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Multiple Forms of Uridine Kinase in Normal and Neoplastic Rat Liver

BY GERALD KRYSTAL AND THOMAS E. WEBB
Department of Biochemistry, McGill University, Montreal, Canada and Department of Physiological Chemistry, Ohio State University, Columbus, Ohio 43210, *U.S.A.*

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Two species of uridine kinase with molecular weights of approximately 120000 (I) and 30000 (II) have been identified in the rat liver system. Species I predominates in the 7-day postnatal and adult rat liver and increases in the regenerating remnant of the latter after partial hepatectomy; the concentration of species II is low in these tissues. Species I also predominates in the slow-growing hepatomas 5123D and 7800. In contrast, II is the predominant form in the foetal rat liver and accounts for 40% of the total activity in the rapidly growing Novikoff ascites hepatoma. In contrast to species II, which was stable, species I was inactivated by preincubation for 30min at 37°C, before assay at 23°C.

Multiple forms of several enzymes or isozymes have been identified in several mammalian tissues including the rat liver system. For example, DNA polymerase activity in rat hepatomas is separable into two enzymes, which exhibit different template specificities; only one of these forms predominates in the resting and regenerating rat liver, the other predominating in foetal liver (Ove, Brown & Laszlo, 1969). Similarly, the normal adult form of hepatic aldolase (B form) appears to be partly or completely replaced in the slow and rapidly growing hepatomas respectively by type A, characteristic of embryonic and muscle tissue, or by type C, characteristic of brain tissue (Schapiro, Reuben & Hatzfeld, 1970; Sugimura, Sato & Kawabe, 1970). Further examples of hepatic proteins that appear in different molecular forms during development, or before and after neoplastic transformation, include guanine deaminase (Kumar & Krishnan, 1970), ATP–AMP phosphotransferase (Criss, Litwack, Morris & Weinhouse, 1970), hexokinase (Sato, Matsushima & Sugimura, 1969), serine dehydratase (Inoue & Pitot, 1969) and ferritin (Linder, Munro & Morris, 1970).

The present paper presents evidence for two forms of hepatic uridine kinase (ATP–uridine phosphotransferase, EC 2.7.1.48); the results further indicate that the relative proportions of the two forms vary in embryonic, adult, regenerating and neoplastic rat liver. Uridine kinase forms part of the salvage pathway of nucleotide metabolism and appears to catalyse a rate-limiting step in the anabolism of uridine to the successive nucleotide derivatives (Anderson & Brockman, 1964). The results of a separate study (G. Krystal & T. E. Webb, unpublished work) indicate that two species of uridine kinase are also present in Ehrlich ascites-tumour cells.

MATERIALS AND METHODS

Materials

Chemicals. [5-^{3}H]Uridine (sp. radioactivity 20Ci/mmol), purchased from the New England Nuclear Corp., Boston, Mass., U.S.A., was purified by descending chromatography on Whatmann 3MM paper in 86% (v/v) butan-1-ol–aq. NH_3 (sp.gr. 0.88) (94.5:5.5, v/v). The radioactive spot corresponding to uridine was eluted with 50% (v/v) ethanol, evaporated to dryness and redissolved in water.

Non-radioactive uridine and ATP were purchased from Calbiochem, Los Angeles, Calif., U.S.A., and Schwartz Bioresearch, New York, N.Y., U.S.A., respectively. Streptomycin sulphate was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Sepharose 6B and DEAE-cellulose discs (2.5cm diam.) were purchased from Pharmacia Fine Chemicals, Montreal, Quebec, Canada, and from Reeve Angel Co., Clifton, N.J., U.S.A., respectively.

Animals. The hepatomas 5123D and 7800, carried intramuscularly in both hind legs of rats of the Buffalo strain, were kindly supplied by Dr H. P. Morris, Howard University, Washington, D.C., U.S.A. The Novikoff ascites hepatoma, obtained from Dr L. Poirier of the Montreal Cancer Institute, Montreal, Quebec, Canada, was carried intraperitoneally in Sprague–Dawley rats. The morphological and biochemical properties of the slow-growing highly differentiated Morris hepatomas 5123D and 7800 and the undifferentiated rapidly growing Novikoff hepatoma have been described (Morris & Wagner, 1968; Davidson, 1961). Regenerating liver was obtained from 250g rats of the Sprague–Dawley strain, which were partially hepatectomized according to the procedure of Higgins & Anderson (1931). All rats received

Purina Chow and water *ad libitum*; lighting was controlled from 6a.m. to 6p.m.

Methods

Purification of uridine kinase. The purification of uridine kinase was performed at 0–4°C. The cells in the ascitic fluid from 9-day Novikoff ascites cells were washed (800*g* for 20s) four times with iso-osmotic saline (0.9% NaCl) to remove contaminating erythrocytes. After sedimentation from 0.9% NaCl at 800*g* for 2min, the packed-cell volume was recorded and the cells were resuspended in 200mM-tris–HCl buffer, pH7.5, to give a 30% (v/v) suspension. The cells were lysed by sonication for 90s, in 30s bursts, with a Blackstone Ultrasonic Probe (BP2). The livers from foetal, postnatal and young adult rats (250g) were washed with 0.9% NaCl, minced, and then gently homogenized before sonication. The solid hepatomas (hepatomas 5123D and 7800) were dissected free of fibrous fatty and necrotic tissue before homogenization and sonication.

The $105000g_{av.}$ ($r_{av.} = 5.9$cm) supernatant obtained by centrifuging the sonicate in a Beckman Ti50 rotor for 60min was treated with 10% neutralized streptomycin sulphate (0.05ml/ml of supernatant), with stirring for 30min, and then centrifuged at 15000*g* for 30min. The resulting supernatant was brought to 30% saturation with $(NH_4)_2SO_4$, stirred for 30min and then centrifuged at 105000*g* for 30min. The concentration of $(NH_4)_2SO_4$ in the supernatant was increased to 50% saturation and the suspension was centrifuged as before. Finally the 30–50%-saturation $(NH_4)_2SO_4$ fraction was dissolved in a minimum volume of 200mM-tris–HCl buffer, pH7.5, containing 20mM-mercaptoethanol and 20% (v/v) of glycerol. Portions of the 30–50%-saturation $(NH_4)_2SO_4$ fraction were applied to a Sepharose 6B column (0.635cm^2 × 56cm) equilibrated with 2 column volumes of a medium containing 200mM-tris–HCl buffer, pH7.5, 20mM-mercaptoethanol and 20% (v/v) of glycerol. The protein was eluted with the same buffer, by using a downward flow rate of 2ml/h, and 1ml fractions were collected; portions of the latter were analysed for protein (Warburg & Christian, 1941) and uridine kinase activity (see below).

Assay of uridine kinase. The standard assay mixture contained (final concentrations) 180mM-tris–HCl buffer, pH7.5, 10mM-mercaptoethanol, 10mM-$MgCl_2$, 1mM-ATP, 0.05mM-[5-^{3}H]uridine and 50μl of protein solution (as the enzyme preparation), in a final volume of 100μl. After 20min of incubation at 23°C, 5ml of the ice-cold water was added to stop the reaction and the diluted assay mixture was filtered through a Millipore filter containing DEAE-cellulose discs, which were pre-washed with 0.01M-HCl. The discs were washed with water and dried at 60°C, and then their radioactivities were counted in 10ml of liquid scintillant consisting of 5g of 2,5-diphenyloxazole and 100mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl) benzene/l of toluene. The rate of phosphorylation of uridine was linear over the period studied. The activity of uridine kinase in each 1ml fraction is expressed as nmol of UMP formed during 20min incubation.

Thermal stability of uridine kinase. The crude supernatant was preincubated at 37°C and 10μl portions were removed at various intervals for assay. The assay was identical with that described above except that the vessels were incubated for 30min at 23°C, heated for 3min at 90°C, diluted with 5ml of ice-cold water and then centrifuged and assayed by the DEAE-cellulose-disc method.

RESULTS

The rapid assay for uridine kinase, utilized in the present study, greatly facilitated the monitoring of the eluates from the columns. Before adoption of this assay, which depends on the binding of nucleotides (i.e. UMP) to DEAE-cellulose filters (Furlong, 1963), it was established that the streptomycin and ammonium sulphate purification procedure effected a complete separation of virtually all of the uridine phosphorylase and UMP kinase activities from uridine kinase, at the same time giving a 90% recovery of the latter enzyme. During the initial purification of uridine kinase to remove interfering enzymes, the kinase activity was assayed by spotting portions of the boiled assay mixture (fortified with uracil, uridine, UMP, UDP and UTP as markers) on Whatman 3MM paper; the components in the reaction mixture were separated by developing the chromatogram for 20h in an isobutyric acid–aq. ammonia (sp.gr. 0.88)–water (57:4:30, by vol.) system. Both assays gave identical activities with the enzyme preparation obtained after ammonium sulphate fractionation, on which most of the work to be reported is based. The latter preparation was also devoid of activities that degrade the purine and pyrimidine nucleotides and was stable to storage at −20°C for up to 2 weeks.

In preliminary studies it was shown (G. Krystal & T. E. Webb, unpublished work) that two distinct peaks of uridine kinase activity were obtained by chromatographing the 30–50%-saturation ammonium sulphate fraction of the cytosol from the Ehrlich ascites carcinoma (a mouse tumour), or the 20-day foetal rat liver, on a Sepharose 6B column. A comparative study of the relative concentration of the two species of uridine kinase in several rat tissues was therefore undertaken to determine whether their relative proportions changed during development, or in response to partial hepatectomy and neoplastic transformation. In each experiment the Sepharose 6B column (0.635cm^2 × 56cm) was loaded with 10mg of the 30–50%-saturation ammonium sulphate fraction of the cytosol, which contained 90% of the activity originally present in the crude sonicate. The results from several of the tissues investigated are recorded in Figs. 1 and 2.

The two distinct peaks of uridine kinase activity are particularly evident in the 30–50%-saturation ammonium sulphate fraction of the cytosol from foetal rat liver (Fig. 1). The component of higher molecular weight is centred at fraction 18.5 (species I) and the component of lower molecular weight is

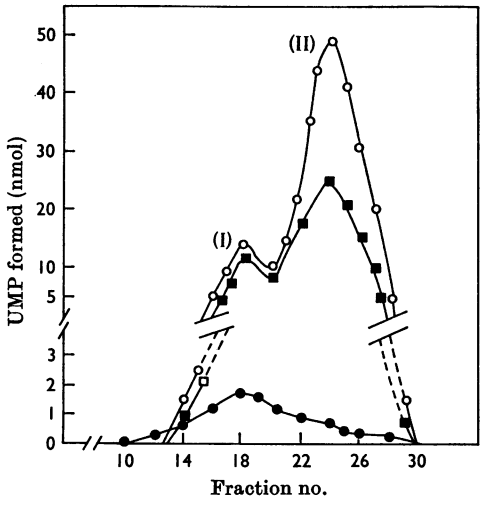

Fig. 1. Sepharose 6B chromatography of the 30–50%-saturation $(NH_4)_2SO_4$ fraction of the cytosol from the 13-day foetal liver (○), the 18-day foetal liver (■) and the 7-day postnatal liver (●). A portion containing 10 mg of protein was applied to a Sepharose 6B column (0.635 cm^2 × 56 cm) equilibrated with 50 mM-tris–HCl buffer, pH 7.5, containing 20 mM-mercaptoethanol and 20% (v/v) of glycerol. Fractions (1 ml) were collected for the determination of uridine kinase activity (nmol of UMP formed/20 min per ml of eluate) and protein (mg/ml).

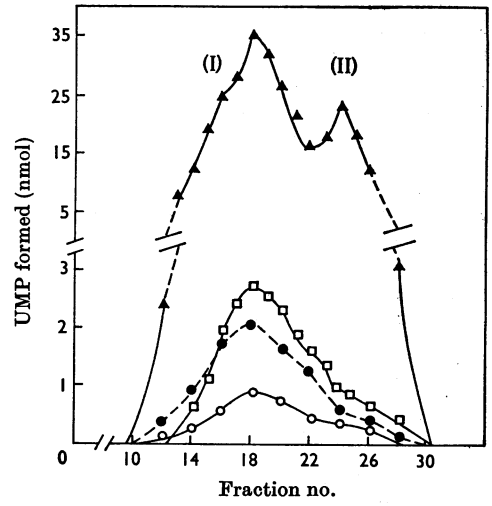

Fig. 2. Sepharose 6B chromatography of the 30–50%-saturation $(NH_4)_2SO_4$ fraction of the cytosol from the normal adult liver (○) and 25 h-regenerating liver (●) and from hepatoma 5123D (□) and the Novikoff hepatoma (▲). Other conditions are identical with those given in Fig. 1.

centred at fraction 24 (species II). Both species of uridine kinase in the embryonic rat liver were stable to prolonged incubation at 23°C. However, as with the Ehrlich ascites carcinoma (G. Krystal & T. E. Webb, unpublished work), when the crude cytosol was incubated at 37°C (see the Materials and Methods section for details) there was a complete loss of a fraction of the activity that corresponded to peak I, within 30 min of the start of the incubation; the residual activity, which corresponded to peak II (G. Krystal & T. E. Webb, unpublished work), was stable to further incubation at 37°C for at least 2 h.

The results in Fig. 1 indicate that species II is the predominant form of uridine kinase in foetal liver and that the concentration of this species decreases dramatically during development. To facilitate a more precise analysis the total units of activity in each peak (1 unit = 1 nmol of UMP formed in 20 min) was estimated by extrapolating each peak to the base-line (assuming symmetry), then totalling the activity in each 1 ml fraction of the eluate from the Sepharose 6B column. Thus the concentration of species II decreased 50% (from 200 units to 107 units) between 13 and 18 days gestation; between 18 and 20 days gestation (not shown in Fig. 1) there was a further 84% decrease in species II to 18.8 units. Development to the 7-day postnatal stage is accompanied by a further 85% decrease in species II (2.45 units total activity). Consequently species II has virtually disappeared at this stage of development. In contrast, species I remains fairly constant (at approx. 50 units) until 18 days gestation, when an 85% decrease in concentration is observed. The total activity in peak I remained relatively constant (at approx. 12 units) until at least 7 days after birth.

Comparison of the results in Figs. 1 and 2 indicates that there is a 50% decrease in species I (to 7.2 units) during the maturation of the 7-day postnatal liver to the resting adult liver. In contrast with the embryonic liver system, where species II predominates, there is a 2-fold increase (from 7.2 to 16.7 units) in the concentration of species I in the cytosol of the 25 h-regenerating liver, obtained by partial hepatectomy of the adult rat; there was no comparable change in peak II.

Hepatomas produced in the livers of adult rats also appear to have higher uridine kinase activity than the corresponding tissue of origin. For example, species I was 2.3-fold (total of 16.6 units) and 4.5-fold (total of 33.0 units) higher respectively in the slow-growing hepatomas 5123D (cf. Fig. 2) and 7800 (pattern not shown) than in the adult liver, whereas species II differed slightly if at all. In contrast, the concentration of species I and II was 30-fold (total of 332 units) and 64-fold (total of

117 units) higher respectively in the rapidly growing undifferentiated Novikoff hepatoma as compared with adult rat liver.

In summary, the results of the comparative study emphasize the marked decrease in species II relative to species I during embryonic and postnatal development of the rat liver. They further emphasize the similarity of the patterns in more highly differentiated tissues, such as the 7-day postnatal, the 25h-regenerating and the resting adult liver, and the slow-growing hepatomas 5123D and 7800. The pattern for the Novikoff hepatoma, which has a significant concentration of species II, appears to fall between those for the normal adult and embryonic liver.

The molecular weights of species I and II were estimated by molecular-exclusion chromatography (Whitaker, 1963) by using columns of Sepharase 6B. The columns were calibrated with myoglobin, bovine serum albumin and thyroglobulin, which are of known molecular weights (Klotz & Darnall, 1969). The molecular weights were estimated from plots of elution volume versus log(molecular weight) for embryonic, postnatal, regenerating and resting adult liver, as well as the Novikoff hepatomas 5123D and 7800 and the Ehrlich carcinoma. On the basis of 25 separate experiments, the average ($\pm$range) molecular weight of species I was 120000 ± 20000 and of species II was 30000 ± 10000.

DISCUSSION

The results of the present study indicate that there are at least two species of uridine kinase in rapidly growing undifferentiated hepatic tissues, such as the Novikoff hepatoma and embryonic rat liver. Species II, which is virtually absent from normal rat liver and the more slowly growing highly differentiated hepatomas, has a molecular weight of approx. 30000. The larger species, which is the predominant form in normal and regenerating liver and in hepatomas 5123D and 7800, and which is preferentially inactivated by incubation at 37°C, has a molecular weight of approx. 120000. The results further indicate that during development from 13 days gestation to the 7-day postnatal stage there is a 98% decrease in species II and an 87% decrease in species I. The possibility cannot be ruled out that a portion of the uridine kinase activity in the embryonic liver, and in particular before 17 days gestation, is derived from haemopoietic tissue, which may represent up to 60% of the liver cells at this time (Oliver, Blumer & Witham, 1963). However, the decrease in species II does not correlate with the disappearance of the haemopoietic tissue. The latter still accounts for about 10% of the cells in the 7 day postnatal liver, whereas species II has virtually disappeared.

The reappearance of a significant concentration of species II in the rapidly growing Novikoff hepatoma is of particular relevance to the phenomenon of tumour progression, whereby embryonic forms of the enzymes appear in addition to, or in place of, the normal adult forms (Potter, 1969; Sato *et al.* 1969). It is tentatively concluded that there has been a de-repression of the embryonic enzyme (species II) in addition to increased activities of species I in the Novikoff hepatoma. It is significant that species I rather than species II increases in the regenerating liver of the adult rat. In general the results of this comparative study on hepatic uridine kinase are comparable with those of a similar study on hepatic DNA polymerase (Ove *et al.* 1969). For example, two species of DNA polymerase were present in hepatomas, one of which predominated in normal liver and increased in regenerating liver whereas the second species predominated in embryonic liver. Further, two species of uridine kinase with properties identical with those of the rat liver system are present in the mouse tumour, the Ehrlich ascites carcinoma (G. Krystal & T. E. Webb, unpublished work). Only the large, more labile species were present in normal mouse intestine.

The identification of multiple forms of uridine kinase in mammalian tissue has implications not only with reference to developmental biology and tumour progression, but also with reference to the problem of cancer chemotherapy. For example, in 5-fluorouracil-resistant Ehrlich ascites cells the activity of uridine kinase is only 5–15% that of the sensitive cells (Reichard, Sköld, Klein, Revesz & Magnusson, 1962). This decrease appeared to occur by a stepwise process. However, during the initial stages there appeared to be a clear lack of correspondence between the appearance of resistance and the decrease in the uridine kinase activity. In addition one subline showed resistance to both 5-fluorouracil and 5-fluorouridine without any decrease in uridine kinase activity or uptake of the drugs. The results of the present study could account for these results if species I was unable to phosphorylate 5-fluorouridine. Sköld (1963) did, in fact, observe changes in the physical properties of uridine kinase of Ehrlich ascites cells during the development of resistance to 5-fluorouracil. These changes, which were thought to be due to a change in the structure of the enzyme, included precipitation at lower ammonium sulphate concentrations, higher affinity for DEAE-cellulose and increased lability to heating at 60°C. The results of the present study offer the alternative possibility, that the low-molecular-weight species, which is stable at 37°C, may possess the exclusive capacity to phosphorylate 5-fluorouridine and that the higher-molecular-weight species becomes the predominant

form. A similar phenomenon might also account for the observation (Vesely, Cihak & Šorm, 1967) that the development of resistance of leukaemic cells to 5-azacytidine, which is probably incorporated into nucleic acid as 5-azauridine (Doskocil & Šorm, 1970), is accompanied by a decrease in the incorporation of the analogue into RNA.

Further studies will be required to determine whether the differential changes in the concentrations of the two species of uridine kinase account for the resistance to the base analogues and whether these species represent true isozymes rather than a monomer–polymer equilibrium that is regulated by the intracellular environment.

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