Dihydrofolate Reductase and Thymidylate Synthetase Activities in the Liver of Rabbit Foetuses during the Haemopoietic Period

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Dihydrofolate reductase activity did not change with variations of mitotic rate in foetal rabbit haemopoietic tissue, whereas the activity of thymidylate synthetase declined with decrease in cell divisions. Both the activities were normal in the brachydactylia strain, in which a failure in folate metabolism was previously assumed.

Folate metabolism is known for its implication in nucleic acid synthesis and cell multiplication (Valencia, 1974). In the foetal liver, mitotic activity is particularly high during the period of hepatic erythropoiesis. Therefore it was considered that the enzymic activities of the folate pathway in the foetal liver during this period were worthy of study. Two enzymes were studied: (a) dihydrofolate reductase (EC 1.5.1.3), which catalyses the reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid; this crucial step conditions the biosynthesis of the folate coenzymes, all deriving from tetrahydrofolic acid (Blakley, 1969); (b) thymidylate synthetase (EC 2.1.1.-), which catalyses the reductive methylation of dUMP to dTMP, by using N^5N^{10} -methylene-tetrahydrofolic acid as methyl-group donor; thymidylate synthetase thus plays a vital role in the biosynthesis of DNA (Blakley, 1969).

In the rabbit foetus, hepatic haemopoiesis begins on day 13 of gestation (Jolly, 1923); the number of haemopoietic elements reaches its maximum on days 18–20; after the day 20, maturation of haemopoietic cells predominates, and the hepatic haemopoietic tissue declines (Sorenson, 1963).

Dihydrofolate reductase and thymidylate synthetase activities were determined in the liver of 16- and 20-day rabbit foetuses and of adult rabbits. In addition, these activities were studied in the foetuses of the rabbit brachydactylia (*br*) strain (Greene & Saxton, 1939). These foetuses have macrocytic blood cells that were assumed to be implicated in the teratogenic process occurring *in utero* in this strain (Petter *et al.*, 1973). This macrocytosis is at least partially prevented by treatment of the pregnant female with folic acid plus vitamin B-12 (Petter, 1975). Therefore the possibility of enzymic disturbance of the folate pathway in this strain has been considered.

Materials and Methods

Rabbits of the 'Géant Normand' and 'brachydactylia' strains were used. Since ovulation in rabbits is induced by mating, this time was considered as the

beginning of gestation. Pregnant females were anaesthetized with pentobarbitol and laparotomized. Foetuses were rapidly removed, bled, and the livers excised. The livers of litter-mate foetuses were pooled. In adult animals, assays were made on individual samples. Tissues were homogenized with a glass/Teflon homogenizer in 0.25M-sucrose in 2mM-Tris/HCl buffer, pH 7.5 (1 g of tissue/3 ml of buffer), at 0°C, and centrifuged for 1 h at 35000g in the cold (4°C). Supernatants were kept frozen at -25°C until assayed.

Dihydrofolate reductase was assayed as described by Mathews *et al.* (1963), by measuring the decrease in E_{340} that occurs when NADPH and dihydrofolate are converted into NADP⁺ and tetrahydrofolate. This decrease was automatically recorded by using a u.v./visible spectrophotometer (Beckman M 24) for 5 min periods. Assays were performed at room temperature ($\approx 25^\circ\text{C}$). Enzyme activity was expressed as nmol of NADPH converted/min per mg of protein.

Thymidylate synthetase was tested by the conversion *in vitro* of [^{14}C]dUMP into [^{14}C]dTMP in the presence of formaldehyde and tetrahydrofolate. [^{14}C]dUMP (1 μCi), tetrahydrofolate (10mM), formaldehyde (30mM), 2-mercaptoethanol (0.2M), magnesium chloride (50mM) and Tris/HCl buffer, pH 8.4, were mixed in a total volume of 250 μl . A portion (20 μl) of this mixture was incubated with 10 μl of extract, adjusted to 0.6–0.8 mg of protein/ml, for 30 min at 37°C. The reaction mixture was then chromatographed overnight on Whatman 3MM chromatography paper with propan-2-ol/water/14M-NH₃ (7:2:1, by vol.). dUMP and dTMP were localized with a Geiger counter (Nuclear-Chicago Actigraph III), and the strips cut and eluted for 30 min with 1 ml of water; PCS scintillation fluid (10 ml; Amersham/Searle, Des Plaines, IL, U.S.A.) was then added, and radioactivity determined in a Nuclear-Chicago mark I liquid-scintillation counter. Enzyme activity was expressed as ng of dTMP formed/h per mg of protein.

The protein concentration of the liver extracts was determined by the biuret method (B. Kadenbach, personal communication).

Table 1. *Enzymic activities in foetal and adult liver*

Results are means $\pm$ s.e.m. Numbers in parentheses represent numbers of experiments.

	16-day normal foetuses	20-day normal foetuses	Normal adults	16-day <i>br/br</i> foetuses	20-day <i>br/br</i> foetuses
Dihydrofolate reductase activity (nmol/min per mg)	2.3 $\pm$ 0.3 (4)	1.98 $\pm$ 0.1 (3)	2.3 $\pm$ 0.2 (4)	2.2 $\pm$ 0.5 (4)	1.98 $\pm$ 0.2 (4)
Thymidylate synthetase activity (ng/h per mg)	14 $\pm$ 1 (5)	9 $\pm$ 1 (3)	127 $\pm$ 5 (4)	14.5 $\pm$ 3 (4)	6.5 $\pm$ 0.5 (4)

Results and Discussion

Results are summarized in Table 1. Dihydrofolate reductase activity showed a remarkable constancy; there was no difference between the three stages studied. Values were of the same order as those reported by McCullough & Bertino (1971) for mouse liver and spleen, and by Zielinska *et al.* (1974) for various mammalian cells; they were smaller than those given by Schulz (1972) for foetal rat liver. Schulz (1972) reported variations in this species between foetal and adult animals, such results contrasting with our findings in rabbits.

By contrast, thymidylate synthetase activity varied considerably. It was greater in 16-day- than in 20-day foetuses (statistically significant) in normal as well as in *br/br* rabbit foetuses. The activity was very high in adult rabbits.

There was no difference between normal and *br/br* foetuses either for dihydrofolate reductase or for thymidylate synthetase activities at foetal stages at which macrocytosis and teratogenic processes occur.

The purpose of the present study was to explore whether the high mitotic activity due to hepatic haemopoiesis is reflected in the activity of folate-dependent enzymes. Dihydrofolate reductase does not seem to be affected by variations of mitotic activity in this tissue. Moreover, in the adult liver, which no longer contains haemopoietic tissue, the activity of this enzyme was the same as in the foetus, regardless of the very different nature of the tissue. Dihydrofolate reductase plays a role in the first step of the folate pathway, and synthesis other than that of DNA precursors depends on tetrahydrofolate. This might explain why its concentration is not related to mitosis rate.

Thymidylate synthetase activity is more increased in the liver of 16-day foetuses, at which time haemopoietic cell divisions are numerous, than in 20-day foetuses, at which time the intense mitotic phase is over. dTMP availability directly conditions DNA synthesis, and the variations of thymidylate synthetase activity between 16 and 20 days of gestation

are probably parallel to the dTMP requirement. These observations are consistent with Kohler's (1972) findings that the activities of thymidine kinase and DNA polymerase are high in the developing rat liver (day 18 of gestation until birth) and decline in the early suckling period. The decrease of thymidylate synthetase activity in foetal rabbits occurs even earlier. The possibility that thymidylate synthetase activity plays a role in the regulation of DNA synthesis could be considered.

Erythropoietin has been reported to enhance the synthesis of DNA (Paul & Hunter, 1969) and RNA (Djaldetti *et al.*, 1972) in erythroid cells of foetal mouse liver, and these cells produce erythropoietin *in vitro* (Zucali *et al.*, 1975). It would be interesting to study the effects of erythropoietin on folate-dependent enzymes such as thymidylate synthetase, to test whether the effect of the hormone on erythropoietic cell division is mediated by the activity of these enzymes.

On the other hand, the present study shows that the macrocytosis of the rabbit foetuses homozygous for the *br* gene does not result from a disturbance in dihydrofolate reductase or thymidylate synthetase activities; the possibility of abnormalities in other enzyme systems of the folate pathway remains open.

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