Boyd for technical assistance and Miss Zina Malevitch and Miss Barbara Emmett for technical advice.

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Received July 29, 1952. P.S.E.B.M., 1952, v81.

Effect of Nucleic Acid Derivatives in the Reversal of Aminopterin Inhibition in the Chick Embryo.* (19766)

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Aminopterin (4-amino-pteroylglutamic acid) is a powerful inhibitor of embryonic development in the chick(1,2). The injection of 10 μg or more into eggs prior to incubation completely inhibits embryonic development. This inhibitory effect is partially alleviated by thymidine and more completely by thymidine plus hypoxanthine desoxyriboside(2). The inhibitory effect is also alleviated by high levels of "leucovorin," but not by folacin itself(3). These results were interpreted to mean that aminopterin acts in the chick embryo, as in other organisms, as an antagonist of folacin and its derivatives; that these substances are directly or indirectly essential for synthesis of thymidine and one or more purine derivatives; and that synthesis of these essential components of nucleic acid is the first process to become limiting in the aminopterin inhibited chick embryo. When thymidine, which is highly specific, and a suitable purine derivative are supplied preformed, these inhibited reactions are by-passed; nucleic acid synthesis, and hence initiation of embryonic development, can then occur in their absence.

The effectiveness of various purine bases and their nucleosides in enhancing the effects of thymidine is compared below. If the viewpoint cited above is correct, each of the effective purine derivatives must be able to serve as a precursor for synthesis of nucleic acid by the chick embryo.

Experimental. The procedures followed in

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Presented at the 121 National Meeting of the American Chemical Society, Milwaukee, Wisc., April 1952.

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TABLE I. Effect of Nucleic Acid Derivatives on Inhibitory Action of Aminopterin in the Chick Embryo.

Group No.	Compound injected, amt per egg*	No. of eggs	% embryos dying 0-5 days	% embryos dying 6-20 days	% hatch
1	None	60	20	5	75
2	Water (.1 ml)	50	20	20	60
3	" (.2 ")	10	20	20	60
4	20 μg aminopterin	30	100	0	0
5	20 " " + 1 mg thymidine	30	83	7	10
6	As in 5 + .9 mg hypoxanthine	30	50	10	40
7	" + 2 inosine	20	25	15	60
8	" + 2 hypoxanthine desoxyriboside	20	60	20	20
9	" + .9 adenine	30	57	20	23
10	" + 2 adenosine	20	70	15	15
11	" + 1 adenine desoxyriboside	10	90	0	10
12	" + 1 guanine	30	57	17	27
13	" + 2 guanosine	20	80	15	5
14	" + 1 guanine desoxyriboside	10	100	0	0
15	" + 1 xanthine	10	80	10	10
16	" + .9 adenine and 1 mg guanine	30	37	37	27
17	" + .9 adenine and .9 hypoxanthine	30	30	33	37
18	" + 1 guanine and .9 hypoxanthine	30	33	23	43

* Thymidine (m.p. 185-86°), hypoxanthine desoxyriboside, adenine desoxyriboside, and guanine desoxyriboside were isolated from an enzymatic digest of desoxyribonucleic acid by slight modifications of published procedures(15,16). Other pure compounds were obtained from commercial sources.

injecting and incubating the eggs have been described(4). Injections were made prior to incubation in a total volume of 0.1 or 0.2 ml per egg. Where insolubility of compounds was a problem, they were dissolved and injected as sodium salts. Ten eggs were used per group in each experiment. At the level used, aminopterin frequently causes death prior to any blood formation. Upon gross examination such eggs cannot be distinguished from infertile eggs. For this reason, some infertile eggs may be included in the 0-5 day embryonic death classification.

Results. The combined results of 3 experiments are given in Table I. The non-injected control group average 75% hatch as compared with 60% for the water-injected control group, indicating a slight depressing effect of injecting water on hatchability. In confirmation of previous results(2), the injection of 20 μg of aminopterin prior to incubation resulted in 100% embryonic mortality prior to the fifth day of incubation, and this "toxic" effect is partially counteracted by simultaneous injection of 1 mg of thymidine. The activity of compounds such as thymidine is evidenced not only by the hatch of live chicks, but also in the definite increase in survival time of embryos which die before hatching.

Of the purine derivatives tested, inosine (hypoxanthine riboside) was easily the most effective in enhancing the effects of thymidine. The mixture completely counteracts aminopterin inhibition. Hypoxanthine desoxyriboside is active, but much less so than inosine. Lower concentrations of the desoxyribosides of adenine and guanine also were ineffective; the possibility remains that higher concentrations might be more effective.

Of the free purine bases, hypoxanthine is most active. Guanine and adenine each show distinct activity, however. Xanthine is inactive under the same conditions. Mixtures of the active purine bases show greater activity than the individual compounds; this enhanced activity may result in part, however, from the increased total concentration present.

Discussion. The participation of folic acid in the synthesis of purine bases and of thymine (or thymidine) was first suggested by the observation that certain lactic acid bacteria grew without folic acid if these nucleic acid derivatives were supplied(5-7). This same nutritional equivalence of nucleic acid derivatives and "leucovorin" can be demonstrated in the chick embryo, under the conditions used herein. The common feature in synthesis of the 2 groups of substances is now known to be

incorporation of a one carbon unit (*e.g.* formate) in their molecules, for which a derivative of folic acid appears essential(7-9).

It is obvious that the small amounts of thymidine and purine bases injected in these experiments are not sufficient for synthesis of all of the nucleic acids of the hatched chick. From the figures of Novikoff and Potter(10) they should suffice, however, to permit development through the 8th day of incubation. It was shown earlier that the amount of aminopterin used here, 20 μg, is insufficient to prevent continued development if injected after the 8th day; the quantitative relationships are hence consistent with the interpretation made. If sufficient amounts of aminopterin were used to inhibit growth during the later phases of incubation, when rapid synthesis of comparatively large amounts of nucleic acids must occur, it is probable that reversal by the amounts of thymidine and inosine used here could not be accomplished.

If these interpretations are correct, the purine and pyrimidine bases that are effective in overcoming aminopterin inhibition must be directly utilizable as precursors of nucleic acids in the chick. The requirement here for thymidine, and the ineffectiveness of free thymine(2), correlates with the findings that bound pyrimidines, but not free pyrimidines are incorporated into the nucleic acids of rats (11,12). The superiority of hypoxanthine and especially inosine over other purine derivatives in potentiating the effects of thymidine correlates with the finding of Schulman *et al.*(13) that hypoxanthine appears in pigeon liver homogenates acting upon 4-amino-5-imidazolecarboxamide, and the postulate of Greenberg(14) that inosinic acid precedes hypoxanthine (which may not be an essential intermediate) in the biosynthetic pathway by which this system forms purine bases. In its preference for hypoxanthine and inosine, the chick embryo differs markedly from the rat, for which free hypoxanthine is not an effective precursor of nucleic acid(11). Adenine also appears readily utilizable by the chick embryo, as it is by the rat(11). In contrast to the rat, which does not utilize guanine for nucleic acid synthesis, the chick appears able to do so, and in this respect resembles the mouse(11). These interesting differences make it appear that the incubating egg would be an excellent additional tool for the elucidation of more intimate details of nucleic acid biogenesis through the use of isotopically marked intermediates.

Summary. Several purine bases and related nucleosides were tested for their effectiveness in potentiating the reversal by thymidine of aminopterin inhibition of the chick embryo. Of the free purine bases, hypoxanthine was most active; adenine and guanine were also active, while xanthine was inactive. Of the nucleosides tested, only inosine was active. Inosine was more active than hypoxanthine; together with thymidine it completely overcame the effects of the low level of aminopterin employed. The results are interpreted to mean that aminopterin prevents embryonic growth by preventing synthesis of compounds essential for formation of nucleic acids; by supplying these substances preformed, growth occurs even though aminopterin is present. It follows that thymidine, and the active purine bases and derivatives listed above, are utilizable for nucleic acid synthesis by the chick embryo.

We are indebted to Lederle Laboratories, Pearl River, N. Y., for the aminopterin.

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Received July 29, 1952. P.S.E.B.M., 1952, v81.

Inhibition of Pancreatic Secretion by the Carbonic Anhydrase Inhibitor, 2-Acetylamino-1,3,4-Thiadiazole-5-Sulfonamide (Compound No. 6063).* (19767)

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Carbonic anhydrase, the enzyme concerned with the hydration of CO_2 and, consequently, the formation of bicarbonate, occurs in significant concentration in the pancreas(1). Tucker and Ball(2) attempted to demonstrate that carbonic anhydrase plays a major role in the formation of bicarbonate by the pancreas. For this purpose, sulfanilamide, an inhibitor of the enzyme *in vitro*, was injected into anesthetized dogs with cannulated pancreatic ducts secreting in response to secretin. Failure of these efforts led the investigators to conclude that this enzyme plays no essential role in pancreatic secretion. Recently, a new carbonic anhydrase inhibitor (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide, designated compound No. 6063) which may be as much as 440 times more potent than sulfanilamide *in vitro*, has become available(3,4). This agent has already been found to reduce the secretion of histamine-induced gastric HCl considerably, when administered intravenously to Heidenhain pouch dogs(5), or when applied to the mucosa topically(6). Hence, we undertook to study the effect of this drug on pancreatic secretion, and the results are reported herewith.

Methods and materials. Observations were made on 3 mongrel dogs, provided with duodenal fistulae for collection of pancreatic juice according to Thomas(7). Experiments were never done less than 4 weeks after operation, nor at intervals of less than one week. The dogs were fasted 24 hours prior to each experiment. Following cannulation of the main pancreatic duct, unstimulated secretion was collected for one hour. Then Secretin, Lilly (5 units/kg) was injected intravenously and the secretory response was followed for one hour (control period, A). Immediately after this second hour, the inhibitor, dissolved in a minimum of NaOH solution, was injected slowly intravenously. Two or 3 hours after the inhibitor had been administered, a second injection of secretin was given and the secretion collected for the following hour (period B). The same procedure was repeated 5 hours after injection of the inhibitor (period C). Pancreatic juice specimens were collected for successive 10-minute intervals and all volumes were recorded; those containing less than one ml were pooled consecutively until a specimen with at least this volume was obtained. The concentration of total CO_2 (*i.e.*, dissolved gas plus bicarbonate) in each sample was determined in duplicate, using the constant volume method of Van Slyke(8). All determinations were performed with as little delay as possible —never more than 5 hours after collection. In all, 18 experiments were performed, using compound No. 6063 in dosages of 2, 5, 10, 30, 40 or 60 mg/kg. There were no general toxic signs following the slow intravenous injection of the drug, in conformity with previous observations(9) in this laboratory.

The extent of inhibition was measured in terms of volume of secretion and bicarbonate concentration, by comparison of the *hourly* response to secretin for period A (without inhibitor) with the responses for periods B and C. Percentage inhibition for each experiment was calculated in terms of the decrease

* This work was carried out with the aid of a grant from The Altman Foundation.

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