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All hexokinase isoenzymes coexist in rat hepatocytes

Alejandro REYES* and María Luz CÁRDENAS†
Departamento de Biología, Facultad de Ciencias Básicas y Farmacéuticas, Universidad de Chile, Santiago, Chile

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The cellular distribution of hexokinase isoenzymes, *N*-acetylglucosamine kinase and pyruvate kinases in rat liver was studied. Hepatocytes and non-parenchymal cells with high viability and almost no cross-contamination were obtained by perfusion *in situ* of the liver with collagenase, with the use of an enriched cell-culture medium in all steps of cell isolation. Separation of hexokinase isoenzymes was done by DEAE-cellulose chromatography, and enzyme activities were measured by a specific radioassay. Cytosol from isolated hepatocytes contained high-affinity hexokinases A, B and C, in addition to hexokinase D. The last-mentioned represented about 95% of total glucose-phosphorylating activity. Only hexokinase A was found associated to the particulate fraction. Isolated non-parenchymal cells contained only hexokinases A, B and C. *N*-Acetylglucosamine kinase was measured with a specific radioassay and was found as a single enzyme form in both hepatocytes and non-parenchymal cells, with higher activities in the former. Pyruvate kinase isoenzyme L was present only in the hepatocytes and isoenzyme K only in the non-parenchymal liver cells, confirming that they are good cellular markers.

In rat liver there are four isoenzymes of hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1), named isoenzymes A, B, C and D (González *et al.*, 1964) or I, II, III and IV (Katzen *et al.*, 1965). Hexokinases A, B and C exhibit K_m values for glucose of 0.2–0.01 mM (high-affinity isoenzymes), whereas hexokinase D has a half-saturation concentration value of about 8 mM at pH 7.5 (low-affinity isoenzyme). [Hexokinase D or IV is often known as glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2)]. As in other tissues, in the liver there is a *N*-acetylglucosamine kinase (ATP:2-acetamido-2-deoxy-D-glucose 6-phosphotransferase, EC 2.7.1.59) that also phosphorylates glucose and, because its low affinity for this substrate, may be mistakenly identified as hexokinase D (Davagnino & Ureta, 1980; Allen *et al.*, 1980; Vera *et al.*, 1984).

Since the liver contains several types of cells (cf. Van Berkel, 1982), it has been a challenge to know the cellular location of each glucose-phosphorylating enzyme. Several authors postulated that hexokinase D is present exclusively in hepatocytes and that the high-affinity hexokinases are confined to non-parenchymal liver cells (Sapag-Hagar *et al.*, 1969; Crisp & Pogson, 1972; Bontemps *et al.*, 1978). However, other investigators have described the presence of one or two high-affinity hexokinase activities in hepatocytes (Morrison, 1967; Werner *et al.*, 1972; Bonney *et al.*, 1973; Guixé *et al.*, 1978). The uncertainty on this subject persists either because the method used for isolation of the cells, the quality of the isolated cells and the estimation of contaminant cells or because the method for detecting the enzyme activities may be open to serious criticisms. In the present work we have been able to circumvent most previous pitfalls and to demonstrate the presence of the three high-affinity hexokinases, in addition to hexokinase D, in isolated rat hepatocytes. In non-parenchymal liver cells, however, only hexokinases A, B and C were found. A single enzyme form of *N*-acetylglucosamine kinase was

Abbreviations used: MEM, Minimum Essential Medium; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

* Present address: Instituto de Bioquímica, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile.

† To whom reprint requests and correspondence should be addressed at the Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

present in both types of cells. Pyruvate kinase isoenzymes (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) were confirmed as good markers for liver cells. Portions of this work have been published in abstract form (Reyes & Cárdenas, 1980; Reyes *et al.*, 1980).

Experimental

Materials

Dithiothreitol, phosphoenolpyruvate, Hepes, glucose, sucrose, glycerol, *N*-acetylglucosamine, glucose 6-phosphate dehydrogenase (EC 1.1.1.49, type VII, from baker's yeast), collagenase (EC 3.4.24.3, type IV, from *Clostridium histolyticum*), Pronase E (proteinase type XIV, from *Streptomyces griseus*), Dowex 1-X8 and Triton X-100 were products of Sigma Chemical Co. DEAE-cellulose (DE-52) was from Whatman. D-[U-^{14}C]Glucose (290mCi/mmol) was a product of New England Nuclear. *N*-Acetyl-D-[1-^{14}C]glucosamine (51mCi/mmol) was obtained from The Radiochemical Centre. Penicillin G and streptomycin sulphate were from Difco. Eagle's Minimum Essential Medium (catalogue no. 410-1100) was obtained from Gibco. All other chemicals were of the highest purity commercially available.

Animals

Albino rats weighing between 200 and 250g were used. They were kept in an animal room at about 23°C with alternating light–dark periods of 12h, and fed on commercial pellet diet and water *ad libitum*.

Preparation of isolated liver cells

The method used was basically the 'two-step procedure' of Seglen (1976), which was derived from that of Berry & Friend (1969). The following two modifications were introduced. First, instead of a phosphate/saline buffer solution used by Seglen (1976), the perfusion solution was enriched MEM, which is MEM (Eagle, 1959) with the addition of 20mM-Hepes buffer (pH7.4), 25mM-glucose (making the final concentration 30.6mM), 25mM-$NaHCO_3$, 100 units of penicillin/ml and 100μg of streptomycin/ml. The perfusion liquid was oxygenated by direct bubbling of O_2/CO_2 (19:1) mixtures. For the initial extraction of Ca^{2+} ions from the liver the same medium was used but with $CaCl_2$ omitted. The perfusion solution was filtered through 22μm-pore-size membranes of nitrocellulose. Secondly, the perfusion was performed *in situ*, as used by Hue *et al.* (1975).

After about 15min of perfusion the liver was carefully removed and transferred to a polyethylene Petri dish containing 30ml of enriched MEM. The Glison capsule was removed and the cells were separated by gentle pressure with a blunt spatula. The suspension was filtered through a nylon net and then divided into two portions. Hepatocytes were obtained from one portion by sedimenting at 50*g* for 2min and resuspending in enriched MEM containing 10mg of gelatin/ml. The centrifugation and resuspension were repeated thrice. Non-parenchymal cells were purified from the other portion by digestion of hepatocytes with Pronase (Berg & Boman, 1973). Briefly, 2–4g of cells was incubated for 1h at 37°C in 100ml of enriched MEM containing 1.5mg of Pronase/ml. The undigested cells were centrifuged down at 300*g* for 3min, and resuspended in the gelatin-containing medium. The centrifugation and resuspension were repeated twice. The procedures for the two cell types were conducted at room temperature.

Cells were counted in a Neubauer haemocytometer, and viability was assessed by the capacity to exclude Trypan Blue (3mg/ml), and occasionally by the retention of lactate dehydrogenase (Baur *et al.*, 1975). Over 90% of hepatocytes and nearly 100% of non-parenchymal cells were found to be viable by these criteria.

Purity of the isolated cells

The contamination of the hepatocyte preparations with non-parenchymal cells was generally less than 2% and the contamination of the non-parenchymal cell preparations with hepatocytes was less than 1%, when examined by phase-contrast microscopy. As a biochemical criterion of cross-contamination pyruvate kinase isoenzymes L and K were assayed in both cell preparations. Our results confirm previous observations (Van Berkel *et al.*, 1972; Crisp & Pogson, 1972; Bonney *et al.*, 1973), since isoenzyme L was found only in hepatocytes and isoenzyme K only in non-parenchymal cells (Fig. 1). However, each isoenzyme was detected in the other type of cell in no more than 0.5–2% of its total activity, values that agree with the cell contamination detected by microscopy. We conclude that isoenzymes L and K of pyruvate kinase are good quantitative markers for hepatocytes and non-parenchymal liver cells respectively.

Preparation of cell cytosols

The purified cells (hepatocytes and non-parenchymal cells) were collected by centrifugation at 300*g* for 5min and suspended in 3vol. of homogenizing solution, which varied as follows according to the enzyme to be assayed. For hexokinases and *N*-acetylglucosamine kinase, buffer A [10mM-potassium phosphate buffer, pH7.0, containing 1mM-EDTA, 2mM-dithiothreitol and 5% (v/v) glycerol] containing 50mM-glucose was used. For pyruvate

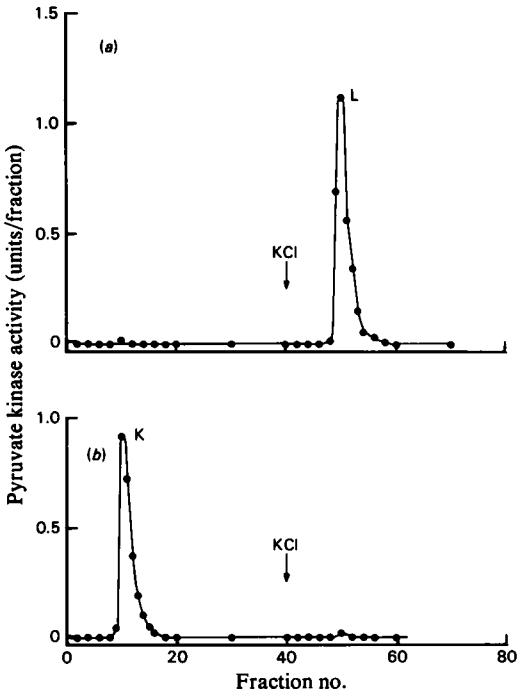

Fig. 1. *Chromatographic separation on DEAE-cellulose of pyruvate kinase isoenzymes from hepatocytes and non-parenchymal liver cells*

Cells were isolated from an animal fed on equilibrated diet and processed as described in the Experimental section. Samples of supernatants from isolated hepatocytes (equivalent to 0.87mg of DNA of the original homogenate) (*a*) or non-parenchymal cells (equivalent to 1.0mg of DNA of the original homogenate) were applied on 2ml columns. Enzyme activities were normalized to 1mg of cell DNA. Fractions of volume 0.37ml were collected. The arrow indicates the addition of 1M-KCl.

kinase buffer B (10mM-potassium phosphate buffer, pH7.5, containing 2mM-dithiothreitol and 0.5M-sucrose) was used. The cells were homogenized by means of two 5s pulses from an Ultrasonic W185 sonicator at half-maximal potency. The homogenates were centrifuged at 105000*g* for 60min in a Beckman L5-50B ultracentrifuge, and the supernatant fluids were used as enzyme sources. When the association of enzymes with particulate fractions was investigated, the sediments were washed twice (resuspension and centrifugation) and associated enzymes were released by incubation for 10min with 0.5% (v/v) Triton X-100 prepared in the homogenizing solution. The supernatant fluid of a 105000*g* centrifugation was then resolved by chromatography on DEAE-cellulose.

Chromatographic separation of isoenzymes

Small DEAE-cellulose columns (7cm × 0.6cm, 2ml bed volume) were equilibrated with either buffer A or buffer B, depending on the enzymes to be studied, and charged with the supernatant fluids. After washing with 10ml of buffer A, hexokinase isoenzymes and *N*-acetylglucosamine kinase were eluted with a linear concentration gradient of 0–0.4M-KCl in buffer A. A total volume of 40ml gave good separation of these enzymes, with collection of 0.37ml fractions. Pyruvate kinase isoenzyme K was found in the fractions (0.3ml each) collected during washing of the columns with 12ml of buffer B (Garnett *et al.*, 1974). Isoenzyme L was retained under the conditions used and was eluted after addition of 1M-KCl to buffer B.

Enzyme assays

Hexokinase activity was measured at pH8.0 either by a spectrophotometric method or by a radioassay. Both methods give the same results with partially purified enzymes after the chromatography on DEAE-cellulose. The spectrophotometric method measures the formation of glucose 6-phosphate by coupling the hexokinase reaction with $NADP^+$ and glucose-6-phosphate dehydrogenase, as described by Cárdenas *et al.* (1978). Usually, two glucose concentrations (100 and 0.5mM) were used. Assay systems with ATP omitted were used as blanks. The radioassay was as outlined by Radojković *et al.* (1978) with [U-^{14}C]-glucose as substrate and a reaction mixture as described in Cárdenas *et al.* (1978) except for the omission of the dehydrogenase and $NADP^+$ and the inclusion of 0.5mM-glucose containing about 400000–600000c.p.m. of [U-^{14}C]sugar. After incubation, the labelled product was separated from unchanged glucose by filtration on small Dowex 1-X8 (formate form) columns. Counting was performed in a Phillips PW 4510/01 automatic liquid-scintillation analyser at 60% efficiency, with a standard toluene-based scintillation liquid plus Triton X-100 (Radojković *et al.*, 1978). *N*-Acetylglucosamine kinase activity was determined by a radioassay derived from the one described by Radojković *et al.* (1978). The reaction mixture was as described above for hexokinases but with *N*-acetyl[1-^{14}C]glucosamine as substrate at a concentration of 0.7mM and containing about 150000–300000c.p.m. Pyruvate kinase activity was measured by the spectrophotometric assay method of Bücher & Pfleiderer (1955), with a reaction mixture (0.5ml) with composition specified by Garnett *et al.* (1974). One unit of enzyme was defined as the amount of enzyme catalysing the transformation of 1μmol of substrate/min at 30°C under the indicated conditions. When radioassays

were used the enzyme activity was calculated by dividing the number of disintegrations/min corresponding to the product of the reaction retained in the Dowex column by the specific radioactivity of the substrate.

Miscellaneous assays

Protein was measured by the method of Bradford (1978), which uses Coomassie Brilliant Blue G, with bovine serum albumin as standard. DNA was determined by the procedure of Giles & Myers (1965) with trivial modifications, with sheep thymus DNA as standard. KCl concentration was determined by conductimetry.

Results

Hexokinase activities, total protein and DNA in crude extracts of hepatocytes

Soluble hexokinase activity was measured spectrophotometrically in crude extracts of isolated hepatocytes. When assayed at two glucose concentrations (100 and 0.5mM) in order to measure total and high-affinity activities, hexokinase D, which would correspond to the difference between them, appeared to represent about 95% of total hexokinase activity. As was expected, velocities measured with the low concentration of glucose were at the limit of sensitivity of the assay. As there is a theoretical contribution of about 3% of activity by hexokinase D itself to the value of 0.5mM-glucose, it was not possible by this method to obtain any reliable conclusion on the presence or absence of high-affinity hexokinases.

Specific activities of total hexokinase were about 10munits/mg of soluble protein in whole livers as well as in hepatocytes. No significant leakage of proteins or DNA from hepatocytes was noticed during their isolation. Soluble proteins and DNA contents were 223 ± 21 ($n = 8$) and 3.1 ± 0.7 ($n = 8$) mg/g wet wt. respectively, values that are similar to those given by other authors (Crisp & Pogson, 1972; Bonney *et al.*, 1973; Seglen, 1976; Dileepan *et al.*, 1979).

Chromatographic separation of hexokinase isoenzymes from isolated liver cells

Fig. 2 shows typical chromatographic profiles of hexokinase isoenzymes from high-speed supernatant fluids of hepatocytes and non-parenchymal cells. Both cell preparations were isolated from the same liver. By using the radioassay with 0.5mM-glucose, four symmetric well-defined peaks were found in chromatographic patterns from hepatocytes (Fig. 2*a*), which corresponded to hexokinases A, B, C and D. The great amount of hexokinase D permits its detection even with this low concentration of glucose, which measures about 3% of the enzyme activity as mentioned above. Hexokinase isoenzymes were identified by their chromatographic mobilities (González *et al.*, 1964). In addition, the high-affinity isoenzymes were recognized by the proportions of their activities at 0.5mM and 5mM-glucose in the radioassay, which typically were 1.05, 1.2 and 0.8 for hexokinases A, B and C respectively. Hexokinase D was also measured at 100mM-glucose by the spectrophotometric method, with which high-affinity hexo-

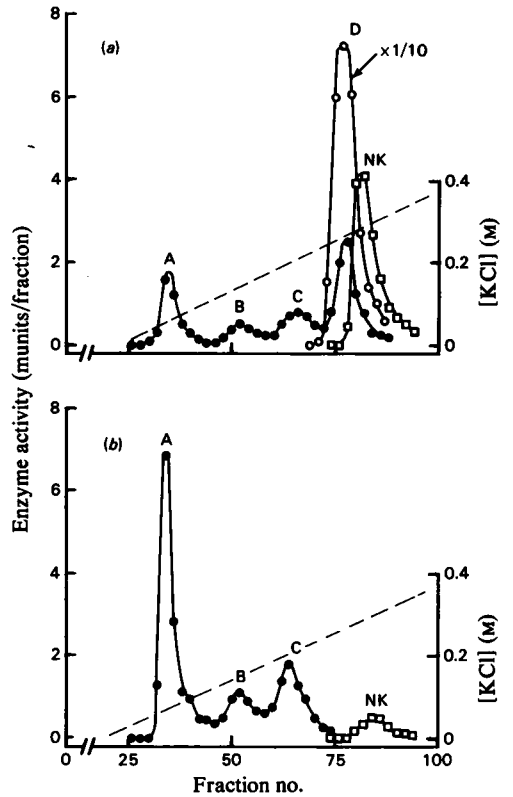

Fig. 2. *DEAE-cellulose chromatography of hexokinases and N-acetylglucosamine kinase from isolated hepatocytes and non-parenchymal liver cells*

Cells were isolated from an adult rat and processed as described in the Experimental section. Samples from high-speed supernatants were applied on 2ml columns. The samples corresponded to hepatocytes (0.63mg of DNA) (*a*) or non-parenchymal cells (0.85mg of DNA) (*b*). Enzyme activities were normalized to 1mg of cell DNA. Hexokinase activities were measured spectrophotometrically at 100mM-glucose (○) or by the radioassay at 0.5mM-glucose (●). For practical reasons the activities at 100mM-glucose of hexokinase D are represented only as one-tenth of the actual values. *N*-Acetylglucosamine kinase activity (□) was also measured. The broken line indicates the KCl concentration gradient.

kinases were almost undetectable. When expressed as the amount of high-affinity hexokinases per mg of cell DNA, as in Fig. 2, it is obvious from simple calculations that isoenzymes found in hepatocytes cannot result from contamination, because this would imply the presence in hepatocyte preparation of more than 50% of non-parenchymal cells, which is against microscopic observations (see the Experimental section). Furthermore, in some preparations in which pyruvate kinase isoenzyme K was undetectable, indicating no contamination with non-parenchymal cells, high-affinity hexokinases were unmistakably found in about the same amounts. The sum of the activities of the three peaks of the high-affinity isoenzymes (about 24munits/mg of DNA) was about 5% of the total hexokinase activity (about 440munits/mg of DNA). In non-parenchymal liver cells only three protein fractions with hexokinase activity, corresponding to isoenzymes A, B and C, were detected (Fig. 2*b*). The striking predominance of isoenzyme A was a constant finding. Hexokinase D was always absent from these cells.

Chromatographic separation of proteins associated to the particulate fraction of hepatocytes, and released by incubation with Triton X-100, showed the presence of hexokinase A (Fig. 3). For comparative purposes, the pattern obtained with an equivalent amount of supernatant fluid from the same preparation is also shown. The particulate fraction contained about half ($46 \pm 8\%$; $n = 3$) of the hexokinase A from rat hepatocytes. No systematic investigation of the subcellular distribution of this particulate hexokinase A was attempted. The sedimentable hexokinase activity could result from association to particulate material during cell preparation, as seems to occur with hexokinase in hepatocytes from guinea pig (Faulkner & Jones, 1979). However, we have also detected hexokinase A in particulate fractions from whole rat liver (Fig. 4). Incubation of rehomogenized particulate fraction from whole

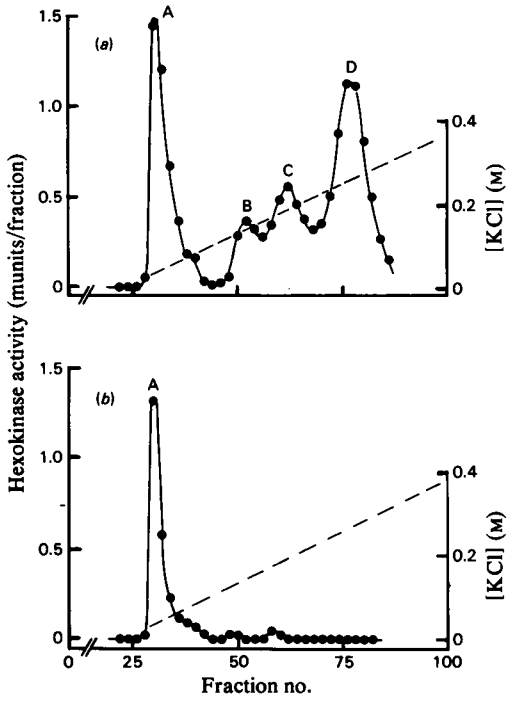

Fig. 3. *DEAE-cellulose chromatography of hexokinase isoenzymes from supernatant and sediment of isolated hepatocytes*

Cells were isolated from a single rat and processed as described in the Experimental section. Samples of the supernatant of the initial centrifugation (*a*) and of the supernatant fluid obtained after treatment of the sediment with Triton X-100 (*b*), each corresponding to approx. 1g of packed cells, were applied on 2ml columns and chromatographed as outlined in the Experimental section. Fractions of volume 0.37ml were collected. Only the activities measured by the radioassay at 0.5mM-glucose are represented. The broken line indicates the KCl concentration gradient.

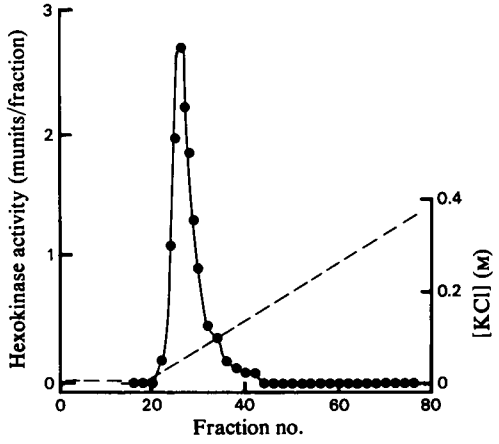

Fig. 4. *Chromatographic resolution of hexokinase A from the particulate fraction of whole liver*

The rat was killed by decapitation, and after thorough bleeding the liver was excised. A 30% (w/v) homogenate was prepared in buffer A in a Potter–Elvehjem homogenizer. The rest of the procedure was as described in the Experimental section for isolated cells. A sample of the supernatant obtained after centrifugation of the Triton X-100-treated sediment, and equivalent to 3g of liver, was chromatographed. Hexokinase activity was detected spectrophotometrically with 0.5mM-glucose as substrate. The broken line indicates the KCl concentration gradient.

liver with 10mM-ATP or 2mM-glucose 6-phosphate did not release hexokinase activity, which is only released by a subsequent incubation with Triton X-100. Other hexokinase isoenzymes and *N*-acetylglucosamine kinase were never found in particulate fractions from either isolated hepatocytes or whole liver, and traces occasionally detected could be attributed to contamination with soluble fraction.

N-Acetylglucosamine kinase distribution in liver cells

Fig. 2 also shows the cellular distribution of *N*-acetylglucosamine kinase in isolated liver cells, A single peak of activity was found in both parenchymal and non-parenchymal liver cells, which was eluted from the columns at higher KCl concentration than hexokinase D, in agreement with the results reported by others (Davagnino & Ureta, 1980; Allen *et al.*, 1980). Fractions collected before the saline gradient was established were thoroughly examined for any *N*-acetylglucosamine-phosphorylating isoenzyme not retained by DEAE-cellulose, but none was found. It should be noted that the radioassay used to detect *N*-acetylglucosamine kinase activity was as sensitive as that used for hexokinases (A. Reyes, M. L. Cárdenas & H. Niemeyer, unpublished work). *N*-Acetylglucosamine kinase was found predominantly in hepatocytes (Fig. 2*a*), but a small amount was always present in non-parenchymal cells (Fig. 2*b*).

Discussion

This work shows conclusively that hepatocytes as well as non-parenchymal rat liver cells contain the three high-affinity hexokinases (A, B and C), and that hexokinase D is present only in hepatocytes. Our results therefore resolve the controversy about the presence of high-affinity hexokinases in the hepatocytes. Thus, whereas the work of some authors suggested the presence of one or two high-affinity hexokinases in the parenchymal liver cells of adult rats (Morrison, 1967; Werner *et al.*, 1972; Bonney *et al.*, 1973; Guixé *et al.*, 1978), other reports had not detected the high-affinity isoenzymes in hepatocytes (Sapag-Hagar *et al.*, 1969; Crisp & Pogson, 1972; Bontemps *et al.*, 1978).

The uncertainty about the presence or absence of high-affinity hexokinases in hepatocytes has persisted because of several limitations of the methods used by previous workers. Some of these limitations are discussed here. Because of their different half-saturation concentration values, the three high-affinity hexokinases as a whole were distinguished from the low-affinity hexokinase D by assaying spectrophotometrically the activity at two glucose concentrations, 0.5mM (it would measure the total of the high-affinity hexokinases) and 100mM (it would measure total hexokinases), as originally proposed by Viñuela *et al.* (1963). This procedure does not permit the identification of the isoenzymes present, and provides only an approximate measurement of total high-affinity hexokinase activity. In fact, when the sample contains a high proportion of isoenzyme D, as happens in normal hepatocytes, its contribution to the velocity measured at 0.5mM-glucose is such that a proportion of high-affinity hexokinases of 5% or less of the total phosphotransferase activity could scarcely be discriminated.

Instead, our approach was to determine each hexokinase isoenzyme separately by using a partial purification procedure, together with an analytical method of great sensitivity to detect minor amounts of high-affinity hexokinases. Small DEAE-cellulose columns were used to isolate the hexokinases, since they permit a rapid separation (3h from charging the sample until column resolution). Furthermore, with addition of protecting reagents such as glycerol, dithiothreitol and KCl, the recovery of enzyme activity was usually over 90% and a very good reproducibility of chromatographic profiles was obtained. The highly sensitive and specific radioassay allowed us to measure accurately each hexokinase present even in small amounts of cells, and to obtain chromatographic profiles of both parenchymal and non-parenchymal liver cells isolated from the same animal.

We found that the detection of hexokinase isoenzymes from hepatocytes depended on a careful isolation of the cells, and especially on the composition of the perfusion medium, since isoenzyme B was not detected when Krebs–Henseleit bicarbonate buffer was used for perfusion (Reyes & Cárdenas, 1980), even in the presence of 1mM-phenylmethanesulphonyl fluoride. When enriched MEM was routinely used as perfusion solution and during the following steps of cell isolation, chromatographic profiles became very reproducible, always showing the four hexokinase isoenzymes. (No hexokinase activity was detected in the perfusion medium.)

Particulate hexokinase activity had been reported for most rat tissues (Crane & Sols, 1953; Colowick, 1973), including foetal and neonatal liver (Ureta *et al.*, 1975; Hommes & Everts, 1978), and for the liver from other mammals (Ureta *et al.*, 1981), but not for adult rat liver (Siekevitz & Potter, 1955; Ureta *et al.*, 1975). The small amount of particulate hexokinase existing in the liver and the high concentration of detergent required for its solubilization could explain why it had not been previously detected. In the case of several rat tissues, most sedimentable hexokinase activity has been located on the mitochondrial outer mem-

brane (Craven & Basford, 1969), from which it can be specifically released by incubation with ATP or glucose 6-phosphate (Hernández & Crane, 1966; Rose & Warms, 1967). As we found that liver particulate hexokinase was refractory to those treatments, it is tempting to speculate that the particulate hexokinase A could be associated with membranous structures, either plasmic or endocellular, as in chick liver (Lagos & Ureta, 1981).

We are aware of the heterogeneity of liver parenchymal cells with respect to their content of hexokinase D and other enzymes (for reviews see Jungermann & Sasse, 1978; Jungermann & Katz, 1982), and thus our determinations refer obviously to an average cell. It would be of considerable interest to investigate the zonal distribution of each hexokinase isoenzyme within the liver lobule as well as their subcellular location in order to gain an insight as to the distinct physiological role that each isoenzyme may have.

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