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Serine metabolism in rat embryos undergoing organogenesis

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SUMMARY

Rat embryos (9·5 days gestation) were cultured for 48 h in heat-inactivated homologous serum containing [3-^{14}C] serine. Analysis of the distribution of the radioactive label in the conceptus demonstrated that almost one half of the incorporated serine was cleaved to provide one-carbon units for the synthesis of purine and pyrimidine nucleotides. Analysis of the free amino acids in the serum, the exocoelomic fluid and the cells of the yolk sac and the embryo showed that there was a variably selective increase in the concentration of amino acids in the exocoelomic fluid compared with the serum and a significant decrease in the specific radioactivity of the free serine within the conceptus which was the highest in the yolk sac and lowest in the embryo. These findings would support the concept of yolk sac lysosomal degradation of medium serum protein as a major source of amino acids supplying almost 86% of that required during this phase of embryonic development.

The specific radioactivity of purine bases in cellular nucleotides, RNA and DNA was similar to that of the free serine in both yolk sac and embryo. This indicated that an alternative, as yet unidentified source of one-carbon units was available for purine nucleotide biosynthesis.

Analysis of the cellular purine nucleotides revealed low ATP/GTP ratios in both the embryo and its yolk sac and this may be related to the undifferentiated state of many of the cells of the conceptus.

INTRODUCTION

The development of techniques for the culture of rat embryos undergoing organogenesis (9·5 to 11·5 days gestation) has provided a useful tool for the study of organ differentiation and for the evaluation of potential mechanisms of action of teratogenic agents (New, 1978; Freinkel *et al.* 1984). Despite the current widespread use of this technique, relatively few basic physiological and biochemical data are available.

Some years ago Beck, Lloyd & Griffiths (1967) proposed that disturbance of yolk sac-mediated embryotrophic nutrition resulted in teratogenic effects on the developing rat. More recent studies (Freeman, Beck & Lloyd, 1981; Freeman & Lloyd, 1983*a*) have confirmed the existence of and clarified to some extent the nature of this nutritional pathway and its susceptibility to modification by the teratogens anti-yolk sac antiserum (Freeman, Brent & Lloyd, 1982) and the lysosomal protease inhibitor leupeptin (Freeman & Lloyd, 1983*b*). This nutritional

Key words: in vitro culture, serine metabolism, one carbon metabolism, rat embryo, amino acids.

pathway involves the yolk sac lysosomal proteolysis of medium serum proteins as a major source of the amino acids required for the rapid growth and development of the conceptus.

We have recently shown, using [1-^{14}C] glycine, that the cultured rat embryo and its associated yolk sac (Fig. 1) synthesize purine nucleotides exclusively via the *de novo* pathway rather than by the salvage of preformed purines (Rowe & McEwen, 1983). The relatively lower specific radioactivity of the purine bases in both the yolk sac and the embryo in comparison with that of the medium isotope suggested the existence of a large glycine pool which was probably derived from the proteolytic degradation of the medium serum protein by the yolk sac.

In this study an analysis has been made of the incorporation of [3-^{14}C] serine by cultured rat embryos undergoing organogenesis. Amino acid analysis has determined the free amino acid content of the culture medium, the yolk sac, embryo and the exocoelomic fluid as well as the specific radioactivity of serine in each component. Similar determinations were made on the serum and cellular proteins. A detailed analysis was undertaken of the content and specific radioactivity of purine bases and thymine in the soluble phases and the nucleic acids of the conceptus.

The overall aim of the study was to directly quantify the extent to which yolk sac proteolysis contributed to the amino acid supply and the extent to which the 3-carbon atom of serine was responsible for the supply of essential one-carbon units required for the synthesis of purine nucleotides.

MATERIALS AND METHODS

The embryo culture technique has been described previously in detail (Rowe & McEwen, 1983). Briefly, embryos were selected at the head-fold stage and cultured in groups of three per glass bottle containing 1·8 ml of centrifuged heat treated and filtered rat serum, 0·1 ml of sterile water, 0·4 μCi [3-^{14}C] serine and 0·1 ml of antibiotics (streptomycin at 0·6 mg ml^{-1} and penicillin at 6 μg ml^{-1}. Bottles were sealed with rubber stoppers and rolled at 30 r.p.m. at 37 °C.

After 48 h culture the embryos within their yolk sacs (Fig. 1) were washed twice in 0·9 M-NaCl. Exocoelomic fluid was withdrawn via a microsyringe and perchloric acid added to a final concentration of 1·0 M. The yolk sac and the amniotic membrane were dissected free of the embryo retaining the amniotic fluid. All tissues were washed in ice-cold NaCl and placed in 1·0 M-perchloric acid at 0 °C. After sonication, the acid-soluble phase, RNA, DNA and protein were separated by the method described by Shibko *et al.* (1967). Half of the acid-soluble phase was retained for amino acid analysis while the nucleotides in the remaining half, after HPLC analysis, and the nucleic acids were hydrolysed to base level in 12·0 M-perchloric acid at 96 °C for 1·0 h. The protein fractions from the embryos, yolk sacs and the culture medium were hydrolysed under vacuum in constant boiling hydrochloric acid for 20 h at 110 °C.

Analyses of the purine nucleotides and purine and pyrimidine bases were carried out by reverse-phase paired-ion HPLC (McCairns, Fahey, Sauer & Rowe, 1983). Amino acids were determined by gas chromatography after acylation of the amino groups with hepta-fluorobutyric acid (Desgres, Boisson & Padeau, 1979). Although this method is very sensitive, glutamine and asparagine are converted to glutamic and aspartic acids respectively, tryptophan is partially hydrolysed, cysteine is oxidized to cystine and histidine is not effectively recovered as a result of incomplete derivatization.

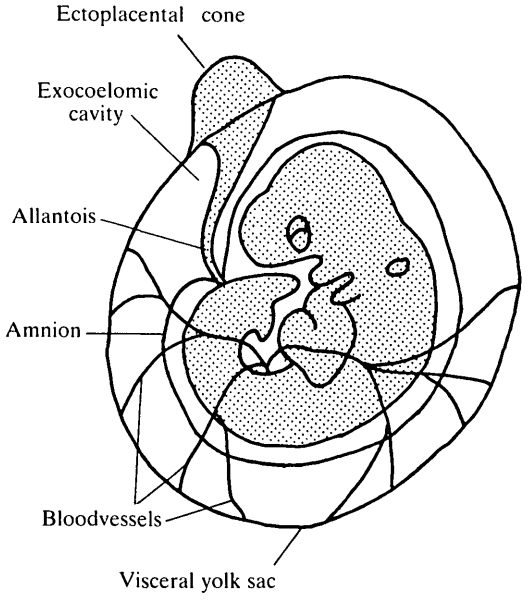

Fig. 1. Rat embryo after 48 h in culture. The embryo contains approximately $3{\cdot}0 \times 10^6$ cells and the yolk sac $0{\cdot}7 \times 10^6$ cells an increase of some two orders of magnitude over the period of organogenesis. (Brown, Goulding & Fabro, 1979).

RESULTS

Amino acid analysis of the culture medium and the exocoelomic fluid (Table 1) revealed a large concentration gradient across the yolk sac. Based on this analysis of 15 amino acids there was a mean overall gradient of 4·5. The relative concentration of individual amino acids in the exocoelomic fluid when compared with that in the culture medium was quite variable ranging from 8·9 for proline through 6·7 for serine down to 1·1 for arginine. While the proportion of serine, proline, aspartic acid and lysine was relatively increased in the exocoelomic fluid, that of alanine, glycine, arginine and glutamic acid was reduced. Amino acid analysis of the total serum proteins (data not shown), which are presumed to supply most of the amino acids, did not provide any guide to these selective changes. In the serum proteins the proportion of valine, leucine, aspartic acid, glutamic acid and arginine was higher while that of serine, proline and lysine was in fact lower when compared with the composition of the amino acids in the exocoelomic fluid. The amino acid compositions of the total proteins of the serum, the embryo and the yolk sac were virtually identical.

The distribution of radioisotope into the various components of the conceptus following 48 h exposure to medium containing [3-^{14}C] serine is illustrated in Table 2. It can be calculated that 17·6 % of the isotope from the medium was incorporated, representing 25·5 nmol of serine per conceptus.

In the acid-soluble phase of the embryo 24 % of the radiolabel was in serine with 39·5 % and 36·5 % in guanine and adenine nucleotides respectively. In the yolk sac

Table 1. *Amino acid concentrations and distribution in the culture medium and exocoelomic fluid**

Amino acid	Culture medium		Exocoelomic fluid	
	Concentration	%	Concentration	%
Ala	474	18·9	1763	15·7
Arg	91	3·6	104	0·9
Asp	51	2·0	350	3·1
Glu	275	11·0	933	8·3
Gly	269	10·7	920	8·2
Ile	65	2·6	245	2·2
Leu	194	7·7	833	7·4
Lys	212	8·5	1302	11·6
Met	15	0·6	40	0·3
Phe	135	5·4	382	3·4
Pro	152	6·1	1346	12·0
Ser	187	7·5	1253	11·1
Thr	143	5·7	605	5·4
Tyr	111	4·4	430	3·8
Val	130	5·2	726	6·5
Total	2504		11233	

* Concentration is expressed as μM. The values for the culture medium were obtained at the end of the culture period. These did not differ significantly from those at the beginning, any loss of amino acids as a result of embryonic uptake being compensated for by the fluid loss during the gassing procedures. The average volume of exocoelomic fluid for each embryo was 21 μl in this study but in other studies this was as low as 12 μl.

soluble phase the distribution was 18 % in serine and 36·1 % in guanine and 45.9 % in adenine nucleotides. In the acid-soluble phases of both the exocoelomic and amniotic fluids 94 % of the label was present as serine.

In the overall detailed analysis of the distribution of radiolabel in the embryo, soluble phase (7·3 %) and protein (35 %) serine constituted 42·3 % with the remainder in the soluble phase purines (23·1 %), in RNA purines (8·8 %) and in DNA purine bases and thymine (25·8 %).

In a similar analysis of the radiolabel distribution in the yolk sac, soluble phase (4·2 %) and protein (45 %) serine constituted 49·2 % with the residue in purine bases in the soluble phase (19·1 %) and in RNA (13·4 %) and in DNA purine bases and thymine (18·3). Of the total serine incorporated by all phases of the culture system one half was utilized to provide one-carbon units for purine and pyrimidine nucleotide synthesis.

The specific radioactivity of serine in each of the components of the conceptus (Table 3) showed that there was a fourfold dilution of the isotope when it entered the cells of the yolk sac and that further dilution occurred within the exocoelomic fluid. The serine specific radioactivity in the embryo was similar to that in the exocoelomic fluid. It would appear that this dilution occurred within the yolk sac.

Table 2. *Incorporation of [3-^{14}C] serine into components of the conceptus**

	Acid soluble	RNA	DNA	Protein	%
Embryo	99692	29006	84875	115067	28·8
Yolk sac	132724	76056	103873	255504	49·8
Exocoelomic fluid	158838	6640	6630	17500	16·6
Amniotic fluid	28720	4485	1879	8540	3·8

* The culture medium for 20 embryos in this experiment contained 2·9 μCi of [3-^{14}C] serine at a specific activity of 1·0 Ci Mole^{-1}. The residual 1% of radioactivity was incorporated into amniotic membrane. Incorporation is expressed as d.p.m.

Table 3. *Serine content and specific radioactivity in various phases of the conceptus**

Phase	Specific radioactivity Ci.Mol^{-1}	Content nmol.20 embryos^{-1}
1. Culture medium	1·00	2390
2. Yolk sac acid soluble	0·24	45
3. Yolk sac protein	0·15	786
4. Exocoelomic fluid	0·12	530
5. Embryo acid soluble	0·13	82
6. Embryo protein	0·11	471

* These values were based on the same experiment as for Table 1.

The specific radioactivity and content of the purine bases and thymine in the soluble phase nucleotides, RNA and DNA are shown in Table 4. As expected the specific activity of the purine bases either as the free nucleotides or in the nucleic acids was virtually identical both in the embryo and the yolk sac. The significant difference between the levels in the embryo and the yolk sac reflected the difference in the specific activity of the free serine pools (Table 3). Thymine specific activity was approximately half that of the purine bases.

In order to assess the relative contribution of other potential sources of one carbon groups equivalent studies were undertaken with [2-^{14}C ring] histidine, [2-^{14}C] glycine and [methyl-^{14}C]choline. No radiolabelling of thymine was observed with any of these suggesting that there was little catabolism of any of these compounds at this stage of embryonic development. A study with [2-^{14}C ring] tryptophan (results not shown) indicated that tryptophan breakdown did occur but the contribution to the one-carbon pool from this source was only one quarter that of serine.

HPLC analysis confirmed that most of the soluble-phase purines were present as their nucleoside triphosphate derivatives and accordingly the adenine/guanine purine base ratios largely reflected the ATP/GTP ratios. These ratios of 0·95 and 1·3 (derived from Table 4) in the embryo and the yolk sac respectively are very low but are similar to those observed but not discussed in an earlier study (Rowe & McEwen, 1983).

Table 4. *Specific radioactivity and content of purine and pyrimidine bases in soluble phase nucleotides and nucleic acids in embryo and yolk sac**

		Adenine		Guanine		Thymine	
		SA	Content	SA	Content	SA	Content
Embryo	Nucleotides	0·13	126	0·13	133		
	RNA	0·135	27	0·14	72		
	DNA	0·13	133	0·125	110	0·06	123
Yolk sac	Nucleotides	0·24	115	0·25	87		
	RNA	0·24	55	0·26	75		
	DNA	0·23	84	0·23	80	0·12	85

* These values were based on the same experiment with 20 embryos as for Tables 1 and 3. Specific radioactivity (SA) is expressed as Ci.mole^{-1} and content as nmol.20 embryos^{-1}.

Although these values were the lowest we observed in ten separate studies, the maximum value observed was 2·0.

DISCUSSION

This study has shown the extent to which yolk sac proteolysis contributes to the amino acid supply required for growth and development of the rat conceptus undergoing organogenesis *in vitro*. The total serine content of the embryo, yolk sac and exocoelomic fluid at the end of the 48 h culture period was 95·7 nmol.conceptus^{-1} (derived from Table 3). Of this only 12·7 nmol (13·3 %) can be calculated to have come from the free amino acid pool of the culture medium. Almost 86 % therefore has arisen from yolk sac lysosomal proteolysis of medium serum proteins. Some minor contribution from amino acid biosynthetic pathways (Popp, 1958) cannot, however, be excluded.

There would appear to be compartmentalization of amino acids in the cells of yolk sac as the specific radioactivity of the free serine was significantly higher than that of the cellular protein and this was a little higher again than that observed in the exocoelomic fluid. Alternatively or additionally, the relatively lower specific radioactivity of the yolk sac proteins could be the result of dilution by the medium serum protein undergoing degradation within the yolk sac lysosomes (Fridhandler & Zipper, 1964; Williams, Lloyd, Davies & Beck, 1971).

There did not appear to be any equilibration of the amino acid pools in the medium and the exocoelomic fluid. The final specific radioactivity of the medium serine was similar to that at the outset as was the overall relative concentration and distribution of the other amino acids.

The relatively high amino acid concentration gradient for serine, valine, proline aspartic acid and lysine could not be explained on the basis of the amino acid composition of the serum proteins and this would indirectly support the concept of

some selectivity in the degradation of these proteins within the yolk sac (Freeman, Beck & Lloyd, 1981; Priscott, Gough & Barnes, 1983). The relatively large size of the exocoelomic amino acid pool would indicate that this constitutes a major reservoir for the various embryonic biosynthetic pathways. Although the average volume of exocoelomic fluid was 21 μl in the study shown on Table 1, there was considerable variation to as low as 12 μl in other studies. Despite this variation the concentration of individual amino acids remained remarkably constant.

The specific activity of the soluble-phase purine nucleotides in both the embryo and its yolk sac was virtually identical with that of the free cellular serine. If the 3-carbon atom of serine was the sole one-carbon source for purine biosynthesis, the purine bases should exhibit twice the specific activity of free serine. Accordingly serine supplies a little more than 50 % of the essential one-carbon units. There is clearly another major source yet to be identified.

Studies with histidine, choline and glycine demonstrated that these were not alternative sources of one carbon units. As glycine cleavage is located within the mitochondrion, it was not surprising that the 2-carbon atom of glycine was not available as mitochondrial function is just being established at this stage of embryonic development (New, 1978). Studies with tryptophan showed that, although it is clearly a significant source of one-carbon units, it cannot account for the balance.

The difference in purine specific activity between the embryo and the yolk sac confirmed earlier observations (Rowe & McEwen, 1983) of distinct non-interchangeable purine pools in the two major components of the conceptus.

Most of the soluble-phase purines were present as their nucleoside triphosphate derivatives. Although there is significant variation in ATP/GTP ratios even in the same type of cell from different species of the same mammal (Henderson, Zombor, Johnson & Smith, 1983) we are not aware of any studies where the ratio was so low. This finding was not an artifact of the culture system as the same ratio was present in embryos at an equivalent stage of development *in vivo*. In a recent study with a line of myeloid precursor cells (HL-60), (Lucas, Webster & Wright, 1983) have shown that induced cellular differentiation is associated with a rise in the ATP/GTP ratio from 3·6 to 6·0. They have suggested that the size of the intracellular guanine nucleotide pools and the rate of biosynthesis of guanine nucleotides may be central to the regulation of terminal maturation of these cells. The cells of the rodent embryo at this stage of development are still undergoing differentiation and the high levels of guanine nucleotides may be related to this process. While a variety of hypotheses could be proposed to account for this high GTP requirement including demands for high rates of protein synthesis or for the activity of a variety of guanine regulatory proteins for information exchange across cell membranes (Houslay, 1984) these would, at this stage, be purely conjectural.

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