critical importance.

All of this evidence, however, still leaves unresolved the question of the nature of the postulated positive toxic defect in the dialyzed materials. In any case, our observations, though limited, suggest that it is unsafe to assume that dialyzed plasma or embryo juice is truly inert. Until further experiments have been carried out to clarify the effects of dilution on dialyzed extract, we feel that these materials should not be used in the preparation of substrata on which to test the nutritive value of supernatants. We believe that bare glass or cellophane are still the only really safe bases for such studies.

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17232. Effects of 4-Amino-Pteroylglutamic Acid in Dogs with Special Reference to Megaloblastosis.*

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Experimental production of macrocytic anemia and megaloblastosis of bone marrow has been achieved by administration of diets deficient in folic acid (PGA). Miller and Rhoads[1] observed in dogs fed the Goldberger

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† Post-doctorate fellow of the National Cancer Institute; Neale Research Pathologist, University of Adelaide. S. Australia.

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diet macrocytic anemia with megaloblasts in the purple marrow, leucopenia and diarrhea. The same authors described later a hypercellular, megaloblastic marrow in pigs receiving a similar diet.[2] Wills and Stewart[3] reported an experimental macrocytic anemia with megaloblasts in bone marrow in their classical studies with monkeys fed purified diets. In the light of present knowledge the diets employed in the above studies were probably deficient in PGA.[4-9] Subsequent studies in pigs receiving PGA-deficient diets supplemented with a folic acid antagonist, x-methyl-PGA, and sulfasuxidine provided further evidence of the role of folic acid in the maintenance of hematopoiesis. The addition of x-methyl-PGA enhanced the development of macrocytic anemia and leucopenia which responded rapidly to PGA and its conjugates. The bone marrow of deficient pigs showed hyperactive erythropoiesis with megaloblastosis similar to that seen in pernicious anemia of man in relapse.[10,11] With the synthesis of 4-amino-pteroylglutamic acid (4-amino-PGA)[12] an antagonist was provided which proved more potent and more rapid in inducing signs of folic acid deficiency in mice and rats[13-16] than x-methyl-PGA. In view of this finding it was of interest to determine whether the administration of 4-amino-PGA to dogs would also elicit signs of PGA-deficiency similar to the syndrome described by Miller and Rhoads.[1]

Procedure. Seventeen adult, mongrel dogs were used (Table I). The antagonist‡ was prepared for daily use by dissolving with 2 molar equivalents of $NaHCO_3$ or NaOH to achieve a final pH of less than 8.0. Complete hematological examination including volumetric estimations[17] of blood drawn from the jugular vein into heparinized syringes and of sternal bone marrow aspirations were carried out prior to administration of 4-amino-PGA and at intervals thereafter. The animals were anesthetized briefly with 10 to 20 mg/kg pentothal sodium, intravenously, to facilitate sampling of bone marrow. Blood and marrow films were stained in Jenner-Giemsa buffered at pH 6.85. At autopsy marrow was collected from sternum and femur, smeared, and sectioned. Tissues were taken from all internal organs except nervous system, fixed in Vandergrift's, sectioned, and stained with H and E and Giemsa. In 10 dogs plasma chloride[18] and total nitrogen and NPN in trichloracetic acid filtrates using the micro-Kjeldahl procedure and blood glucose[19] were followed. Plasma protein was calculated to be 6.25 times protein nitrogen.

Clinical course. In dogs 1 through 9 (Table I) receiving single, large doses of 4-amino-PGA no adverse signs were noted until after

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TABLE I.
Effect of 4-Amino-PGA on Cells in Blood and Bone Marrow of Dogs.

Dog	Dosage mg/kg/day	Dosage No. of inj.	Day	Wt, kg	Herit., %	PMN	MNC	Differential count of nucleated cells in bone marrow: Myeloid, %	Lymphoid, %	Erythroid, %	Differential count of nucleated erythroid cells in bone marrow: Nuclear remnants, %	Normoblasts, %	Erythroblasts, %	Megaloblasts, %	Primitive stem cells, %
			0	18.0	45.0	6,950	1,650	38	5	57	10	90	0	0	0
1	56iv	1	3S	16.3	52.0	700	250	32	5	63	29	50	11	10	0
								19F	2F	79F	50F	23F	10F	17F	0F
2	37iv	1	0	16.2	28.2	14,200	2,500	47	10	43	10	90	0	0	0
			3S	14.8	33.0	2,150	300	29F	21F	50F	51F	24F	0F	25F	0F
4	20iv	1	0	14.1	44.3	6,700	550	89	3	8	6	90	4	0	0
			3S	11.7	60.0	650*		69	3	28	15	34	10	41	0
			0	12.2	32.9	12,900	1,900	69	8	23	18	80	2	0	0
5	20iv	1	3S	11.1	47.0	4,150	200	78	11	11	26	62	4	8	0
								18F	8F	74F	51F	28F	2F	19F	0F
			0	12.1	32.5	14,600	950	58	3	44	15	80	5	0	0
6	20iv	1	3S	10.9	37.0	4,000	450	78	11	11	26	54	4	8	8
								54F	8F	38F	32F	11F	7F	44F	6F
7	10iv	1	0	13.0	46.0	13,100	1,600	65	6	29	3	96	1	0	0
			4S	10.2	58.0	900	300	23	pr	77	29	44	2	25	0
			0	20.9	41.0	14,600	1,100	66	8	26	2	98	0	0	0
8	10iv	1	3	18.3	46.7	5,750	650	68	9	23	28	43	8	21	0
			4S	16.6	60.5	1,850	100	45	2	53	24	40	4	0	32
9	5iv	1	0	16.7	29.2	12,400	2,350	72	4	24	10	90	0	0	0
			3S		43.1	11,700	0	83	7	10	31	43	16	10	0
10	0.1im	5	0	10.5	47.0	19,800	1,250	80	7	13	6	92	2	0	0
			5S	8.5	60.5	9,600	2,100	28	15	57	17	53	0	0	30
12	0.1im	5	0	10.1	36.5	16,600	4,200	40	3	57	5	94	1	0	0
			5S	7.9	58.0	1,600	550	38	7	55	39	36	0	25	0
	0.05im	14	0	11.3	30.1	10,400	650	46	7	47	13	86	1	0	0
13	0.1im	8	14	11.8	39.0	14,200	750	39	6	65	3	97	0	0	0
			21	10.6	43.0	7,350	400	82	2	16	28	72	0	0	0
			24S	9.1	50.0	3,700	100	87F	3F	10F	55F	45F	0F	0F	0F

14	0.05im	13	0	10.6	51.0	5,550	1,500	48	7	45	19	79	2	0	0	
			9	10.2	48.0	4,750	600	73	1	26	13	77	5	5	0	
			13†	9.2	59.5	11,100	100	81	8	11	2	70	25	0	3	
15	0.05im	13	0	10.6	41.0	7,450	550	54	3	43	10	90	0	0	0	
			9	8.9	39.0	8,950	1,250	82	7	11	26	67	1	0	6	
			13†	7.9	62.0	2,700	200									
16	0.05im	8	0	9.7	44.4	7,850	700	84	6	10	20	76	4	0	0	
			7†	8.2	55.0	9,600	400	97	3	pr	8‡	12‡	16‡	0‡	0‡	
17	0.05im	6	0	9.3	30.5	12,200	400	14	5	81	12	88	0	0	0	
			6S	9.1	46.0	6,850	600	87	5	8	50	25	15	5	5	
								92F	2F	8F	56F	21F	0F	9F	14F	

* Total leucocyte count per cmm.
† Dogs 14, 16, and 16 succumbed during the 14th, 14th, and 9th day, respectively.
‡ Only 25 cells counted.

F, femoral marrow.
Herit, hematocrit.
im, intramuscularly.
iv, intravenously.
MNC, lymphocytes and monocytes per cmm.
PMN, polymorphonuclear granulocytes per cmm.
pr, present.
S, sacrificed.

the first 24 hours when the animals refused food. Diarrhea began shortly after 48 hours. At first yellow fluid, later progressively darker, bloody, and more copious stools, and finally gross blood clots were passed almost continuously. Loss of weight, evident by 48 hours, progressed until time of sacrifice (Table I) when all animals were severely weakened or in coma and dehydrated in appearance. Dog 3 (not shown in Table I) was found dead at 72 hours. In dogs 10 through 17 receiving repeated doses of 4-amino-PGA (Table I) a more prolonged though otherwise similar course was observed. Dog 11 (not shown in Table I) was found dead at 96 hours after receiving 4 daily intramuscular injections of 0.1 mg/kg.

Results. The increased values of the hematocrit noted in Table I confirmed the impression of terminal dehydration. Consistent with this finding was the fact that at time of sacrifice plasma chloride was found decreased 8 to 25 m eq/l below initial values. Simultaneously plasma protein had increased in 8 of 10 dogs. The moribund state of animals 4, 12, and 15 was reflected in elevated plasma non-protein nitrogen, *i.e.*, 92, 107, and 84 mg %, respectively. Changes in blood glucose were not significant.

Neither anemia nor significant changes in erythrocyte volume or content of hemoglobin were observed in animals at any time during the course of intoxication or time of sacrifice.

Reticulocytopenia developed in all but dogs 1 and 8. The peripheral leucocyte count fell in all dogs except 14 and 16 which had granulocytosis due to ascending infections from damaged intestinal tracts. The respective decreases in polymorphonuclear and lymphocytic cells may be seen in detail in Table I.

As noted in aspiration smears and tissue sections of sternal and femoral marrow the total cellularity of the bone marrow decreased involving reduction of both myeloid and erythroid elements. In dogs receiving single, large doses of 4-amino-PGA depletion of bone marrow could be detected after 24 hours and was marked after 48 hours. After 72 to 96 hours only small islands of active hematopoiesis could be found in the fatty, fluid marrow. Differential counts of all nucleated cells and sep-

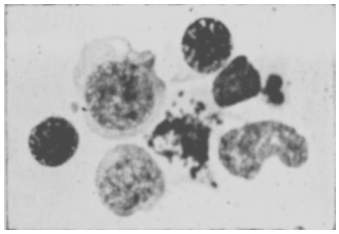

FIG. 1.
Bone marrow aspiration of dog 7 four days after 10 mg/kg of 4-amino-PGA, intravenously. Two megaloblasts, a nuclear "explosion," and a normoblast with nuclear remnant are included in field.

arate differential counts of nucleated erythroid cells ars listed in Table I.

Lesions in myeloid tissues. Peripheral granlocytopenia was associated with depletion of metamyelocytes and neutrophils in bone marrow. However, myelocytes and myeloblasts were uniformly present often maintaining their initial relative proportions in spite of general depletion of myelopoiesis. Only dogs 14 and 16 evidenced active myeloid regeneration. In some cases nests of eosinophils were found in depleted marrows similar to previous findings in rats[16]—an observation still unexplained. Of additional interest was the appearance of giant metamyelocytes, not present prior to administration of 4-amino-PGA, and an increased proportion of hypersegmented neutrophils.

Thrombocytoblasts decreased in numbers with general marrow depletion but were not specifically affected.

Lesions in erythroid tissues. Within 24 to 48 hours after large single doses numerous mature normoblasts were seen to be in pathological mitosis with disintegration of nuclei, chromosomal segregation, and chromatin expulsion into the cytoplasm often simulating nuclear explosions. This process resulted in increased numbers of erythrocytes with nuclear remnants (Fig. 1). During the same period many of the remaining normoblasts and erythroblasts underwent a change in nuclear pattern. The naturally lumpy chromatin was transformed into a distinct network. In the more primitive erythroid elements with basophilic cytoplasm a purple parachromatin appeared consistent with the structure of megaloblasts. Later even hemoglobinized cells with parachromatin were observed. After 48 to 72 hours the proportion of normoblasts was much reduced. In dog 6, for example, at this stage 44% of nucleated erythroid elements in femoral marrow showed nuclear parachromatin and 32%, nuclear remnants. Later with continued depletion primitive stem and sinus endothelial cells become prominent in smears and sections (dogs 6 and 8).

In dogs 10 through 17 megaloblasts were observed in only 3 cases. However, depletion of hematopoiesis was extreme in all dogs receiving repeated injections and megaloblasts might have been present at some stage of intoxication when marrow samples were not taken. The lesions found in dogs 10 through 17 were otherwise identical to those described above.

Lymphoid tissues of spleen, mesenteric nodes, and intestinal tract appeared to be reduced in quantity but no pyknosis or necrosis was seen. By comparison to depleted bone marrows lymphoid tissues were relatively unaffected.

Lesions of the digestive tract were confined to jejunum, ileum, and colon. In all animals at autopsy marked edema and swelling of the mucosa was found throughout small and large intestines. Villi were thickened and blunted by capillary dilatation near the tips. The cytoplasm of superficial epithelial cells was enlarged. In almost the entire colon and ileum epithelium had desquamated leading to denudation of villi and superficial ulceration. In glands and crypts there was evidence of cytoplasmic enlargement and desquamation of epithelium with abnormal regeneration of cells containing irregular nuclei and cytoplasm. Lumina of the deeper glands were frequently filled with plugs consisting of desquamated epithelium and leucocytes. The villi appeared to be matted together resulting from loss of superficial portions and infiltration with leucocytes and fibroblasts below ulcerated areas.

The widespread involvement and necrosis can in large part be attributed to a primary action of 4-amino-PGA on intestinal mucosa. Intestinal damage probably developed early

in intoxication and fully explains the bloody diarrhea which was evident 1 to 2 days prior to sacrifice and before hematocrit values were significantly elevated in dogs receiving acutely lethal doses. Ileitis and colitis in the present animals resembled findings in rats in which microscopic evidence of intestinal damage was detected as early as 6 hours after poisoning with fatal doses although death did not ensue for 3 to 4 days.[16] The pathology of the gut was judged to be more extensive and severe than the focal ulceration known to occur in dogs dying of shock.[20-22] Furthermore, unlike the secondary sequellae of local vasoconstriction in shocked animals, focal ulceration did not occur in duodenum, stomach, or esophagus.

With the exception of dogs 14 and 16 no other lesions of significance were detected in other internal organs. The exceptional animals manifested the effects of ascending infections of probable origin in their damaged intestines.

Discussion. The observations described above provide further evidence that 4-amino-PGA acts as an antagonist of PGA and, thereby, confirm previous deductions from studies in rats and mice.[16] The syndrome produced in dogs of ileitis and colitis with diarrhea, peripheral leucopenia, and depletion of bone marrow with appearance of megaloblasts corresponds to observations of the effects of folic acid deficiency in dogs, pigs, and monkeys. The diarrhea and changes in hematopoiesis resemble sprue in man, a disease responding readily to PGA therapy (see recent review[4]).

A comparison of bone marrow from dogs receiving 4-amino-PGA with marrow from cases of pernicious anemia in man reveals certain features in common. These include increased numbers of hypersegmented polymorphonuclears and giant metamyelocytes, megaloblasts and irregularly shaped nuclear remnants in erythrocytes. However, differences between the two marrows are apparent. In pernicious anemia bone marrow exhibits uniform hyperplasia of both erythroid and myeloid elements. In the present dogs general cellularity was reduced and confined to islands of active marrow. In untreated pernicious anemia erythropoiesis is predominantly megaloblastic whereas in the present animals erythropoiesis was a mixture of normoblastic and megaloblastic elements thus corresponding to marrows reported in sprue.[23-28]

The megaloblasts produced in dogs resembled those found in pernicious anemia and sprue as well as those found in humans treated with 4-amino-PGA[29] possibly more closely than the megaloblastic-like cells observed in swine receiving either PGA-deficient diets supplemented with x-methyl folic acid[10] or injections of 4-amino-PGA.[30,31] From studies now available it would appear that megaloblastic response of erythropoiesis to PGA-deficiency varies in different species. Megaloblasts have not been observed in mice and rats,[16,32,33] with the exception of a single report.[34]

Summary. The administration of 4-amino-PGA to dogs produced a sprue-like syndrome with diarrhea, peripheral leucopenia, depletion of bone marrow with increased numbers of hypersegmented polymorphonuclears and giant metamyelocytes, abnormal nuclear dis-

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integration of normoblasts, change of nuclear pattern in erythroid elements and megaloblasts. Doses given were lethal and animals succumbed with anorexia, weight loss, dehydration, and hemorrhagic diarrhea following severe ileitis and ulcerative colitis. It is suggested that the syndrome results from antagonism of folic acid.

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17233. Chronic Toxicity of Gamma Isomer of Hexachlorocyclohexane in the Albino Rat.

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Included in the report by Slade[1] on the discovery of the potent insecticide, "Gammexane," the gamma isomer of benzene hexachloride (γ-BHC)* were investigations of the acute and chronic toxicity of the drug in laboratory rats. His report indicates that the gamma isomer is much more toxic than the α, β, or δ isomers on oral administration, but, when mixed with the diet, levels of γ-BHC up to 30 mg per rat per day had no effect over a 5 week period. Other acute experiments have demonstrated by oral, subcutaneous and intravenous administration, the toxicity of γ-BHC,[2,3] while Laug[4] reported that the gamma isomer was non-toxic when fed in the diet at 20, 500, and 1000 p.p.m. for periods up to 114 days.

Other workers have been interested in the anti-inositol properties of the gamma isomer, since it was reported to resemble the structure of inositol.[1] Their results indicate that the inhibitory effect of γ-BHC is reversed by inositol in the growth of a microorganism,[5] in the α-amylase enzyme system of the pancreas,[6] and in the growing onion root.[7]

A study of transmethylation and lipotropic agents stimulated in this laboratory by the work of Griffith indicated that it might be of interest to examine the anti-inositol and toxic properties of the gamma isomer in the rat, possibly to make available another method of studying inositol metabolism in the intact animal.

Weanling male rats of the St. Louis University strain, 21-24 days old, weighing 44-49 g, were housed in screen cages and allowed food and water *ad libitum*. The animals were weighed twice weekly. The diet to which supplements of γ-BHC and inositol were added was as follows: Casein, 15.0; l-cystine, 0.3; salts 3A,† 2.0; salts 3B,‡ 2.0; celluration, 2.0; B-complex mixture,§ 1.0; choline chlor-

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* The term, gamma benzene hexachloride, has been approved by the Committee on Insecticide Nomenclature as the accepted name for the gamma isomer of hexachlorocyclohexane (*Science*, 1949, **109**, 330).

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† The salt and vitamin mixtures were devised and thoroughly tested by Dr. Wendell H. Griffith. Since the compositions of these supplements have not been published, the authors wish to express their thanks to Dr. Griffith for his permission to present them here. Salts 3A: $CuSO_4$, 0.5; $CoSO_4 \cdot 7\ H_2O$, 0.1; $ZnSO_4 \cdot 7\ H_2O$, 0.9; $MnSO_4 \cdot 4\ H_2O$, 4.0; $K_2Al_2(SO_4)_3 \cdot 24\ H_2O$, 0.07; NaF, 0.05; Ferric citrate, 24.38; $CaCO_3$, 100; Ca citrate, 185; $MgCO_3$, 45.0; K_2HPO_4, 140 g.

‡ Salts 3B: KI, 0.8; NaCl, 169.2; K_2HPO_4, 100; $CaHPO_4$, 230 g.