Uridine Kinase in Embryonic Rat Liver

MODULATION OF ENZYME ACTIVITY BY 5-AZACYTIDINE

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Uridine kinase activity in rat liver decreases during embryonic and postnatal development. Administration of 5-azacytidine enhances liver uridine kinase activity in adult rats, but depresses it in embryos. The liver enzymes from the foetus and the adult are precipitated at different $(\text{NH}_4)_2\text{SO}_4$ concentrations although they are eluted at about the same position on chromatography on a column of Sepharose 6B.

Previously we have studied uridine kinase in mouse and adult rat liver under different experimental conditions. Although the activity of the enzyme from livers of leukaemic mice made resistant to 5-azacytidine is diminished in comparison with its counterpart from livers of the parental leukaemic line of mice (Vesely *et al.*, 1970), the activity of uridine kinase from normal adult rat liver is markedly enhanced after the administration of 5-azacytidine (Čihák *et al.*, 1972). Both enzyme preparations are more stable on heating than the respective controls. Further, although the enhancement after administration of 5-azacytidine occurs in the rat liver, uridine kinase activity in the thymus under identical conditions is diminished (Čihák & Vesely, 1973).

It has been observed that thymidine kinases from the foetal liver and from rhabdomyosarcoma differ from their counterparts isolated from adult tissues in various respects, especially by decreased stability on heating (Taylor *et al.*, 1972; Stafford & Jones, 1972). However, two species of uridine kinase have been reported to exist in rat liver, depending on the age of the animals. Species II is the predominant form in foetal rat liver and its stability on heating is apparently greater than that of species I, which is the predominant form in the adult animal (Krystal & Webb, 1971). Consequently we decided to investigate the activity of uridine kinase in rat embryonic liver after the transplacental administration of 5-azacytidine.

Materials and Methods

Materials

Chemicals. 6-Aza[4,5- ^{14}C]uridine (80 mCi/mmol) was delivered by the Institute for Research, Production and Uses of Radioisotopes, Prague, Czechoslovakia. ATP was obtained from Calbiochem, Los Angeles, Calif., U.S.A. Streptomycin sulphate was a product of Jenapharma, Jena, German Democratic

Republic; Sephadex G-200, Sepharose 4B and 6B were purchased from Pharmacia, Uppsala, Sweden.

Animals. Wistar female rats kept under standard conditions were used throughout the experiments. The animals were killed between 08:00h and 10:00h. The rats with positive vaginal smears were isolated and regarded as at day zero of pregnancy. Unless otherwise stated, 5-azacytidine (8 $\mu\text{mol}/100\text{g}$) was injected intraperitoneally 48 and 24h before the animals were killed. The experiments were performed with the 18-day-old foetuses and with the adult animals weighing 160–180g.

Methods

Assay of uridine kinase. The activity of uridine kinase was determined in a standard reaction mixture (0.3 ml) containing (final concentrations) 50 mM-Tris-HCl buffer, pH 7.5, 4 mM-ATP, 2 mM-MgCl₂, 0.1 mM-6-aza[4,5- ^{14}C]uridine and 0.1 ml of enzyme preparation obtained by centrifugation (105000g, 4°C, 120 min) of the sonicated material. The resulting supernatant was used for the assay of the enzyme. The sonicated material was prepared as described below. After 10 min of incubation at 37°C the mixture was heated in a boiling-water bath for 1 min and analysed by descending chromatography on Whatman no. 1 paper in butan-1-ol-acetic acid-water (10:1:3, by vol.). The radioactive zones were cut out by comparison with standards and their radioactivity was counted in a Packard Tri-Carb liquid-scintillation spectrometer after the addition of 10 ml of scintillation fluid [4g of 2,5-diphenyloxazole and 0.15g of 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene made up in 1 litre of toluene]. The rate of phosphorylation was linear over the period studied.

Purification of uridine kinase. The liver was homogenized in a glass homogenizer with a motor-driven Teflon pestle in 4 vol. of 0.01 M-Tris-HCl buffer, pH 7.5, containing 0.25 M-sucrose. The cells were lysed

by ultrasonic disintegration, and further steps of the procedure were essentially as described by Orengo (1969). The sonicated material was centrifuged at 40000g (Spinco, 4°C, 20min), and the resulting supernatant was centrifuged at 105000g (Spinco, 4°C, 120min). The final supernatant was adjusted to 8–10mg of protein/ml with 0.01 M-Tris-HCl buffer, pH7.5, containing 0.1 mM-dithiothreitol, and streptomycin sulphate was added (5% solution, 20ml/100ml of the supernatant). The suspension was stored overnight at 4°C and centrifuged at 40000g for 20min. The resulting supernatant was adjusted to 30% saturation with $(\text{NH}_4)_2\text{SO}_4$. After centrifugation the supernatant fraction was adjusted to 39 or 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ in the adult or embryonic liver, respectively. The sediment was dissolved in 0.01 M-Tris-HCl buffer, pH7.5, containing 0.1 mM-dithiothreitol, and a portion of this fraction (15–20mg of protein) was layered on a Sepharose 4B or 6B column (1 cm $\times$ 50 cm). The elution was carried out with the same buffer that was used for equilibration. The flow rate was 2.5 ml/h and fractions of 1.2 ml were collected. Sometimes the sample was applied to a Sephadex G-200 column (3 cm $\times$ 40 cm), eluted with the buffer described above at a flow rate of 15–20 ml/h; 2.5 ml fractions were collected. The E_{280} was recorded and the enzyme activity of individual fractions was determined as described by Veselý *et al.* (1971).

Fig. 1. Uridine kinase activity from foetal rat liver at different periods of gestation

The enzyme was assayed in a standard reaction mixture which contained 0.1 ml of 105000g liver supernatant fraction (generally 2 mg of protein). The results are expressed as specific activity (nmol of 6-azauridine 5'-phosphate/h per mg of protein) (○) and as total activity (μmol of 6-azauridine 5'-phosphate/h per total liver) (●). The broken line indicates the time of birth.

The fractions with maximal enzyme activity were pooled. All operations were done at 4°C.

Results

Uridine kinase activity in embryonic rat liver during the final stages of gestation is shown in Fig. 1. Although the specific activity of the enzyme falls with the age of the embryo, the total enzyme activity per liver rises. Salting out of the enzyme molecules from embryonic and adult tissue indicated that uridine kinase activity from embryonic liver was precipitated at higher concentrations of $(\text{NH}_4)_2\text{SO}_4$ than its counterpart from adult liver (Fig. 2). This finding was reproduced in three separate experiments carried out on different embryonic litters. Although adsorption of one protein to another is usually slight at high salt concentrations, it cannot be excluded that the range of concentration over which the enzymes are

Fig. 2. Precipitation of uridine kinase from adult (●) and embryonic (○) rat liver at different concentrations of $(\text{NH}_4)_2\text{SO}_4$

Liver supernatant fractions (105000g) were treated with streptomycin; after centrifugation (40000g for 20min) the resulting supernatants were adjusted to the same concentration of enzyme activity and divided into 1 ml portions that were saturated with $(\text{NH}_4)_2\text{SO}_4$ added at the indicated concentrations. After standing for 1 h at 4°C the samples were centrifuged at 40000g for 20min. The sediments were dissolved in 0.2 ml of 0.01 M-Tris-HCl buffer, pH7.5, containing 0.1 mM-dithiothreitol, and used as a source of enzyme activity, which was expressed as nmol of 6-azauridine 5'-phosphate formed.

precipitated may be affected by adsorption of the enzymes to other proteins that differ by their nature and amounts in adult and embryonic livers. After birth uridine kinase molecules were precipitated in 4–5-day-old rats in a manner indistinguishable from that in adult animals. When adult and embryonic liver uridine kinase preparations were compared on a Sepharose 6B column their elution patterns were identical. Essentially the same results were obtained after the application of the enzymes to Sepharose 4B or to Sephadex G-200 columns. However, it should be stressed in this connexion that only about 50% of the total homogenate uridine kinase from embryonic liver was found in the 105000g supernatant fraction (results not shown); thus the failure to demonstrate different molecular forms of embryonic uridine kinase could result from incomplete solubilization.

Whereas 5-azacytidine enhances uridine kinase activity in adult rat liver, in foetal liver it leads to the depression of its activity. Fig. 3 indicates that the diminished activity of the enzyme after 5-azacytidine treatment occurs until the first day of postnatal life. The usual stimulation normally observed in adult animals gradually commences around the third day after birth even though at that time the enhancement is less marked than later on.

Because of the different responses of embryonic and adult rat liver uridine kinase to 5-azacytidine we

Fig. 3. Effect of 5-azacytidine on uridine kinase in embryonic and postnatal rat liver

Groups of two or three pregnant rats were injected intraperitoneally with 8 μ mol of 5-azacytidine/100g body wt. (●) and killed 24h later; neonatal and postnatal rats were injected intraperitoneally with the same dosage of the drug 24h before being killed at various time-intervals after delivery. Each point represents the mean for six to eight animals. Controls (○) received 0.9% NaCl. Conditions of uridine kinase assay were the same as in Fig. 1. The results are expressed as μ mol of 6-azauridine 5'-phosphate/h per g of liver.

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investigated their properties further on a Sepharose 6B column. This was previously employed by other authors for the study of uridine kinase from normal and neoplastic rat liver (Krystal & Webb, 1971), but with a different eluent. The elution pattern of the embryonal enzyme isolated from 18-day-old foetuses treated with 5-azacytidine was similar to that of the control (Fig. 4). Both enzymes were eluted in a single peak centred around the same fraction although the activity of the control was considerably higher. The profile of protein elution was similar in both cases. The pooled fraction with highest activity from both preparations showed the same K_m (0.16mM) when plotted with respect to the phosphate acceptor, and the V_{max} values were 133 and 100nmol of 6-azauridine 5'-phosphate for the control and the treated enzyme preparation, respectively. When crude extract, after the treatment with streptomycin (see under 'Methods'), isolated from normal embryonic livers and its 5-azacytidine-treated counterpart was precipitated with $(\text{NH}_4)_2\text{SO}_4$ as described in Fig. 2, the profiles of enzyme activities at different salt concentrations were in good agreement (results not shown). Thus uridine kinase from embryonic rat

Fig. 4. Chromatography on Sepharose 6B of the 30–50%-satn. $(\text{NH}_4)_2\text{SO}_4$ fraction of cytosol from 18-day foetal rat liver untreated (○) and treated with 5-azacytidine (●)

A portion containing 15–18 mg of protein was layered on a Sepharose 6B column (1 cm $\times$ 50cm) previously equilibrated with 0.01M-Tris-HCl buffer, pH 7.5, containing 0.1mM-dithiothreitol. The elution was carried out with the same buffer and 1 ml fractions were collected at a flow rate of 2.5 ml/h. The enzyme activity of individual fractions was determined in a standard reaction mixture and the results were expressed as nmol of 6-azauridine 5'-phosphate/ml of fraction. —, E_{280} .

Fig. 5. Effect of 5-azacytidine on uridine kinase in adult rat liver at different ages

The drug ($8 \mu\text{mol}/100 \text{ g}$ body wt.) was administered intraperitoneally 24h before the animals were killed. Conditions of uridine kinase determination are given in Fig. 1. Standard deviations of enzyme activities in relation to different body weights of experimental groups of animals are indicated and the results are expressed as μmol of 6-azauridine 5'-phosphate/h per g of liver. $\square$, 5-Azacytidine-treated animals; $\blacksquare$, controls; $\bullet$, ratio of enzyme activities.

liver treated with 5-azacytidine differed from the control enzyme merely by its lower specific activity.

In adult rats the specific activity of hepatic uridine kinase diminished progressively in older animals, and the enhancement brought about by the administration of 5-azacytidine was about the same at different periods of life (Fig. 5). Chromatography on a Sepharose 6B column, by analogy with embryonic liver, did not reveal molecular modification of uridine kinase isolated from livers of 5-azacytidine-treated animals (Fig. 6).

Discussion

The mammalian liver undergoes different metabolic adaptations during development from the foetus to the adult in response to changes in external and internal environment. Uridine kinase is a 'salvage' enzyme which is especially active in rapidly proliferating tissues, and its content is often considered to reflect the relative efficiency of the system to utilize preformed pyrimidine precursors (Sköld, 1960b).

Fig. 6. Chromatography on Sepharose 6B of 30-39% satn.-(NH_4) $_2\text{SO}_4$ fraction of cytosol from adult rat liver untreated ($\circ$) and treated with 5-azacytidine ($\bullet$)

The experimental procedure was as described in Fig. 4. —, E_{280} .

The enzyme has attracted considerable interest because of its pharmacological importance in experimental cancer chemotherapy with uridine (Pasternak *et al.*, 1961; Reichard *et al.*, 1962) and cytidine (Vesely *et al.*, 1970) analogues that are converted into biologically active 5'-phosphates. During the development of resistance towards uridine and cytidine antimetabolites uridine kinase activity has been found in some cases to be partially deleted, and this impairment is often considered as the metabolic deviation underlying the change to resistance (Brockman, 1963). It has also been observed that uridine kinase purified from Ehrlich ascites tumours made resistant to 5-fluorouracil is precipitated by (NH_4) $_2\text{SO}_4$ under conditions different from those of its counterpart from the parental cell line; the mutant enzyme is also less stable on heating (Sköld, 1963).

However, uridine kinase purified 100-fold from mouse leukaemic cells resistant to 5-azacytidine is more stable towards heating and *p*-chloromercuribenzoate treatment than the enzyme from 5-azacytidine-sensitive cells (Vesely *et al.*, 1971). Rapidly growing Novikoff rat hepatoma appears to have two molecular forms of uridine kinase, namely high-molecular-weight species I, which prevails in normal adult rat liver and is the heat-labile species, and low-molecular-weight species II, normally found in embryonic rat liver, which is more stable towards heating than species I (Krystal & Webb, 1971). We were unable to confirm the existence of multiple forms of uridine kinase in embryonic and adult rat livers by Sepharose 6B chromatography (Figs. 4 and 6). The application of 6-azauridine as the substrate for an assay of uridine kinase is more convenient

(Kára *et al.*, 1963) than the application of the natural substrate that was used by Krystal & Webb (1971), since the analogue is not degraded by the phosphorylase, and 6-azauridine 5'-monophosphate is not phosphorylated to higher 5'-phosphates. Moreover, Sköld (1960a) showed that, of a number of related nucleosides used as substrates for uridine kinase, 6-azauridine is as active as uridine. Also, 6-azauridine 5'-triphosphate is fully as active as uridine 5'-triphosphate in exerting feedback inhibition of uridine kinase (Anderson & Brockman, 1964).

However, the embryonic enzyme required 50% saturation of $(\text{NH}_4)_2\text{SO}_4$, whereas the adult enzyme required only 39%, implying that the solubility of enzyme molecules from embryonic liver was increased. This difference disappeared in 4-day-old rats, in which liver uridine kinase was salted out from the crude extract at the same concentration of $(\text{NH}_4)_2\text{SO}_4$ as in the adult. The reaction of hepatic uridine kinase towards 5-azacytidine changes during the course of the postnatal period; the inhibition that is observed in the embryonic liver ceases after birth, and thereafter the drug induces the stimulation of the enzyme activity (Figs. 3 and 5).

It has been shown that in the 17-day-old foetal rat 60% of the liver is composed of haemopoietic and vascular tissue, but the percentage falls in an almost linear fashion to about 10% at 5 days after delivery. After this time few haemopoietic cells can be seen in liver sections (Oliver *et al.*, 1963). This phenomenon may explain the different solubility of uridine kinase molecules at high concentration of $(\text{NH}_4)_2\text{SO}_4$ as well as the different reactions of the enzyme towards 5-azacytidine treatment during ontogeny. The drug has a powerful action against haemopoietic and lymphatic tissue (Vadlamudi *et al.*, 1970), and leads to the depletion of various proliferating cells (Langman & Shimada, 1971), whereas it does not kill the resting cells such as mature hepatocytes. Thus uridine kinase in rat thymus is inhibited by 5-azacytidine (Čihák & Veselý, 1973), and at the same time the drug causes the depletion of thymocytes (Šorm & Veselý, 1965). Accordingly it is probable that 5-azacytidine has a differential effect on 18-day-old foetal rat liver, which is composed at that time of more than 50% proliferating haemopoietic tissue, the rest of the cells being hepatocytes.

Liver uridine kinase has its highest specific activity at the earlier stages of gestation (Fig. 1), and postnatally its activity is steadily declining (Fig. 5). It

seems that in the liver the utilization of uridine is gradually declining, depending on slowing down of the cell proliferation, which becomes negligible in the adult animal when liver haemopoiesis disappears and hepatocytes divide only rarely. In this connexion it is noteworthy that the activity of orotidine 5'-phosphate decarboxylase in postnatal rat liver is also age-dependent, since in the neonatal liver it is increased by a factor of 3.3 compared with the corresponding value in adult animal (Pausch *et al.*, 1971).

Further study of the 'salvage' enzymes as well as of those responsible for the formation of pyrimidines *de novo* in embryonic rat liver during gestation would undoubtedly be rewarding.

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