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# *De novo* purine synthesis in cultured rat embryos undergoing organogenesis

(glycine incorporation/purine base incorporation/serum enzymes)

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ABSTRACT **The cultured rat embryo undergoing organogenesis (9.5–11.5 days of gestation) together with its associated yolk sac synthesize purine nucleotides via the *de novo* synthetic pathway. Although both the embryo and its yolk sac contain significant levels of the purine base salvage enzymes adenine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase, the culture medium that consists largely of rat serum contains no measurable quantities of salvageable purine bases or nucleosides but high activity levels of purine catabolic enzymes. Short-term pulse–chase experiments with adenine and guanine, carried out under virtually serum-free conditions, confirmed that purine base salvage mechanisms were active and that there was no significant net transfer of purines between the embryo and its yolk sac. A comparison between the specific radioactivities of the [$^{14}$C]glycine added to the culture medium for the studies of the *de novo* synthetic pathway and the purine bases in both the cellular nucleotides and the nucleic acids indicated the existence of a large glycine pool, which almost certainly was derived from the degradation of medium serum proteins by the yolk sac. Although there are no clear-cut data available on the *in vivo* plasma levels of purines that could be potentially utilized to meet the demands of the embryo, it is evident that the *de novo* pathway is adequately developed to meet these needs.**

Rat embryos explanted at the early head-fold stage (9.5 days of gestation) maintain rates of growth, organ differentiation, and protein synthesis over 48 hr that are indistinguishable from those *in vivo* (1). Over this time period, the embryo develops from the relatively undifferentiated neurula stage with 0–2 somites to almost the tail bud stage with 24 or 25 somites (Fig. 1 *A* and *B*). The tissues become segregated into the primordia of the neural, cardiac, circulatory, and hepatic organs. These changes are associated with rapid growth of the yolk sac. Cell number increases approximately two orders of magnitude, the embryo containing $\approx 3 \times 10^6$ cells and the yolk sac $\approx 0.7 \times 10^6$ cells at 11.5 days (2).

During this stage of rodent development *in vivo* the yolk sac is the major organ for the transfer of material between mother and fetus as the vascular connection via the chorioallantoic placenta has not yet been established. The yolk sac surrounds the embryo and, with Reichert's membrane intervening, is virtually in direct contact with the uterine blood sinusoids. The cells lining the yolk sac are continuous with those that form the fetal foregut and histologically resemble those of intestinal and proximal renal tubular epithelium, cells which are designed for absorptive, digestive, and secretory functions (3). The nutrient role of the yolk sac is further increased by the development of the yolk sac circulatory system, which feeds into the developing embryonic circulation. In cultured embryos the primitive foregut remains exposed to the culture medium for the first 24 hr—i.e., from 9.5 to 10.5 days of gestation.

There is a high rate of synthesis of purine nucleotides during embryonic growth. The preimplantation mouse embryo contains high levels of activity of both purine salvage enzymes hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) and adenine phosphoribosyltransferase (EC 2.4.2.7). After implantation the levels of activity increase in parallel with the embryonic weight (4). These enzymes provide an ideal mechanism for the potential salvage of purine bases from oviduct and uterine fluids prior to implantation. However, rodent serum contains significant purine catabolic enzymatic activity, including that of adenosine deaminase (EC 3.5.5.5), purine nucleoside phosphorylase (EC 2.4.2.1), guanine deaminase (EC 3.5.4.3) (5), and xanthine oxidase (EC 1.2.3.2.) (6). This raises a significant question about the nature of and the supply of preformed purine bases or nucleosides from the maternal circulation to the embryo.

In this study we have shown that under culture conditions in which no preformed purine bases are available, the rodent embryo and its surrounding yolk sac synthesize purine nucleotides *de novo* and there is no net purine transfer between them at this stage of development. Both the embryo and the yolk sac are capable of purine base salvage but this is limited by the purine catabolic enzyme activity in the serum of the culture medium. This study also confirms that the degradation of serum proteins in the yolk sac is a major source of amino acids for embryonic growth and development.

## MATERIALS AND METHODS

**Embryo Culture.** The embryo culture technique was based in that originally described by New *et al.* (7). Pregnant Sprague–Dawley rats were killed 9½ days after overnight mating (12 noon of the day when sperm was present in a vaginal smear was taken as day 0.5 of gestation). Each uterus was removed and washed in sterile Tyrode's solution. Under sterile conditions, embryos were freed from maternal decidua, keeping the ectoplacental cone intact, and the surrounding Reichert's membrane was split and removed. Embryos at the head-fold stage (Fig. 1*A*) were selected and cultured in groups of three per glass bottle containing 1.8 ml of centrifuged, heat-treated (30 min, 56°C), and filtered (0.45-$\mu$m filter) rat serum; 0.1 ml of sterile water; and 0.1 ml of antibiotics (streptomycin at 0.6 mg/ml and penicillin at 6 $\mu$g/ml). Bottles were sealed with rubber stoppers and rolled at 30 rpm at 37°C. Culture medium was gassed with 5% $O_2$ in 5% $CO_2$ in nitrogen for 10 min prior to the addition of embryos. Subsequent 1-min gassings were 5% $O_2$ at 3 hr, 20% $O_2$ at 19 hr, and 30% $O_2$ at 27 and 43 hr.

After culture for varying time periods, embryos within their yolk sacs (Fig. 1*B*) were washed three times in 0.9 M saline at

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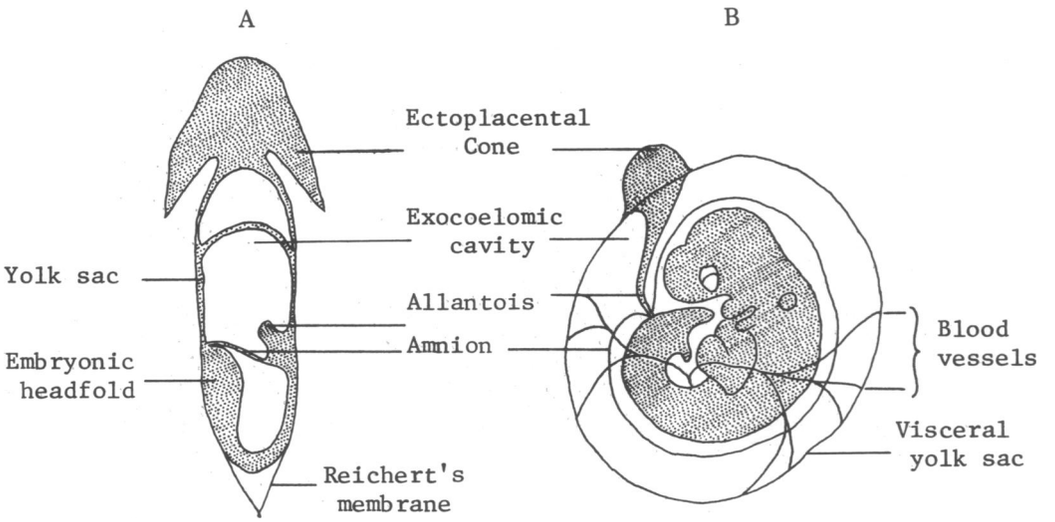


FIG. 1. (*A*) Head-fold-stage embryo. The actual length is 1 mm and the protein content is 30 μg of protein per embryo. (*B*) Embryo after 48 hr of culture. The actual diameter of yolk sac is 4–5 mm and the protein content is 200–250 μg. The embryo contains 190–220 μg.

37°C. The yolk sac was pierced and the exocoelomic fluid together with the relatively small volume of amniotic fluid were drawn off through a micropipette. The yolk sac and embryo were separated by microdissection and washed with ice-cold saline. This wash was added to the exocoelomic fluid, to which was added perchloric acid to a final concentration of 1.0 M. The embryos and yolk sacs were processed in groups of three. After sonication in ice-cold 1.0 M perchloric acid, the acid-soluble phase, total nucleic acids, and protein were separated by the method of Shibko *et al.* (8). Part of the acid-soluble-phase nucleotides and the nucleic acids was hydrolyzed to base level in 12.0 M perchloric acid at 96°C for 1 hr.

**Purine Analysis.** Analysis of the acid-soluble phase nucleotides was carried out by reversed-phase paired-ion HPLC (9), whereas purine bases were analyzed with a different paired-ion system (10). *De novo* purine synthesis was measured by the rate of incorporation of [1-$^{14}$C]glycine into acid-soluble and nucleic acid purine nucleotides from the medium containing 360 μM glycine with a specific activity of 5.7 Ci/mol (1 Ci = 3.7 × $10^{10}$ Bq). The concentration of glycine and other amino acids was measured by gas/liquid chromatography (11).

**Purine Base Salvage.** These studies required modification of the culture system due to the presence of purine catabolic enzymes in the serum-based medium. After 20 hr of routine culture, the embryos were transferred, without washing, to an equivalent volume of RPMI 1640 medium containing 0.25% bovine serum albumin and either [8-$^{14}$C]adenine or [8-$^{14}$C]guanine for 3 hr, the maximal period permissible to achieve continued normal development. Some embryos were immediately processed while others were returned to the original culture medium for a further 3 or 6 hr prior to processing. Embryos transferred to this largely artificial medium earlier in development failed to survive, whereas longer periods of transfer resulted in abnormal development. During the time of the pulse the embryonic foregut is still in direct contact with the culture medium.

**Enzyme Assays.** Hypoxanthine and adenine phosphoribosyltransferase activities in embryonic tissue were assayed by the micromethod described by Epstein (4) with the modification that product identification was carried out on polyethylenimine cellulose thin-layer plates developed with 2% NaCl. Serum assays were carried out with unbuffered culture medium. Xanthine oxidase and xanthine dehydrogenase were measured by the rate of oxidation of 0.4 mM [8-$^{14}$C]hypoxanthine in the absence and presence of 0.4 mM NAD with subsequent product analysis on cellulose thin-layer plates developed with butanol/acetic acid/water (5:3:2). Adenine oxidation by xanthine oxidase was measured at 0.4 mM adenine with analysis of the products, 8-OH adenine and 2,8-dihydroxyadenine, by HPLC (10). Guanine deaminase was assayed by measuring the rate of degradation of 0.4 mM [8-$^{14}$C]guanine with product analysis on the cellulose thin-layer system. The end product was uric acid as a result of the presence of high levels of xanthine oxidase activity. Adenosine deaminase and nucleoside phosphorylase were measured by the rate of conversion of 0.4 mM [8-$^{14}$C]adenosine or 0.4 mM [8-$^{14}$C]inosine, respectively, to uric acid. Product analysis was again undertaken on cellulose thin-layer plates.

## RESULTS

***De Novo* Purine Synthesis.** The incorporation of glycine into the acid-soluble phase, the nucleic acids, and protein of the exocoelomic fluid, the yolk sac, and the embryo throughout the 48-hr culture period is shown on Table 1. HPLC analysis of the unhydrolyzed acid-soluble phases revealed that most of the isotope was present as glycine in the exocoelomic fluid with small amounts in AMP and GMP. However, in the acid-soluble phase of both yolk sac and embryo, the radioactivity was more evenly distributed between glycine and the purine nucleotides, predominantly in ATP and GTP. This was confirmed by HPLC analysis after acid hydrolysis (Table 2). The only major difference between the acid-soluble phases of the yolk sac and the embryo was that there was more extensive labeling of the guanine nucleotide pool in the embryos at the expense of the free glycine pool. The specific radioactivity of adenine and guanine

Table 1. Incorporation of [1-$^{14}$C]glycine into embryonic tissues

| Tissue | Incorporation, nmol per embryo | Isotope distribution, % | | |
|---|---|---|---|---|
| | | Acid soluble | Nucleic acid | Protein |
| Exocoelomic fluid | 3.4 | 91.8 | 2.5 | 5.7 |
| Yolk sac | 11.5 | 30 | 19 | 51 |
| Embryo | 5.6 | 30 | 20 | 50 |

Data are from a typical experiment in which two flasks, each containing three embryos, were cultured for 48 hr.

Table 2. Distribution of [$^{14}$C]glycine in hydrolyzed acid-soluble phases

| Tissue | Distribution, % | | |
|---|---|---|---|
| | Adenine | Guanine | Glycine |
| Exocoelomic fluid | 1.75 | 1.75 | 96.5 |
| Yolk sac | 28.5 | 27.8 | 43.7 |
| Embryo | 29.5 | 38.1 | 32.4 |

Part of the acid-soluble phase from the experiment shown in Table 1 was hydrolyzed in 12 M perchloric acid for 1 hr at 96°C and analyzed by HPLC.

in the yolk sac-soluble phase was of the order of 1/10th of that of the medium glycine, whereas in the embryo-soluble phase it was 1/15th.

In the nucleic acid fractions of both embryo and yolk sac the isotope was evenly distributed between adenine and guanine and the specific radioactivity was identical with that of their respective soluble phase purines.

**Purine Salvage.** In the rat serum that was essential for the growth of cultured rat embryos, no salvageable purine bases or nucleosides—adenine, guanine, hypoxanthine, xanthine, adenosine, inosine, or guanosine—could be detected by HPLC. This was not surprising in view of the high levels of purine catabolic enzymes present. Assayed directly in the medium used for culture, serum contained (in nmol/ml per hr) xanthine oxidase (with hypoxanthine as substrate), 696; xanthine oxidase (with adenine as substrate), 30; guanine deaminase, 346; adenosine deaminase, 124; and purine nucleoside phosphorylase, 57. No xanthine dehydrogenase activity was detected.

The embryos, at head-fold stage, contained significant levels of the purine salvage enzymes hypoxanthine phosphoribosyltransferase (1.48 nmol per embryo per hr with hypoxanthine as substrate; 1.29 nmol per embryo per hr with guanine as substrate) and adenine phosphoribosyltransferase (0.39 nmol per embryo per hr). After 24 hr of culture there was an ≈10-fold increase in the total activity of both enzymes, with the activity being fairly evenly distributed between the embryo and the yolk sac.

In a largely defined medium, which nevertheless contained a small amount of residual serum, both embryo and yolk sac incorporated adenine into purine nucleotides and nucleic acids (Table 3). Although most of the radiolabel was present in adenine nucleotides, 10% was detected in guanine nucleotides, indicating that AMP was cycling through IMP to GMP. The relatively lower incorporation into the embryo is probably secondary to its relatively lower surface exposure and its progressive isolation from direct contact with the medium at the time of the isotope pulse. The pulse–chase phase in this experiment illustrated that there was no net transfer of labeled purine between embryo and yolk sac but merely a progressive shift from the acid-soluble nucleotides into the nucleic acids (Table 3). The ratios of the distribution of the radiolabel between the soluble phase and the nucleic acids were 4.3, 1.16, and 0.82 for the yolk sac at 23, 26, and 29 hr, respectively. The equivalent ratios for the embryo were 3.5, 1.25, and 0.86. There was negligible oxidation of the adenine by xanthine oxidase in the residual serum in the medium during the course of the 3-hr pulse. A similar result was obtained with [C-$^{14}$]guanine, although the rate of incorporation was lower due to the catabolism of 50% of the guanine to uric acid by the residual serum in the culture medium.

Table 3. Adenine incorporation by embryonic tissues

| Tissue | At 23 hr | | At 26 hr | | At 29 hr | |
|---|---|---|---|---|---|---|
| | Soluble phase | Nucleic acids | Soluble phase | Nucleic acids | Soluble phase | Nucleic Acids |
| Yolk sac | 1.33 | 0.31 | 0.84 | 0.72 | 1.03 | 1.26 |
| Embryo | 0.25 | 0.07 | 0.20 | 0.16 | 0.18 | 0.21 |

In this experiment nine embryos were cultured for 20 hr in the serum-based medium. After transfer to RPMI 1640 containing 0.25% bovine serum albumin, they were pulsed for 3 hr with 50 μM [8-$^{14}$C]adenine. One group of three embryos was then processed as described in the *Materials and Methods*, whereas the other two groups of three were returned to the original serum medium for a further 3 or 6 hr of development prior to processing. Data on exocoelomic fluid are not included because of significant cellular contamination that occurred during the extraction of its relatively small volume at this stage of embryonic development.

## DISCUSSION

There is still considerable debate concerning the relative utilization of *de novo* synthetic and salvage pathways for purine nucleotide synthesis by different types of mammalian cells. Most cultured mammalian cell lines are capable of *de novo* purine synthesis but these cells often do not resemble, biologically, their cells of origin.

The original hypothesis that the liver is the major site of *de novo* synthesis in mammals, providing purine bases or nucleosides for export to other tissues (12), has been questioned for some years. Several tissues, including isolated perfused heart (13) and isolated skeletal muscle (14), and lectin-transformed human peripheral blood lymphocytes (15) are capable of *de novo* synthesis. This does not preclude the relative importance of the purine salvage system, but the nature of the purines available in the blood or plasma will vary between species due to the presence of purine catabolic enzymes in the circulation. The serum of most animal species contains adenosine deaminase, nucleoside phosphorylase, and guanine deaminase, whereas xanthine oxidase, also present in most species, is not present in the serum of man, sheep, or pig (6). Accordingly, it is very difficult to assess *in vivo* serum levels of purine nucleotide catabolites, as there are rapid changes both qualitatively and quantitatively once blood is withdrawn from the circulation (16, 17). In these experiments there was an insufficient amount of any salvageable purine catabolite available in the culture medium for embryonic development.

Studies on the energy metabolism of rodent embryos undergoing organogenesis have been summarized by New (1). Three important changes occur during this period: (*i*) a decrease in the relative contribution of the glycolytic pathway, which up to this point has been the major energy source; (*ii*) a concomitant decrease in the activity of the pentose shunt; and (*iii*) an increase in activity of the Krebs cycle and the electron transport system with its higher net energy yield. One might expect that the purine salvage pathway might be more efficient in terms of energy utilization, but it is clear that, *in vitro*, the *de novo* synthetic pathway is the major mechanism of purine nucleotide synthesis for both the embryo and its yolk sac.

It would have been anticipated that, given the huge increase in cell number, the specific activity of the purine bases in both soluble-phase nucleotides and the nucleic acids would have been equivalent to that of the medium glycine with some reduction due to glycine synthesis from serine and possibly from threonine. The observation that the purine base specific radioactivity is greatly reduced to levels of 1/10th and 1/15th in the yolk sac and the embryo, respectively, of the medium glycine indicates that another major source of glycine is available. This is almost certainly derived from the extensive proteolysis that is known to take place in the yolk sac (18, 19) and may at least partly explain not only the high medium serum requirement

but the relative unimportance of free amino acids for embryonic development *in vitro* (20).

It would appear that, at this stage of embryonic development at least, there is little interchange of purine bases between the embryo and its yolk sac, as evidenced by the adenine pulse–chase studies. This was confirmed by the difference in purine base specific activity between the embryo and yolk sac, implying the existence of separate glycine pools.

The question relating to which cells in either the yolk sac or the embryo are capable of *de novo* purine synthesis remains to be answered. Although the yolk sac contains both the hemopoetic cells of the blood islands and the endothelial cells of its developing circulatory system, it would not be unexpected for its basic structural secretory type cells to possess this synthetic capacity as they are initially continuous with the primitive foregut from which the embryonic liver is derived. While the liver is at an early developmental stage, it is possible that other differentiated embryonic cells are also synthesizing purine nucleotides.

There are still major technical problems in maintaining embryos in culture from blastocyst to the limb bud stage of development (21), but it is evident that this approach has a great deal to offer as an experimental tool for the study of the biochemistry of cell differentiation and organogenesis.

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