17220. Effect of Folic Acid, "4-Amino" Folic Acids and Related Substances on Growth of Chick Embryo.*

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(Introduced by C. P. Rhoads.)
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Pteroylglutamic acid (folic acid) has an essential role in the growth of bacteria, protozoa, insects, birds and mammals.[1] Several weak folic acid antagonists were first described in 1947,[2,3] and shortly afterwards Seeger *et al.*[4] discovered 4-amino pteroylglutamic acid, an extremely powerful antagonist to folic acid by the *Streptococcus fecalis* R test. Franklin *et al.*[5] found that 4-amino PGA was toxic to mice, and that folic acid offered only slight protection, and this within a narrow dose range. 4-amino PGA and some of its related componnds produced a toxic picture in mammals somewhat similar to that of other systemic cell poisons, such as x-rays and nitrogen mustard, with lymph node regression, leucopenia and thrombocytopenia with bone marrow aplasia, diarrhea with sloughing of the intestinal mucosa, and death within 3 to 4 days after a single acute dose. Philips, Thiersch *et al.*[6,7,8] have reviewed the pharmacology of these compounds and they conclude that the "4-amino" compounds are true folic acid antagonists, but are unique as anti-metabolites in that they induce an acute and absolute folic acid deficiency physiologically, even in the presence of folic acid. Because of the actions of the "4-amino" folic acids, a study was made of the effect of these and related compounds on the growth of the chick embryo.

Methods. Fertile White Leghorn eggs were obtained from a commercial source. The eggs were incubated at 100°F, and a relative humidity of 75%. In most instances the embryos were injected with the test agent at 4 days of incubation. The chemicals were dissolved in sterile saline and injected into the yolk sac by introducing a No. 25 needle through a small hole at the blunt end of the egg. The total volume injected into each egg ranged between 0.1 and 0.2 cc; larger volumes were sometimes necessary for the large doses of the relatively non-toxic drugs. When most of the embryos in a given group were dead, those remaining were sometimes sacrificed for detailed examination. The toxicity experiments were usually concluded by sacrificing the embryos 8 to 10 days after injection, when the embryos were 12 to 14 days of age.

The 15 compounds used in this study are listed in Table I.

Results. After considerable exploration, the 4 day old embryo was selected as most

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TABLE I. Toxicity of Folic Acid and Allied Compounds to the 4 Day Old Chick Embryo.

Compound	Dose mg	No. embryos	Embryo deaths, or sacrifices () or[1] age embryo, days										Approx. LD_{50}, mg/egg	Abnormality incidence in embryos surviving beyond 9 days of age
			5	6	7	8	9	10	11	12	13	14		
I. Pteroyl glutamic acid	20	15	0	0	2	1	0	0	0	0	(9)	(3)	20	0/12
II. " diglutamic acid	20	5	0	0	0	0	1	0	0	0	(4)	—	20	0/4
III. " triglutamic "	20	8	0	0	0	1	0	1*	0	0	(6)	—	20	1/7*
IV. 4-amino pteroylglutamic acid	.008-.010	29	0	14	10	2	(1)	1[1]	—	—	—	—		2/2
	.004-.006	37	0	5	7	2	5[1]	4	5[1]	1[3]	0	(3)	.003	8/17
	.002-.003	16	0	0	0	0	2	4	2	1	1[5]	(1)		7/14
V. 4-amino N^{10}methyl pteroylglutamic acid	.009-.013	16	1	7	0	1	2	(2)	0	2	1	—		5/5
	.005-.006	16	2	3	1	0	2	1	0	(1)	(3)	1[2]	.005	1/8
	.003	6	0	0	0	0	0	0	0	0	(6)	—		1/6
VI. 4-amino 9 methyl pteroylglutamic acid	.010	11	0	4	4	1	0	0	0	0	(2)	—		0/2
	.004-.005	17	1	3	3	3	2	2	1	1	(1)	—	.003	2/5
	.001-.003	21	2	2	2	2	0	0	1	1	1[9]	(1)		2/13
VII. 4-amino 9-10 dimethyl pteroylglutamic acid	.004-.005	23	0	4	9	2	4	1[1]	0	(2)	—	—		3/4
	.002-.003	20	1	1	1	1	2	2	2	1[7]	1	(1)	.003	4/14
VIII. 4-amino pteroylaspartic acid	.400-.600	15	2	9	0	2	2	—	—	—	—	—		
	.150-.250	15	0	6	4	3	1	0	(1)	—	—	—	.075	1/1
	.050-.100	26	1	5	7	1	3	0	0	0	(5)	(4)		7/9
	.025	9	0	0	0	0	0	0	0	0	0	(9)		0/9
IX. 4-amino pteroylthreonine	4-5	8	0	1	2	4	0	1	—	—	—	—		1/1
	2	9	0	2	0	2	0	0	1	2[2]	—	—	1.5	5/5
	0.5-1	10	2	0	0	0	0	0	0	1	2	(5)		1/8
X. 4-amino pteroylalanine	2	5	1	0	2	2	—	—	—	—	—	—		
	1	5	0	1	1	0	0	0	0	0	0	(3)	1.5	1/3
	0.5	5	0	0	1	0	1	0	0	0	0	(3)		0/3
XI. N^{10}methyl pteroic acid	5-6	21	6	8	1	2	0	0	(1)	0	(3)	—		0/4
	4	5	1	1	0	0	0	0	0	0	0	(3)	4	0/3
	2	15	2	2	0	1	0	0	(2)	(4)	(4)	—		0/10
XII. Pteroylaspartic acid (dextro)	20	6	1	0	0	0	0	1	0	0	(4)	—	20	0/5
	10	6	0	0	0	0	0	0	0	0	(6)	—		0/6
XIII. Pteroylaspartic acid (racemic)	20	5	0	0	1	0	0	0	0	0	(4)	—	20	0/4
	10	6	0	0	0	1	0	0	0	0	(5)	—		0/5
XIV. 9-methyl pteroylglutamic acid	10	17	2	5	1	0	2	1	0	(1)	(3)	(2)	10	0/7
	4-6	43	8	11	0	4	2	0	0	1[1]	(16)	—		0/18
	2	12	0	0	0	1	1	0	(1)	0	1[8]	—		0/10
XV. 2,6-diaminopurine	4-5	41	6	5[1]	5	3	8	6[7]	—	—	—	—		See
	2-3	27	2	1	2	1	6[3]	1	2[2]	2[5]	—	—	1	description
	1-1.5	15	1	0	0	1	0	1	0	2	2	4[4]		below
	.25-.50	6	0	0	0	0	1	0	0	2	1	(2)		(in text)

* A stunted, anophthalmic embryo, not typical of "4-amino" PGA; this probably represents a spontaneous abnormality.

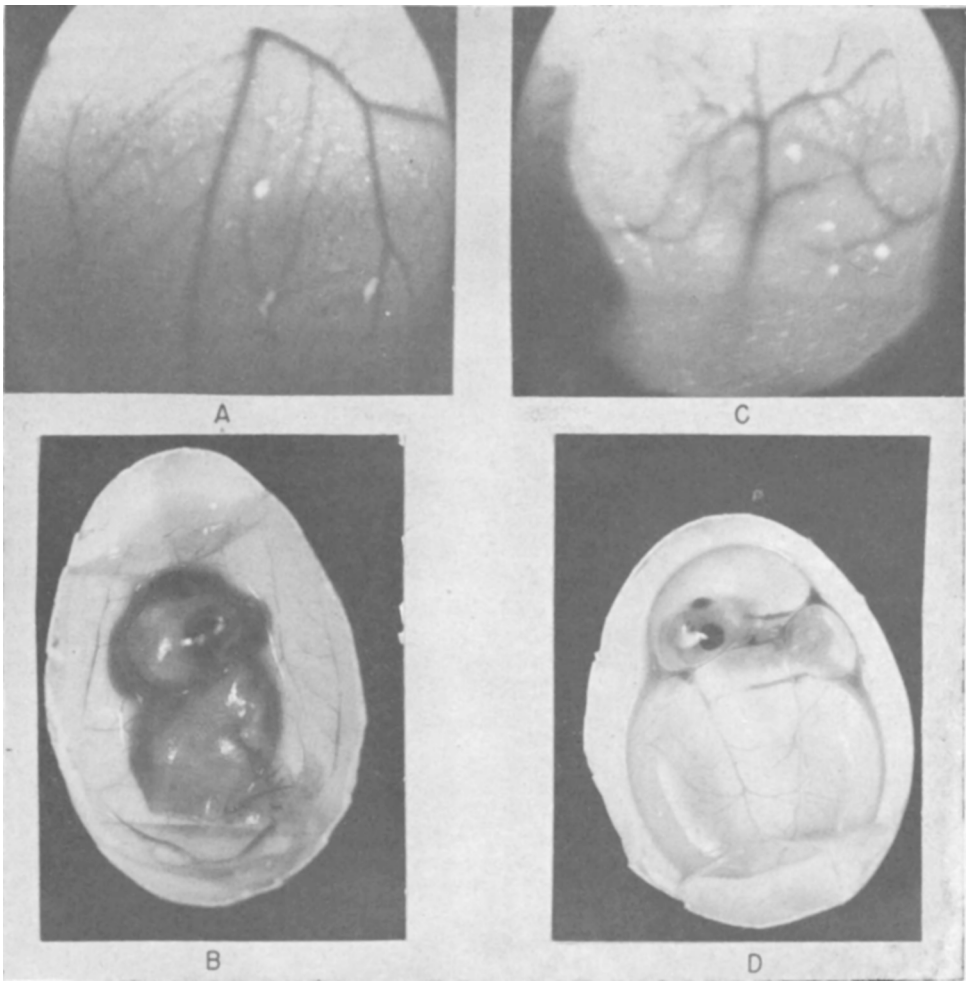

FIG. 1.

Appearance of a normal and stunted embryo, on candling, and in the opened egg. The normal appearing embryo received 0.003 mg/egg of 4-amino 9-methyl PGA at 4 days of age, sacrificed at 11 days of age. A) The vascular pattern, as seen by candling, appears normal. B) The embryo appears to be normally developed, and the allantois has extended laterally to envelop the contents of the egg. The abnormal embryo received 0.004 mg/egg of 4-amino 9-methyl PGA at 4 days of age, sacrificed at 11 days of age. C) The vascular pattern of the allantois, as seen by candling, is limited to a small area, about 3 × 3 cm, and it is separated from the yolk vessels. D) The embryo is severely stunted and abnormal, the amniotic sac is relatively enlarged, and the allantois is a large sac full of clear liquid.

satisfactory for routine use. Viability was easily determined by candling. The 4 day old embryos seemed to survive toxic doses of the "4-amino" folic acids for longer periods than the younger embryos, and developmental abnormalities were more conspicuous in those embryos surviving for longer periods. Older embryos required larger doses of the drugs, and developmental abnormalities were not as apparent.

Toxicity determinations in the chick embryo are subject to considerable variability, which may be attributed to some extent to differences in the genetic and nutritional background of the embryo, the season of the year, the age of the embryo at treatment, and the trauma associated with the injection. The LD_{50} figures are, therefore, approximate and represent the minimum dose range in which the compound shows activity. The

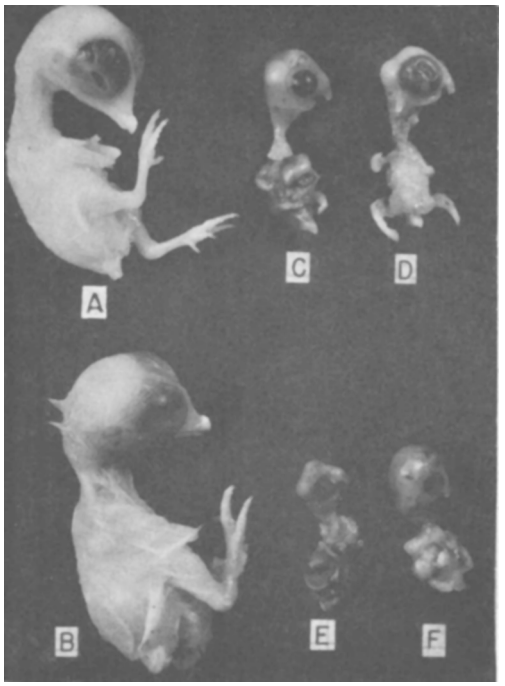

FIG. 2.
Appearance of normal and "4-amino" folic acid treated embryos. These were treated at 4 days of age and sacrificed 8 to 9 days later.
A. 12 day, control.
B. 13 day, control.
C. 2 mg 4-amino pteroylthreonine, sacrificed at 12 days.
D. 0.005 mg 4-amino 9-10 dimethyl PGA, sacrificed at 12 days.
E. 0.010 mg 4-amino N^{10} methyl PGA, sacrificed at 13 days.
F. 0.100 mg 4-amino pteroylaspartic acid, sacrificed at 13 days.

toxicity data for the compounds tested are summarized in Table I

Folic acid and its conjugates, pteroyl mono- (I), di-(II) and tri-(III) glutamic acids do not have an appreciable toxicity in the chick embryo at doses up to 20 mg/egg.

The "4-amino" folic acids produce a uniform toxic effect on the chick embryo, but alterations in the "4-amino" anti-folic molecule may affect the LD_{50} dosage. The LD_{50} of 4-amino PGA (IV) is 0.003 mg/egg; this LD_{50} dose is not appreciably altered by placing a methyl group in the N^{10} position (V) and in the 9 position (VI), and in the 9 and 10 position (VII). Substituting aspartic acid (VIII), threonine (IX) and alanine (X) for glutamic acid, however, increases the LD_{50} to approximately 0.075, 1.5 and 1.5 mg/egg, respectively.

The abnormalities produced in the embryo by the "4-amino" compounds have been remarkable and fairly uniform. The embryos dying during the first 4 days after treatment are severely stunted, but they are usually too autolyzed to permit a useful examination. Those embryos surviving for 8 to 10 days show the most remarkable changes. The appearances of the allantoic vessels in an 11 day normal and abnormal embryo are shown in Fig. 1, A,C. On opening these eggs the reason for the vascular abnormality is apparent. (Fig. 1, B, D). The normal embryo weighed about 4 g and the chorioallantoic membrane extended widely. The treated embryo was severely stunted, weighing about 0.8 g. The amnion was distended with clear fluid. The allantois remained as a large fluid-filled sac, which was in connection with the shell over a relatively small area, and its blood vessel network was appropriately restricted.

Several abnormal embryos and controls of the same age are shown in Fig. 2. These embryos were all sacrificed. Whereas at 12 to 13 days of age the chick embryo normally weighs from 5 to 7 g, the treated embryos weighed from 0.8 to 1.2 g; the weight of a 7 day embryo. Besides the profound stunting, the abnormalities usually present were a flattened head, small eyes which frequently had a clear sac over the lens suggesting an enlarged anterior chamber, absent or underdeveloped lower beak, a long and usually twisted neck, exteriorization of the viscera, a fluid-filled sac surrounding the heart, abnormal or absent toes and various degrees of generalized edema. The abdominal organs appeared to be growing relatively more rapidly than the rest of the embryo. Occasionally, intermediate stages were seen, with larger embryos showing generalized edema, abnormal beaks, absence of feathers and limb abnormalities. Usually, however, those embryos surviving 8 to 10 days after treatment showed either the typical stunting shown in Fig. 2 or they were apparently normal. The oldest stunted embryo we have seen was 14

days old; it is unlikely that many can survive beyond this age.

These embryo abnormalities were not produced by pteroyl aspartic acid (dextro) (XII) and (racemic) (XIII), by 9 methyl pteroylglutamic acid (XIV), also weak folic acid antagonists[2,3] and by N^{10} methylpteroic acid (XI).

2,6-diaminopurine is included in this report because Hitchings *et al.*[9] found that it acted as an antagonist of folic acid over a limited range, and as an antagonist of adenine over a more extended range, in the growth of *Lactobacillus casei*. It also damages the hematopoietic system of laboratory animals,[10] and prolongs the survival time of mice with transplanted leukemia,[11] actions qualitatively similar to those of the "4-amino" antifolics.[6,12]

In the 4-day-old chick embryo, 2,6-diaminopurine is a moderately toxic compound, the LD_{50} being in the range of 1 mg/egg. The mortality pattern seems to differ appreciably from the "4-amino" compounds in that, in the LD_{50} range, many of the embryos may survive for longer than 5 days. At this dose, the embryos surviving for longer than 5 days did not appear appreciably stunted, but they were extraordinarily pale and a few random hemoglobin determinations showed values much lower than the normal. Probably as a result of the anemia, cardiac enlargement was also conspicuous in some of these embryos. Other findings were less constant, and they included generalized edema, abnormal lower beaks, and necrotic areas in the liver. Daniel *et al.*[13] found that 2-amino-4-hydroxy-6,7-dimethyl pteridine and 2-amino-4-hydroxy-6,7-diphenylpteridine inhibited growth and hemoglobin formation in the chick, but the effect could be counteracted by folic acid. The toxicity of 2,6-diaminopurine in the egg, however, could not be altered by adenine (20 mg/egg) or by folic acid (10 mg/egg).

Attempts to determine whether folic acid provided the chick embryo with some protection against the "4-amino" compounds have been unsuccessful. Twenty mg of folic acid were given almost simultaneously with different dose levels (multiples of the approximate LD_{50}) of 4-amino PGA (IV) and 4-amino N^{10} methyl PGA (V), a weight ratio of 1000 to 4000 to 1, but no clear-cut protection could be demonstrated. There were several experiments in which folic acid protected the chick embryo against 4-amino pteroyl aspartic acid, but this could not be demonstrated consistently. There is no evidence in these experiments that folic acid, within the closed system provided by the egg, gave any significant protection against the "anti-folic" compounds; in fact, the stunted embryos, produced by the "4-amino" antifolics, occurred in the presence of folic acid.

Discussion. It is not clear whether the profound stunting of the chick embryo produced by the "4-amino" anti-folics is due to a general interference with the growth of the embryo or due to injury to a specific tissue which leads to retardation in the growth of the embryo. Wagley and Morgan[14] found that the "4-amino" PGA's in the chick embryo produced marked cytological changes in the yolk sac blood islets, and we have observed that the vascular network of the chorioallantoic membrane is considerably reduced in size in the stunted embryos. Hertz and Tullner,[15] in demonstrating that "4-amino" PGA inhibits the response of the chick oviduct to estrogen stimulation, also implied that it acted on specific tissues. We tend to favor the more general interpretation, however, that the "4-amino" folic acids interfere with the growth of tissues—those growing more rapidly being most obviously affected—and the thesis that it interferes with the growth of certain tissues, in a highly specific fashion, remains to be proved.

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The inability of folic acid to protect the chick embryo from the toxic action of the "4-amino" PGA's, even when given in ratios of 1000 to 4000 to 1, is further evidence of the difficulty in protecting against or reversing the activity of these compounds. Franklin *et al.*[5] have obtained slight and limited protection against "4-amino" PGA with large doses of folic acid, and Hertz and Tullner,[15] using a ratio of folic acid to 4-amino pteroylglutamic acid of approximately 300 to 1, prevented the "anti-folic" inhibition of the chick oviduct response to estrogenic stimulation. In frogs, however, a folic acid to "4-amino" PGA ratio of 100 to 1 did not prevent the "4-amino" PGA inhibition of the oviduct response to estrogens.[16] It appears clear, therefore, that folic acid may prevent the toxic effects of the "4-amino" folic acids but the protective ratio necessary will undoubtedly differ quantitatively for the specific "anti-folic" compound and the biological system used.

The apparently specific action of 2,6-diaminopurine on blood formation merits further study. It is of interest that Bendich and Brown[17] have shown that 2,6-diaminopurine may serve as a precursor of nucleic acid guanine, an observation which cannot, as yet, be related to the mechanism of blood formation.

Summary. The "4-amino" pteroylglutamic acids profoundly inhibit the growth of the chick embryo, with the production of developmental abnormalities. These compounds are active in the range of 0.003 to 0.005 mg/egg, and their actions are not prevented by large doses of folic acid. The "4-amino" folic acids with aspartic acid, threonine and alanine substituted for the glutamic acid are considerably less toxic but produce similar toxicological effects. Other compounds allied to folic acid are relatively non-toxic and do not possess the growth-inhibitory activity of the "4-amino" compounds.

2,6-diaminopurine, at LD_{50} doses, does not seem to be an active growth inhibitor, but it produces a pale embryo, presumably deficient in hemoglobin.

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17221. Distribution of C'^4, Administered as CaC'^4O_3, in Rat Liver Glycogen and Blood Glucose and Lactate.*

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In prior studies[1-5] it has been shown that the distribution of isotopic carbon in rat liver glycogen following the feeding of a labeled compound may serve as an indicator of intermediary pathways. Under the proper experimental conditions, the distribution of isotope in blood glucose and lactate might reasonably be expected to mirror that in the liver glycogen. Should this expectation be realized, it would be possible to obtain information from the degradation of the carbohydrates from the blood of larger animals and man comparable

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