Purine Metabolism in the Chick Embryo; Effects of Uricogenesis and Xanthine Oxidase Inhibition

D. E. DUGGAN, K. H. PUA, AND G. ELFENBEIN

Merck Institute for Therapeutic, Research, West Point, Pennsylvania 19486

(Received June 26, 1967, and in revised form October 6, 1967)

SUMMARY

The *de novo* synthesis and subsequent utilization of nucleotide by anabolic and catabolic pathways in the chick embryo have been studied using formate-^{14}C and glycine-1-^{14}C. The normal embryo produces soluble nucleotide far in excess of its requirement for polynucleotide synthesis, the major portion of newly synthesized purine being catabolized to uric acid largely via deamination and subsequent oxidation of guanine and its soluble anabolites. The untreated embryo thus appears to provide a biochemical model for essential hyperuricemia.

The uricogenic agent 2-aminothiadiazole increases urate synthesis, presumably by inhibiting incorporation of nucleotide into nucleic acid, thus triggering a compensatory increase in *de novo* synthesis.

The xanthine oxidase inhibitor 4-hydroxy[3,4-*d*]pyrazolopyrimidine specifically reverses the effect of aminothiadiazole upon uric acid labeling, while having no effect upon acid-soluble or nucleic acid purine. The absence, in the chick embryo, of an adequate salvage pathway for oxypurine bases is suggested as a basis for these and other observations.

INTRODUCTION

In birds, as in man and the anthropoid primates, uric acid is the end product of purine catabolism. In man, uric acid is believed to be derived largely from the catabolism of polynucleotide purines, both endogenous and dietary, and to a lesser but unknown extent, via a direct degradation of newly synthesized nucleotide (1, 2). Comparison of the respective time courses of incorporation of labeled precursors into uric acid in normal subjects on the one hand, with those of primary gouty individuals (1) or normals treated with the uricogenic agent ethylaminothiadiazole (3, 4) suggests that the overproduction of urate observed in the latter instances relates to an accentuation of the normally vestigial "shunt pathway," whereby excess nucleotide is degraded directly to urate.

The demonstration that ethylaminothiadiazole (EATDA) and several related compounds elicit substantial increases in uric acid synthesis in the developing chick embryo (5) suggested a possible utility for the EATDA-treated egg as a convenient model for study of the biochemical defect(s) associated with essential hyperuricemia. Stetten (6) has proposed that the chronic overproduction of urate relates to an abnormal vestige, in man, of the normal avian uricotelic mechanism.

Preliminary isotope experiments in this laboratory suggested that the major portion of purine synthesized in the untreated egg is catabolized directly to uric acid, bypassing polynucleotides, and that EATDA effect is merely a quantitative one, augmenting a synthesis of inosinic acid (IMP), which is normally far in excess of that required for polynucleotide synthesis. The present paper describes the results of a detailed study of the normal temporal and quantitative relationships between the anabolic and catabolic processes for purine in the chick embryo, and the effects upon

these of aminothiadiazole and inhibitors of xanthine oxidase.

MATERIALS AND METHODS

Sodium formate-^{14}C (24.8 μC/μmole) and glycine-1-^{14}C (22 μC/μmole) were purchased from Calbiochem Corporation and diluted with carrier to 10 μC/μmole. Stock solutions were diluted to 50 μC/ml and autoclaved immediately before use. White Leghorn hens' eggs at 10 days' incubation were received from Wallace-Hycross Hatcheries, Doylestown, Pennsylvania and maintained at 38° and 90% relative humidity until used.

Injection. At 13 days' incubation, 0.1 ml of labeled precursor in normal saline was injected by chorioallantoic artery under sterile mineral oil, and the eggs were immediately returned to the incubator until sacrifice. Before sacrifice, each egg was inspected by candle, and any showing evidence of abnormal bleeding at the injection site were discarded, as were those embryos whose weights differed from the mean by more than 10%.

Isolation of uric acid. Since preliminary experiments had indicated that only negligible amounts of urate were contained in the embryo, yolk sac, and albumen sac, only the chorioallantoic and amniotic fluids were collected. This was accomplished by carefully stripping the chorioallantoic and amniotic membranes away from the embryo, and rinsing the embryo with a small volume of cold water above a petri dish containing the remainder of the egg. The fluid was decanted through a single layer of gauze, and the intact yolk and albumen sacs were twice washed with 30-ml portions of cold water by decantation. The combined fluid and washings were diluted to 100 ml with water. A small volume was set aside for determination of total urate by the colorimetric method (Technicon Autoanalyzer), and a 20-ml aliquot was treated immediately with 2.0 ml each of 0.66 N H_2SO_4 and 10% sodium tungstate. Silver urate was precipitated from the clear tungstic acid filtrate according to Folin (7), and the uric acid was reextracted by suspending the precipitate in 5 ml of boiling 1 N HCl. The extract was evaporated to dryness at reduced pressure, the last traces of HCl were removed at high vacuum, and the residue was dissolved in 0.3 ml of 0.1 M Li_2CO_3. Three or four superimpositions of 10 μl each were applied to Whatman No. 3MM paper and developed by descent in *n*-propanol:NH_3H_2O::75:5:20. The uric acid spots were located under 254 mμ light ($R_F \simeq 0.19$), cut out, and extracted by heating in 0.05 M Li_2CO_3. Urate concentration was determined by absorption of 292 mμ, and 2.0-ml aliquots were transferred directly from the cuvettes to counting vials. Five drops of 10 M NaOH and 20 ml of Cab-o-Sil-dioxane scintillator (8) were added for counting. Specific activities determined by this procedure were found to be reproducible to within 2%.

Isolation of acid-soluble and polynucleotide purines. The rinsed embryos were blotted dry, weighed, and homogenized in 9 volumes of cold, 10% trichloroacetic acid. Aliquots of the homogenate were taken for measurement of total radioactivity in the embryo, and of the supernatant, following acid hydrolysis, for isolation of acid-soluble adenine. The isolation of the individual purines of the acid-soluble and nucleic acid fractions, and determination of their specific activities were accomplished as described previously (9). For conversion of polynucleotide purine specific activities to total radioactivity incorporated, values for total nucleic acid were calculated using a constant of 3.7 μmoles A and G per gram of embryo, derived from the standard curves for nucleic acid per unit weight of embryo according to Reddy (10), and confirmed by preliminary experiments here.

RESULTS

The respective time courses of labeling by formate-^{14}C of nucleic acid purines, acid-soluble adenine, and uric acid in the untreated egg are summarized in Fig. 1. Comparison of the curves for acid-soluble and polynucleotide purine demonstrates the anticipated precursor-product relationship between these two pools. After an initially

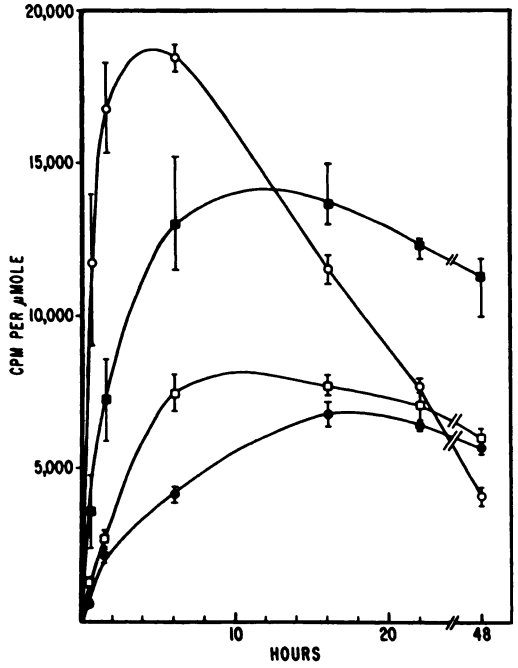

FIG. 1. *Specific activities of purine fraction in normal embryos following formate-^{14}C*

Thirteen-day embryos received 5 μC formate-^{14}C at zero time. Each point represents mean for three embryos with ranges indicated. Nucleic acid adenine, ●—●; nucleic acid guanine □—□; acid-soluble adenine, ○—○; uric acid ■—■.

high rate of increase, uric acid specific activity attained a maximum between 10 and 20 hr; at this time soluble adenine specific activity was declining, suggesting that a significant portion of urate is derived via deamination of the nucleosides and nucleotides of adenine and/or guanine, followed by oxidation, in addition to a direct degradation of IMP and subsequent oxidation. However, the respective sizes of the urate and soluble adenine pools[1] is such that *total* radioactivity as adenine did not exceed total ^{14}C as uric acid, even at the earliest time points.

In Fig. 2 are plotted the respective accumulations of radioactivity (specific activity × pool size) in the polynucleotide

[1] Total acid-soluble adenine was found by isotope dilution to be quite reproducible at 2.4 μmoles per gram of embryo; total uric acid, which was routinely measured for each egg, averaged 9.6 ± 1.0 μmoles per gram of embryo.

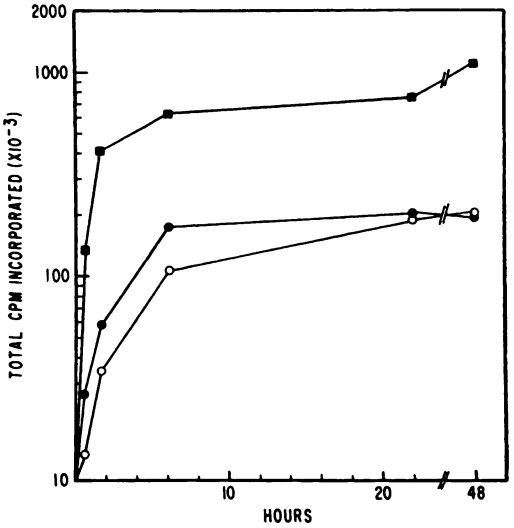

FIG. 2. *Cumulative labeling of purines in normal embryos following formate-^{14}C*

Values represent means of products of individual specific activities times respective pool sizes. Nucleic acid adenine, ○—○; nucleic acid guanine, ●—●; uric acid ■—■.

purine and urate pools. The magnitude of the continued net accumulation of urate-^{14}C over the interval 24–48 hr, during which the polynucleotide adenine and guanine pools were in a steady state with respect to radioactivity, and during which the order of decline in acid-soluble adenine-^{14}C indicated that little labeled purine was being synthesized *de novo*, suggests that an additional portion of the total urate synthesized during this stage of embryonic development derives from the catabolism of nucleic acid.

The distribution, among the various purine pools, of the label from glycine-1-^{14}C was, in the control egg, qualitatively similar to that of formate-^{14}C. Quantitatively, the overall efficiency of glycine-1-^{14}C as precursor for purine was only about one-fifth that of formate (Fig. 3).

For purposes of studying the uricogenic effect of the 2-substituted thiazoles, 2-aminothiadiazole (ATDA) was found to have a significantly greater ratio of activity to toxicity than EATDA, and was therefore chosen for use. The effects of ATDA and of 4-hydroxy[3,4-*d*]pyrazolopyrimidine (4-HPP), a xanthine oxidase inhibitor, are

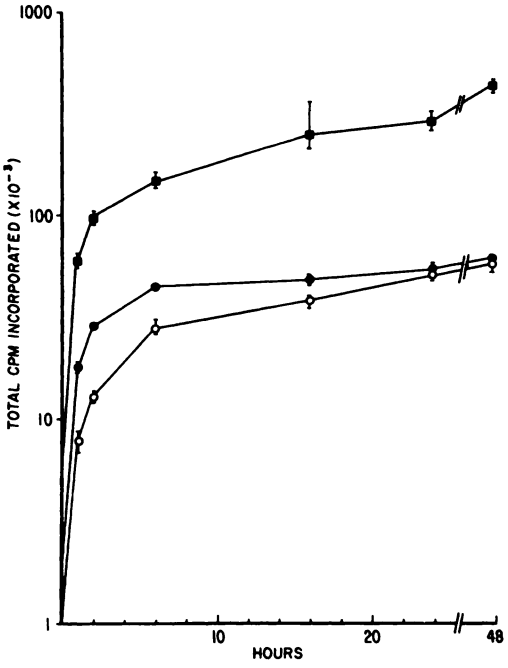

FIG. 3. *Cumulative labeling of purines in normal embryos following glycine-1-^{14}C*

Thirteen-day embryos received 10 μC glycine-1-^{14}C at zero time. Notations as for Fig. 2.

summarized in Table 1. The uricogenic effect of ATDA, which can readily be demonstrated after 24 hr by direct measurement of total uric acid, was evidenced at earlier intervals by a corresponding increase in urate labeling. The maximum effect of ATDA upon both urate and purine labeling required a pretreatment interval of several hours. When formate-^{14}C was administered at 1 hr following ATDA, a small inhibition of guanine labeling was the only discernible result; after 4 hr pretreatment, guanine labeling was almost completely inhibited, polynucleotide adenine was reduced approximately 50% and acid-soluble adenine by 15%.

The effect of 4-HPP appeared to be specifically upon uric acid synthesis, inhibiting urate labeling more than 60% while effecting no changes in the various purine pools. When given together with ATDA, it not only reversed the uricogenic effect of the latter, but decreased urate labeling relative to control values. It was completely without effect in reversing the ATDA-mediated inhibition of adenine and guanine labeling.

When glycine-1-^{14}C was used as precursor, the effects of ATDA and 4-HPP were virtually identical to those observed in the formate-^{14}C experiments (Table 2).

The failure of 4-HPP to affect polynucleotide purine labeling was unexpected

TABLE 1

Effects of aminothiadiazole and 4-HPP upon utilization of formate-^{14}C in the chick embryo

Each 13-day egg received inhibitors, where indicated, at 1 mg total per egg at the indicated intervals prior to label. Formate-^{14}C (5 μC in 0.1 ml of saline) was injected by chorioallontoic artery at zero time, and eggs sacrificed 2 hr later. Each value represents mean plus or minus mean deviation for best three of four eggs (duplicate eggs in two individual experiments).

	Cpm per μmole				Total cpm incorporated ($\times 10^{-3}$)			
Pretreatment	A	G	Urate	Acid-soluble A	A	G	Urate	Urate/(A + G)
None	1208 ±40	2470 ±30	6560 ±230	14,450 ±150	32 ±0.6	63.4 ±2.2	378 ±32	3.9
ATDA, at 1 hr	1110 ±70	1705 ±200	9500 ±1800	15,000 ±800	32.1 ±2.1	49 ±6.0	715 ±120	8.8
ATDA, at 4 hr	600 ±60	89 ±40	12,100 ±2200	12,400 ±0	14.9 ±1.5	2.2 ±1.0	960 ±200	55
ATDA + HPP, at 4 hr	510 ±20	74 ±30	2080 ±40	10,600 ±600	14.0 ±0.5	1.95 ±1.0	148 ±17	9.3
HPP, at 4 hr	1115 ±70	2475 ±75	2500 ±870	14,500 ±200	33.1 ±2.2	71.5 ±2.0	205 ±80	1.9

TABLE 2

Effects of 2-aminothiadiazole and 4-HPP upon utilization of glycine-1-^{14}C in the chick embryo

Experimental design and notations as for Table 1. Drugs given at −4 hr, and eggs sacrificed 2 hr after injection of 5 μC of glycine-1-^{14}C.

	Cpm per μmole				Total cpm incorporated ($\times 10^{-3}$)			
Pretreatment	A	G	Urate	Acid soluble A	A	G	Urate	Urate/(A + G)
None	231 ±41	532 ±45	1340 ±130	2410 ±220	5.9 ±.9	13.8 ±2.0	99.3 ±8.0	5.0
ATDA	123 ±14	50 ±10	2290 ±170	2340 ±77	3.3 ±.3	0.9 ±.3	205 ±15	49
ATDA + HPP	108 ±7	42 ±13	560 ±40	2460 ±90	2.6 ±.4	0.9 ±.1	40 ±0	11.4
HPP	253 ±21	562 ±44	535 ±25	2700 ±310	6.3 ±1.1	14.2 ±2.1	44 ±11	2.1

in view of its ability to enhance utilization of purine bases in mammals (11, 12). Dilution experiments were therefore devised to determine whether, in the chick embryo, preformed purine is utilized for polynucleotide synthesis. The injection of hypoxanthine, xanthine, adenine, and guanine, resulted in no case in a significant dilution of polynucleotide labeling by formate-^{14}C (Table 3).

TABLE 3

Lack of effect of preformed purines upon incorporation of formate-^{14}C into nucleic acid

Eggs were pretreated by yolk sac injection of 0.5 ml of purines, 2 mg/ml in 0.1 M Na_2HPO_4 at −30 min. Formate-^{14}C, 5 μC by choriallantoic artery at zero time, and eggs sacrificed at 4 hr. Notations as in Table 1.

	Cmp per μmole	
Pretreatment	Adenine	Guanine
None	1435 ± 120	2440 ± 300
Adenine	1475 ± 125	2450 ± 80
Guanine	1660 ± 80	2800 ± 270
Hypoxanthine	1375 ± 65	2550 ± 220
Xanthine	1605 ± 85	2850 ± 190

DISCUSSION

Prior to the demonstration of the prompt appearance of labeled purine bases in the urine of human subjects receiving glycine-^{14}C, uric acid was believed to derive largely from the breakdown of tissue nucleic acids; and during restricted dietary intake of purine, almost exclusively from this source. The synthesis of uric acid in man was thus assumed to differ qualitatively from that in uricotelic species. It is now recognized that a "shunt-pathway," vestigial of the mechanism by which birds and reptiles dispose of nitrogenous catabolites, is a normal, though apparently minor component of the overall pathway(s) for uric acid in man (1), and that in primary gout, this pathway contributes disproportionately to the increased synthesis of urate (2, 6, 13).

The uricogenic 2-substituted thiazoles have been found to elicit similar increases in the uricotelic activities of man (3, 4) and birds (5), in the case of man, the increased uricogenesis being manifested by an apparent specific increase in the shunt-pathway. The opposing effects of the xanthine oxidase inhibitor 4-HPP have also been found similar in man (14) and in birds (15), substantially decreasing total urate production in each instance. The overall decrement in man has been ascribed in part to a pseudo-feedback inhibition of *de novo* purine biosynthesis, probably at the stage of 5-phosphoribosylamine formation (16).

While the various pathways for purine interconversions in man and birds cannot

be considered identical in all respects, the results cited suggest that the differences in urate synthesis among normal and gouty man and uricotelic species are basically quantitative ones, determined by respective rates of *de novo* synthesis of nucleotide, and evidenced by quantitative differences in the subsequent utilization of newly formed nucleotide for anabolic and catabolic processes, respectively. Preliminary experiments in eggs revealed a remarkable reproducibility in the labeling patterns in control and ATDA-treated embryos following pulse-labeling with formate-^{14}C, confirming that this system provides a convenient model for study of certain of the biochemical events involved in experimentally induced uricogenesis.

A number of conclusions can be drawn from the formate-^{14}C and glycine-^{14}C experiments with untreated eggs. Comparison of urate and polynucleotide purine labeling confirms the traditional assumption that the bird produces soluble nucleotide far in excess of its anabolic requirements. The magnitude of the increment however, was unexpected in view of the comparable order of specific activities for polynucleotide purines and urate in the newly hatched chick (17), whose turnover of nucleic acids is high. In a single experiment, several eggs were incubated beyond 48 hr post-injection, and a half-life for the labeled nucleic acid pool was calculated to be 4 days. From this value and the rate of accumulation of labeled urate (Figs. 2 and 3) and total urate (5, 15), it can be approximated that of the 80 μmoles of urate synthesized per egg between days 14 and 16 of incubation, a maximum of 20 μmoles derives from polynucleotide catabolism.

Of the remaining 75% of urate synthesized, how much derives from a direct hydrolysis of IMP and subsequent oxidation, and how much via deamination of soluble anabolites of adenine and guanine cannot be precisely calculated from the data at hand. Although the temporal relationships between acid-soluble adenine and urate specific activities suggest a precursor–product relationship between these pools, correction for respective pool sizes reveals that the total quantity of labeled adenine never exceeds that of urate-^{14}C. Comparison of the time courses for polynucleotide adenine and guanine specific activities indicate that acid-soluble guanine compounds probably attain a higher, and earlier, peak than the adenine nucleotides. Thus, deaminations of guanine at the level of nucleotide, nucleoside, and/or free base might provide a major pathway for uric acid. In this context, it might be recalled that the labeling of urinary guanine compounds in overproducers of uric acid was both higher and earlier than in normal man (1, 4).

While the precise mechanism by which the uricogenic thiadiazoles increase uric acid synthesis is not known, the available evidence strongly indicates that in the chick embryo, as in man, they effect a marked increase in *de novo* purine synthesis. Further evidence that ATDA augments *de novo* purine synthesis is provided by the observations that folic acid and serine antagonists, whose sites of action have been localized within the *de novo* pathway, block its uricogenic effect, and that it produces a marked increase in the labeling of soluble oxypurines by formate-^{14}C (5). Such an effect upon soluble adenine and guanine compounds, however, was not demonstrated.

The proposal that the ADTA effect is initiated by a blocking of incorporation of nucleotide into nucleic acid, thereby stimulating a compensatory increase in *de novo* synthesis (5), is more consistent with the present data. The inhibition of polynucleotide adenine labeling might reasonably be attributed to a simple isotope dilution effect, which is suggested by the consistent decrease in acid-soluble adenine specific activity, and by the results of a single experiment in which total acid-soluble adenine was measured and found to be elevated in response to ATDA. The almost quantitative inhibition of polynucleotide guanine labeling on the other hand, cannot be explained in terms of dilution. The 10- to 20-fold increase in the soluble guanine pool, which would be required for such an effect, was never manifested by a significant in-

crease in the absorption of the guanine spot (which is ordinarily too small to be precisely quantitated) on the chromatogram of the acid-soluble fraction. Assuming, however, that the usual ratio of total hydrolase and deaminase activities with respect to guanine and adenine compounds (18–20) obtains in the chick, the apparent failure of guanine and/or its soluble anabolites to accumulate in the absence of incorporation into polynucleotide is not surprising. This apparently rapid turnover of soluble guanine components suggests that in the chick embryo, as in man (1), the hydrolysis and deamination of guanine compounds is the major pathway constituting the shunt mechanism. Since the present experimental design precludes the measurement of soluble oxypurines and guanine, inhibition of the IMP → XMP → GMP interconversion by ATDA is not excluded as a possible explanation of the present results. Indeed, the incorporation of ATDA into pyridine nucleotide (21), the specific requirement for NAD by IMP dehydrogenese, and the well known reversal of the ATDA effect by nicotinamide, all suggest such a possibility.

At the dosage levels employed in these studies, 4-HPP has been reported to inhibit urate production in the chick embryo almost quantitatively over the period 8–14 days' incubation, and to a progressively lesser extent thereafter (15). The magnitude of the acute inhibition of urate labeling in the present experiments is consistent with that predictable from available data for the period 13–14 days, and confirmed by direct measurement of 24-hr increments in total urate synthesized.

Of greater interest is the complete lack of effect of HPP upon polynucleotide and acid-soluble purine labeling in either control or ATDA-treated embryos. On the one hand, a decreased labeling might be anticipated in light of the proposed pseudofeedback inhibition of *de novo* synthesis by 4-HPP (16). On the other, an increased labeling would be expected in view of the known ability of 4-HPP to increase the utilization of preformed oxypurine via the salvage pathway in mammals (11, 12). The absence of such an effect, together with the failure of added purine bases to dilute polynucleotide purine labeling, suggest that the chick embryo possesses an inadequate salvage pathway for oxypurine bases. Consistent with this concept is the inactivity in the embryo of 6-mercaptopurine (5), which is known to block avian glutamine PRPP amidotransferase (16) and adenylosuccinate synthetase (22), but only after conversion to its ribotide by IMP pyrophosphorylase (23). Similarly, the inhibition of purified 5-phosphoribosylamine synthetase is not effected by 4-HPP itself, but by its nucleotide, which is formed via IMP pyrophosphorylase (16). Thus, a quantitative, or even qualitative difference with respect to salvage pathway activities might exist between the avian embryo and mammalian systems. Teleologically, the absence of an anabolic sytsem opposing a pathway with so high a capacity for catabolism does not appear unreasonable. Studies of the utilization of preformed purine bases in eggs, in the presence of xanthine oxidase inhibitors have therefore been undertaken in an attempt to clarify this point.

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