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Ontogeny of Chick Liver Hexokinase Isozymes*

Ontogenia de las Isoenzimas de Hexoquinasa de Higado de Pollo

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Glucose phosphorylating activities were measured in liver extracts from chicks at several developmental stages. Enzyme activity levels in supernates were low (about 0.16 units/g liver) from day 10th of egg incubation until the 17th day, at which time a transient increase to 0.5 units/g was observed. At hatching, the levels were again low (0.15 units/g) compared to adult levels (0.9 units/g). Particulate hexokinase activity was rather constant from day 10th to adulthood (about 0.3 units/g).

Chromatography of liver supernates in DEAE-cellulose columns revealed the presence of four hexokinases in embryos up to day 15th of incubation. From that day onwards, the least retained form (hexokinase 4) was no longer found. The most retained form (hexokinase 1) disappeared at hatching, at which time a pattern consisting of hexokinases 2 and 3 was found to be very similar to the adult profile. The four isozymes were characterized as low $K_{m\ glucose}$ hexokinase of broad sugar specificities and molecular weights of about 100,000.

Particulate hexokinase activity of embryonic chick liver was found to be composed of the same isozymes observed in cytosolic extracts. Incubation of particles with glucose 6-P or ATP failed to release hexokinase activity.

The isozymic system catalyzing glucose phosphorylation (ATP: D-hexose 6-phosphotransferases, EC 2.7.1.1, hexokinases) in vertebrates has been the subject of several reports in the last years (for reviews see 1-3). A major conclusion emerging from those studies is that hexokinases from aves and higher reptiles are strikingly different to those of mammals, turtles and amphibians. For instante, two hexokinases of low $K_{m\ glucose}$ values, broad sugar specificities and molecular weights of about 100,000 are present in the liver of most birds (4,5). Neither of those isozymes are inhibited by excess glucose as in the case of mammalian or amphibian hexokinase C (6,7) nor display sigmoidal kinetics with high $K_{0.5\ glucose}$ values and molecular weight of 50,000 as in the case of hexokinase D, the so-called glucokinase from mammals (8-11). Differences are also observed when comparing some parameters of the avian hexokinases with those of the mammalian isozymes A and B, *e.g.*, $K_{m\ glucose}$ values, chromatographic mobilities, and ability to phosphorylate fructose (3-5).

The purpose of the observations to be reported in this paper was to gather more information about the differences mentioned above through studies on the developmental behavior of the hexokinase isozymes from chick liver. Apart from the intrinsic value of the description of ontogenetic changes of the isozymic repertory in avian liver (a field largely neglected), the comparison of the results with those obtained in a similar investigation from this laboratory on the rat liver hexokinases

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(12), further substantiates the marked differences existing in both isozymic systems. Portions of this work were previously published in abstract form (13).

MATERIALS AND METHODS

Materials. ATP, ADP, NADP, NADH, DTT*, pyruvate kinase (containing lactate dehydrogenase), phospho*enol*pyruvate, yeast glucose 6-P dehydrogenase, and Sephadex G-200 were obtained from Sigma Chemical Co. DEAE-cellulose (DE-52) was a Whatman product. [U-^{14}C] glucose was purchased from the Radiochemical Centre, Amersham, England. Other reagents were of the highest purity commercially available.

Animals. Embryonated eggs of the chick Leghorn strain obtained from Instituto Bacteriológico de Chile were incubated for definite periods of time. Embryos were examined for developmental stage according to Hamburger and Hamilton (14) but, for convenience, results are expressed as uncorrected incubation times.

Preparation of crude liver extracts. Animals were killed by decapitation, thoroughly bled, and the liver excised, weighed, and placed on cracked ice. All further operations were performed at 2-4°C unless otherwise stated. Homogenates (3.3 per cent, w/v) were prepared in a buffer containing 100 mM KCl, 25 mM Tris-HCl, 1 mM DTT, 6 mM EDTA and 6 mM $MgCl_2$, pH 7.5 (buffer A). Aliquots of homogenates were treated with Triton X-100 (final concentration 0.1 per cent), incubated during 10 min and centrifuged at 10,000 x *g*. Untreated aliquots of homogenates were centrifuged at 105,000 x *g* in a Beckman L5-B ultracentrifuge for 60 min. Enzyme activity was measured in detergent-treated homogenates and in high speed supernates. The difference between the activity values of homogenates and those of the soluble fraction is referred to as 'particulate' activity.

Chromatographic separation of the glucose phosphorylating isozymes. The procedure of Ureta *et al.* (15) was used. The liver of individual animals were used in all experiments except at the earliest stages where two or more livers were pooled. Livers were homogenized in 3 vols of a buffer containing 10 mM Tris-HCl, 1 mM DTT, 1 mM EDTA, pH 7 (buffer B), and centrifuged at 105,000 x *g* for 60 min. Aliquots of supernatant liquid corresponding to 75 mg of liver were charged in 2-ml DEAE-cellulose columns (0.5 x 10.5 cm) and the enzymes eluted with a linear gradient of KCl from 0 to 0.5 M in buffer B. Gradient volume was 34 ml and fractions of 0.3 ml were collected and used for the measurement of enzyme activity. The linearity of the gradients and KCl concentrations were assessed by conductivity measurements using a CDM 2f Radiometer (Copenhagen) apparatus. Chromatographic mobility is defined as the KCl concentration at the tube of maximal glucose phosphorylating activity of the isozyme.

In order to characterize kinetic parameters and molecular weights of the isozymes, high speed supernates from the livers of 250 embryos at the 14th day of incubation, and from the livers of 10 hatching chicks were chromatographed in 14-ml DEAE-cellulose colums (1.1 x 14.2 cm). Gradient volume was 240 ml and fractions of 2 ml were collected.

Enzyme assays. ATP-dependent glucose phosphorylating activities were determined either spectrophotometrically or by a radioassay. In the former (15) the rate of glucose 6-P formation was measured by coupling the reaction with $NADP^+$ and glucose 6-P dehydrogenase. The reaction mixtures contained (final concentrations): 84 mM Tris-HCl, pH 7.5, 12.6 mM $MgCl_2$, 1.6 mM EDTA, 5 mM ATP, 0.5 mM $NADP^+$, 0.2 I.U. of glucose 6-P dehydrogenase, substrate and enzyme in a final volume of 0.5 ml. Two glucose concentrations (100 and 0.5 mM) were routinely used. A similar reaction mixture with ATP omitted acted as blank. NADPH formation was followed at 340 nm in a Gilford model 2400, or in a Zeiss model PM QII spectrophotometer thermostatted at 30°C.

For the radioassay [U-^{14}C] glucose was used as substrate (16). The reaction mix-

* Abbreviations used are: DEAE-cellulose: diethylaminoethyl-cellulose; EDTA: ethylene diaminetetracetate; DTT: dithiothreitol.

TABLE I

Some Properties of the Hexokinases from Chick Liver*

Parameter	Hexokinase			
	4	3	2	1
Michaelis constant for glucose (mM)	0.13	0.15	0.10	0.10
$v_{fructose}/v_{glucose}$	1.2	1.4	1.1	1.9
Chromatographic mobility (mM KCl)	38 ± 9	110 ± 10	130 ± 11	150 ± 21
Molecular size	–	100,000	97,000	97,000

* Isozyme preparations were obtained by DEAE-cellulose column chromatography, dialyzed and rechromatographed. Apparent K_m values were calculated from double reciprocal plots. Fructose and glucose phosphorylation was measured at 100 mM concentrations. Chromatographic mobilities are expressed as the means ± S.D. of ten experiments. The values for the relative molecular size were obtained from the experiment of Fig. 4.

ture was similar to the one described above except for the omission of $NADP^+$ and dehydrogenase, and the inclusion of 0.5 mM glucose containing about 400,000 cpm. The labeled glucose 6-P formed after 10 min of incubation was separated from unreacted glucose by filtration through small Dowex 1 columns and eluted with 0.4 M ammonium formate. Aliquots of the eluate were counted in a Philips PW 4510/01 automatic liquid scintillation spectrometer using a standard toluene-based scintillation liquid plus Triton X-100. This assay was used to measure enzyme activity in the effluent of small columns and kinetic values when the amount of enzyme available was scarce.

In either assay one unit of ATP: hexose 6-phosphotransferase activity is defined as the amount of enzyme catalyzing the phosphorylation of 1 μmol of glucose in 1 min at 30°C under the specified conditions.

Estimation of relative molecular mass. Two columns (column a: 0.8 x 18.5 cm; column b: 1.5 x 26.3 cm) containing Sephadex G-200 equilibrated with buffer B plus 10 mM 2-mercaptoethanol and 200 mM KCl were used. Fractions of 0.1 ml (column a) or 0.3 ml (column b) were collected. The columns were calibrated with the following markers: ovalbumin, rat brain hexokinase, horse liver alcohol dehydrogenase, bovine serum albumin, rabbit immunoglobulin, blue dextran and labeled leucine.

Subcellular fractionation. Subcellular fractionation was accomplished by differential centrifugation according to de Duve *et al.* (17, 18). Liver extracts were prepared in a buffer containing 5 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 0.25 M sucrose, pH 7.5 (buffer C). Catalase, glutamic dehydrogenase and NADPH: cytochrome c reductase were measured according to Leighton *et al.* (18) and Hogeboom and Schneider (19).

Other assays. Protein was measured by the method of Miller (20) and glycogen by the procedure of Montgomery (21).

RESULTS

Hexokinase activities in crude liver extracts from chick embryos

Glucose phosphotransferase activity was measured at 100 mM glucose in homogenates and in high speed supernates from the liver of chick embryos at several stages of development. "Particulate" enzyme activity *(vide infra)* was calculated by difference. Glucose phosphorylating levels of homogenates (Fig. 1, *top*) showed two small transient increases, the first at about the 13th, and a second at the 20th day of egg incubation. Enzyme levels at hatching (0.4 units/g liver) were about one third of the adult values. Hexokinase activity in the supernates was very low (0.1 units/g) and constant during egg incubation up to day 18th when the levels began to increase reaching a maximum of about 0.5 units/g at the 20th day. Thereafter, enzyme ac-

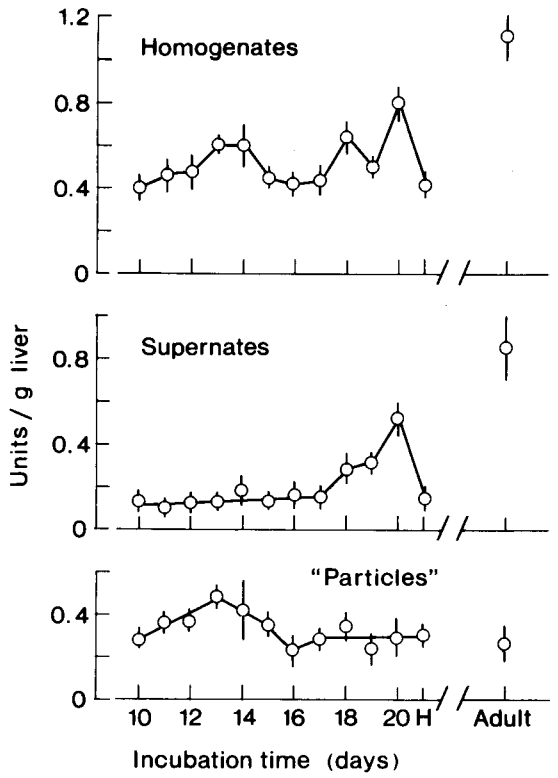

Figure 1: Developmental changes in glucose phosphorylating activity in crude chick liver extracts. Ten per cent homogenates of livers from chick embryos at different times of egg incubation were treated with Triton X-100, centrifuged at low speed, and the activity measured in the supernatant fluid (*top*), and also in high speed supernatant fluids of untreated homogenates (*middle*). Differences between the activity of homogenates and supernates are shown as "particulate" activity (*bottom*). Activities were measured at 100 mM glucose using the spectrophotometric assay. Each point corresponds to the mean ± S.E. of three observations.

tivity diminished rather abruptly at hatching time to levels about one tenth those of the adult (Fig. 1, *middle*). Thus, the elevation of enzyme levels of homogenates just before hatching reflects augmented activity of the cytosolic enzyme(s). On the other hand, the first transient increase seems to correspond to enzyme activity of the particulate material (Fig. 1, *bottom*). "Particulate" hexokinase levels were very similar either in embryonic or adult liver.

Glycogen levels during chick development

It was thought desirable to record hepatic glycogen levels during chick embryogenesis to seek any possible relationship between changes of hexokinase levels and polysaccharide accumulation. Liver glycogen content (Fig. 2) gradually increased five-to six-fold from day 15th onwards to attain maximal values (11 mg/g liver) at the 19th day of incubation and then diminished abruptly to the adult levels (3 mg/g) just before hatching. These temporal changes in glycogen content are in good agreemen with those observed by several workers (22-25). It should be noted that the second transient increase of hexokinase activity (Fig. 1) parallels glycogen deposition at the end of the incubation period. This may suggest that glycogen accumulation just before hatching is at least partially dependent upon synthesis from glucose.

Chromatographic separation of chick hexokinases during development

To study the hexokinase isozymes during chick development, chromatography on small DEAE-cellulose columns was used. Recoveries of glucose phosphorylating activity was about 75 ± 10 per cent. A total of about fifty runs were performed. The eight observations shown in Fig. 3 correspond to a series in which all eggs came from the same batch and incubation began at the same day. Then, starting from day 10th a daily experiment was run until hatching. A total of three such surveys were performed with almost identical re-

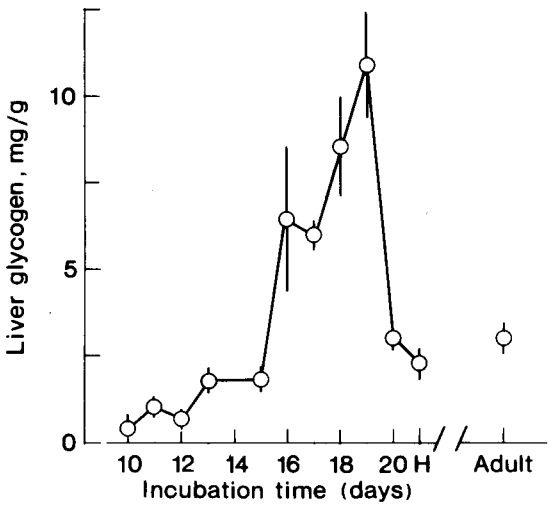

Figure 2: Liver glycogen levels during development of the chick embryo. Each point corresponds to the mean ± S.E. of three observations.

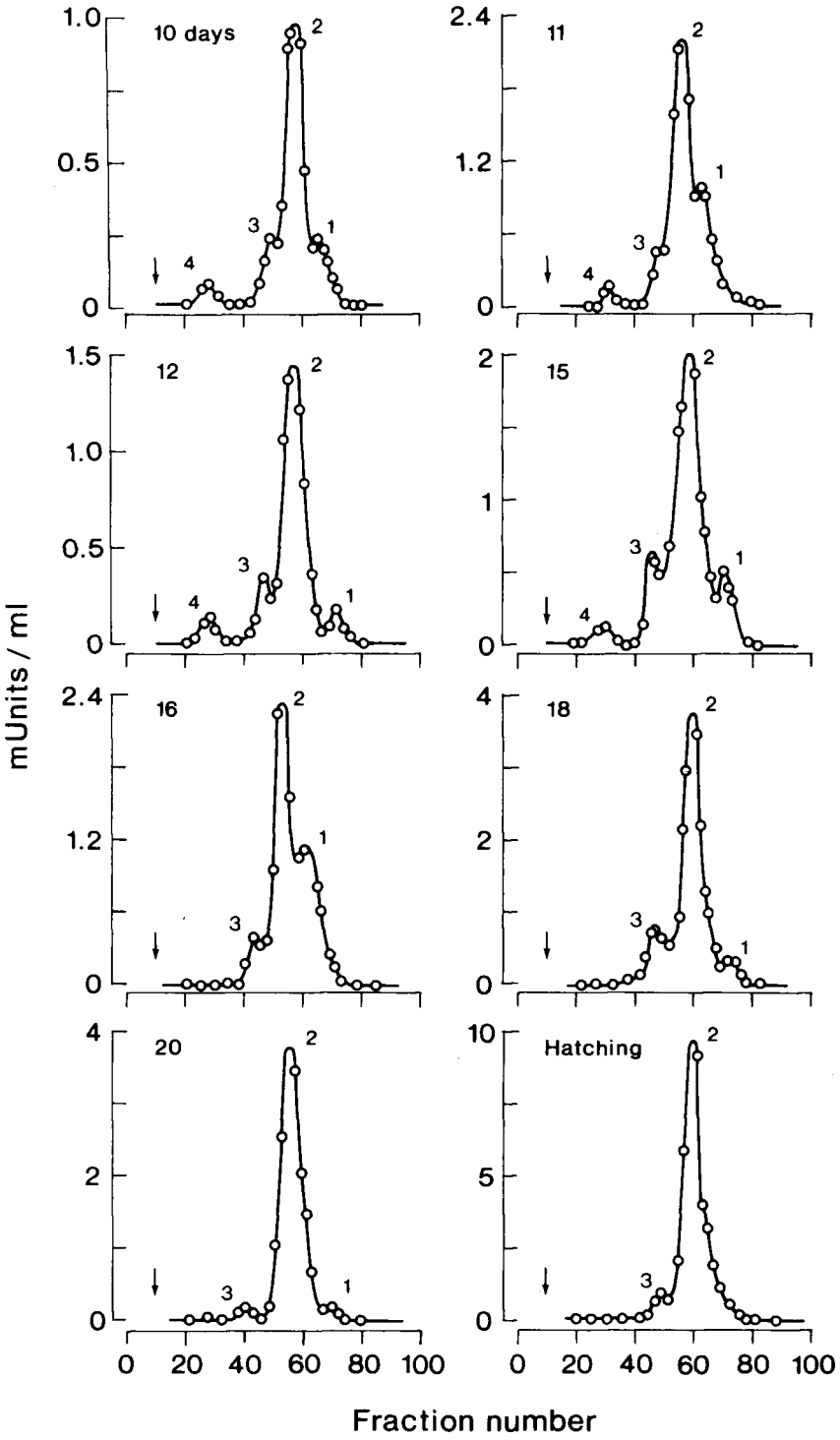

Figure 3: DEAE-cellulose column chromatography of glucose phosphorylating isozymes from the liver of chick embryos at several days of egg incubation. Aliquots of high speed supernatant fluids corresponding to 75 mg of chick liver were charged in 2-ml columns and processed as described under Materials and Methods. Individual livers were used except at days 10, 11, 12 and 15 in which case the livers of 5, 5, 4 and 2 embryos were pooled, respectively. Glucose phosphorylating activities were measured by the radioassay using 0.5 mM glucose as substrate.

sults. For some developmental stages, deemed critical because of the disappearance of some peaks of activity, additional chromatographic runs were performed. Thus, the results of Fig. 3 are indeed typical in the sense that the temptation to assort the best separations in a composite picture was resisted, not without regret.

As stated in the Introduction, two hexokinases are present in the liver of adult chicks (4,5). The same two isozymes were observed in the hatching chick (Fig. 3, *bottom right*). In the liver of chick embryos, however, four hexokinases were found which were only partially resolved, especially those eluting at high KCl concentrations. The use of bigger columns, such as those employed to obtain fractions amenable to characterization (but not suited for analytical work), gave somewhat better separations. Although the levels of some of the embryonic isozymes were always very low, they could be unmistakably documented using the sensitive and specific radioassay of Radojković *et al.* (16).

As discussed later on this paper, any equivalence between the glucose phosphorylating isozymes from mammals and those of chicks (or other birds) is still premature, and consequently it would be impertinent to employ for the present case the nomenclatural conventions used for the fairly well characterized mammalian hexokinases. Thus, for convenience in the description that follows, we will refer to the chick isozymes with numerals, hexokinase 1 being the isozyme most retained in DEAE-cellulose columns and presumably the most anodal form. This provisional designation does not, therefore, convey any parallelism of the avian and the mammalian isozymes which have been called A, B, C, and D as proposed by González *et al.* (26) or, alternatively, I, II, III and IV (the last being the most anodal form) as advocated by Grossbard and Schimke (27)*.

The most retained glucose phosphorylating isozyme (hexokinase 1) eluted from the columns as a well marked shoulder, sometimes as a distinct peak, in the descending limb of the hexokinase 2 peak (Fig. 3). Chromatographic mobility was about 150 mM KCl. From day 18th of egg incubation the levels of this isozyme began to decline and at hatching only traces could be occasionally detected. Hexokinase 1 is absent in the adult chicken (4,5).

The by far predominant isozyme during development of chick liver was hexokinase 2 (Fig. 3), as it is also the case for the adult animal in which it accounts for almost 85 per cent of the glucose phosphorylating activity of the soluble fraction (4,5). In embryos and adults this isozyme eluted from the columns at about 130 mM KCl.

Hexokinase 3 was always present during chick liver development. It eluted from the columns at about 110 mM KCl as a distinct shoulder at the ascending limb of the predominant hexokinase 2. Hexokinase 3 is likewise present in adult animals where it constitudes 15 to 20 per cent of the enzyme activity (4,5).

The first eluting hexokinase 4 (38 mM KCl) is a definitely embryonic isozyme. It was found already at the 10th day of egg incubation. From day 16th onwards it was no longer detectable although bare amounts could be ocassionally found in extracts from animals at day 1 after hatching in columns deliberately overcharged. When present, the levels of isozyme 4 were always very low.

* The nomenclature for the glucose phosphorylating isozymes is approaching a state of utter confusion. We have discussed elsewhere (3) the pitfalls of numbering systems for the identification of hexokinases, and have advocated the use of letters defined by the properties of each isozyme. Nonetheless, most workers in the field use roman numerals as proposed by Grossbard and Schimke (27), hexokinase I being the least anodal enzyme. On the other hand, Masters and Holmes (28) have used arabic numerals in the Enzyme Commission canonical way, e.g., hexokinase 1 being the most anodal form. Recently, Nagayama *et al.* (29) have used a letter system for fish hexokinases, but their hexokinase D seems to be equivalent to the mammalian enzyme which we have called hexokinase C, Grossbard and Schimke (27) nominated as hexokinase III, and Masters and Holmes (28) prefer to call hexokinase 2. It should be remembered that yeast hexokinases have been designated P-I and P-II (30), or I and II (31), or A and B (32), and that a third isozyme is called neither by letter nor by a letter-numeral combination, but instead by the word "glucokinase" even though the enzyme is clearly a multisubstrate hexose kinase (3).

Characterization of the hexokinases from chick embryos

In previous reports (4,5), "adult" hexokinases 2 and 3 from chick liver were characterized as low K_m glucose isozymes of broad sugar specificity and molecular weight of about 100,000. The discovery in chick embryos of two additional isozymes (hexokinases 1 and 4) immediately stimulated a search of their properties inasmuch as the chromatographic mobilities of the new hexokinases are not dissimilar to those of mammalian hexokinases A and D (6,26,33).

Enzyme fractions from a preparative column (charged with high speed supernates from the livers of 250 embryos of 14 days of egg incubation) were rechromatographed in DEAE-cellulose columns and active fractions were used for the measurements of K_m glucose values, relative velocity of fructose phosphorylation, and molecular size (Table 1). Because of the very low levels of the isozymes and the scarcity of starting material no further purification was attempted.

The four hexokinases displayed hyperbolic curves with glucose as variable substrate at a fixed saturating concentration of ATP (data not shown). Apparent K_m values were very similar in all cases (0.10-0.15. mM). The four embryonic hexokinases were able to phosphorylate fructose as effectively as, or slightly better than, glucose.

Estimations of molecular size were possible only with hexokinase 1, 2 and 3. Elution volumes of those isozymes from Sephadex G-200 calibrated columns permit the conclusion that all three have almost identical sizes of about 100,000 (Fig. 4).

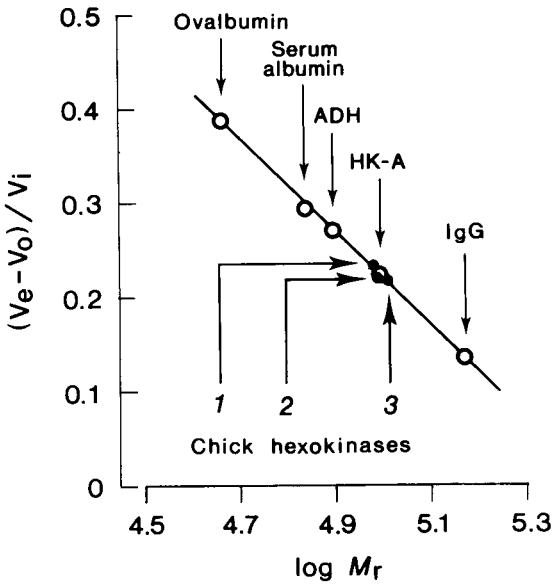

Figure 4: Determination of the relative molecular mass (M_r) of hexokinases 1, 2, and 3 from chick liver. Two calibrated columns of Sephadex G-200 (described under Materials and Methods) were used and the results pooled. Exclusion volume (V_0) is the evolution volume of blue dextran; internal volume (V_i) is the elution volume of [^{14}C] leucine minus V_0. Molecular mass estimation of hexokinase 1 was performed using the enzyme from embryos at 14 days of egg incubation. In the case of hexokinases 2 and 3, the enzymes were obtained from hatching chicks.

The association of chick liver hexokinases with particulate fractions

As shown in Fig. 1, a portion of the glucose phosphorylating activity of chick liver homogenates cannot be recovered in high speed supernatant liquids. A similar phenomenon has been repeatedly described in most rat tissues (30,34) including fetal and neonatal liver (12,35) but not adult liver. In the case of rat tissues, the sedimentable hexokinase activity has been shown to be located in the outer mitochondrial membrane (36) from which it can be specifically released by incubation with ATP or glucose 6-P (37,38).

Fig. 5 shows the results of differential centrifugation of sucrose homogenates from the liver of a 1-day old chick. Fractions are designated according to de Duve *et al.* (39). Most of the glucose phosphorylating activity was recovered in the post-microsomal fraction S. Mitochondria were almost devoid of activity. In fact, the particle bound activity was concentrated in the nuclear and microsomal fractions, a pattern characteristic of plasma membrane markers (40). Incubation of washed particulate material with 10 mM ATP or 2 mM glucose 6-P did not result in release of hexokinase activity (not shown).

Chromatographic patterns of particulate hexokinase activity released with detergent treatment from the liver of a chick embryo

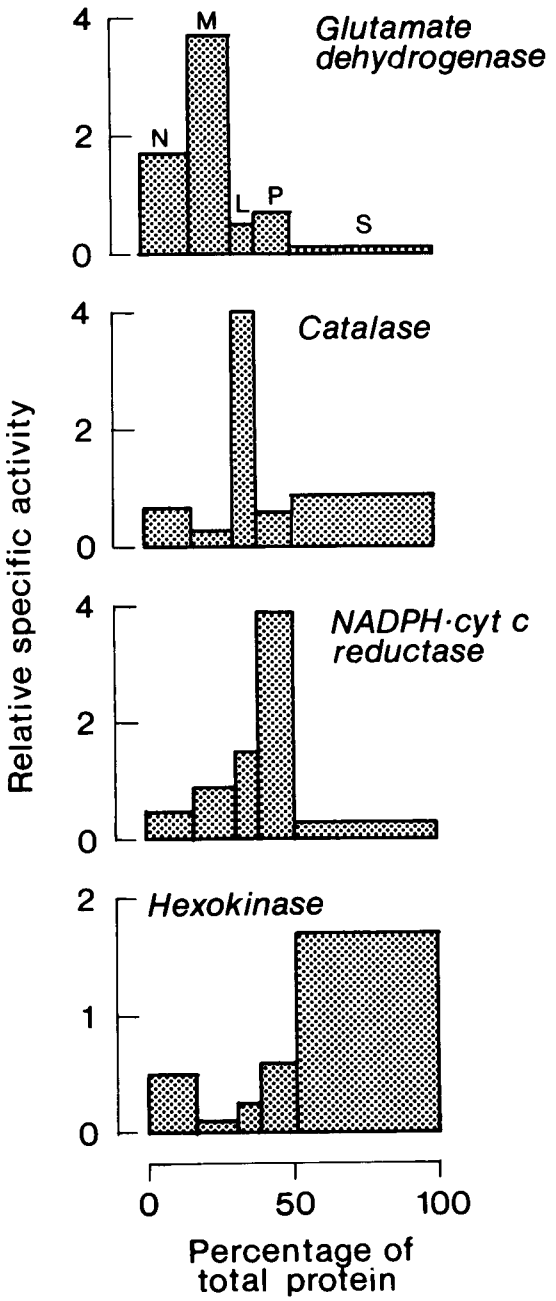

Figure 5: Distribution patterns of hexokinase activity and enzyme markers after fractionation of chick liver by differential centrifugation. Fractions are presented in the standard manner of de Duve *et al.* (17, 39, 40) in the order of sedimentation of subcellular components from left to right: N (nucleus), M (heavy mitochondria), L (light mitochondria, lysosomes and golgi), P (microsomes) and S (soluble proteins). For the assay of hexokinase activity each fraction was treated with Triton X-100 (0.1%), centrifuged at 105,000 x *g* during 30 min and the activity measured in the supernatant liquid with 0.1 M glucose using the spectrophotometric procedure described under Materials and Methods.

at 13 days of egg incubation (Fig. 6 *top*) or from a hatching chick (Fig. 6, *bottom*) revealed similar profiles to those found in the corresponding high speed supernates, except that hexokinase 4 was not observed in the case of the chick embryo.

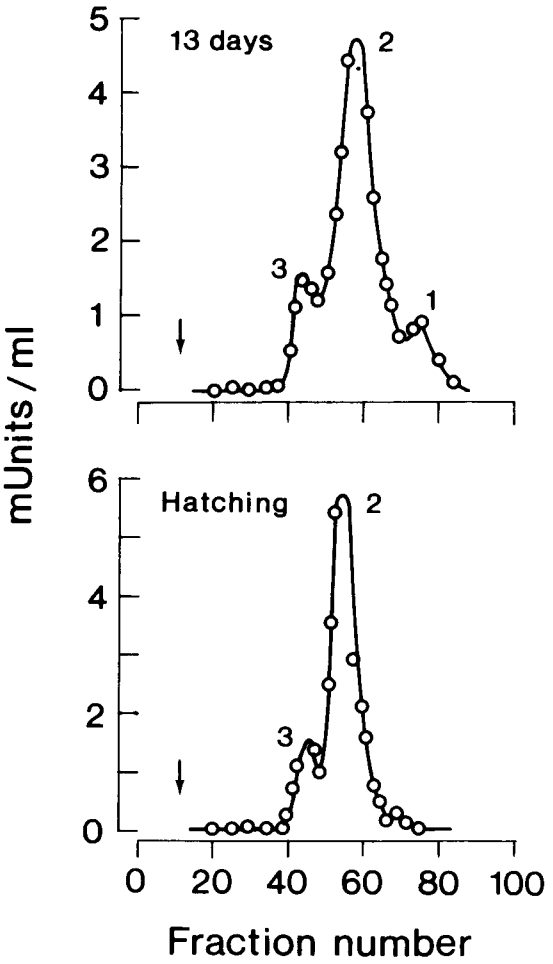

Figure 6: DEAE-cellulose çolumn chromatography of glucose phosphorylating isozymes from particulate fractions of chick embryo and hatching chick. The livers of a chick embryo at 13 days of egg incubation and of a hatching chick were homogenized in buffer B and centrifuged at 105,000 x *g*. The pellet was washed and resuspended in buffer B containing 0.1% Triton X-100 and centrifuged again at high speed. Aliquots of the supernatant liquid corresponding to approximately 100 mg of liver were charged in 2-ml columns and processed as outlined in the legend to Fig. 3.

DISCUSSION

The fact that the isozymic equipment of most fetal tissues differ from that of adults has been amply documented in studies of mammalian development. The same si-

tuation seems to occur during avian organogenesis as shown, e.g. by heart pyruvate kinases (41), muscle AMP deaminases (42), liver and muscle aldolases (43,44), lactate dehydrogenases from a few tissues (45), and skeletal muscle myosins (46). This change of isozymes has been teleonomically explained by the suggestion that fetal isozymes are better geared for functioning in the stable environment prevailing at that time of life. After birth, the need to adjust the metabolic flow to the external whims would require different enzymes. Verification of this hypothesis is not easy since it requires a knowledge of the role of isozymes in metabolism, a problem which is still in the speculative step (47). Nonetheless, studies attempting to document further examples of isozymic replacement during differentiation are important since by themselves they may provide an answer to the question: why isozymes?

A previous study from this laboratory (12) revealed that rat liver cytosolic hexokinases A, B, and C increase sequentially during the first week of extrauterine life to levels about four-fold higher than those of adults and then fall-off, also sequentially, to reach their characteristic adult levels at the time of weaning. At this time, hexokinase D suddenly appears reaching adult levels two weeks later (see also refs. 48 and 49). It is probable that liver hexokinases from other mammalian species behave in a similar way (50).

It is clear from the present work that the developmental pattern of hexokinases in chick liver markedly differs from that of the mammalian enzymes. The predominant isozymes throughout chick liver development are the same hexokinases 2 and 3 seen in adult liver. Moreover, embryonic chick liver displays two minor hexokinases (hexokinases 1 and 4) that are absent in adults.

The finding of four glucose phosphorylating isozymes in embryonic chick liver immediately recalls the fact that four hexokinases are also found in rat liver and makes one wonder whether any equivalence between those isozymes may exist. It has been shown (4,5) that adult chick hexokinases 2 and 3 are low K_m glucose enzymes displaying normal saturation functions for glucose, broad sugar specificities, and molecular weights of about 100,000 *i.e.*, they are not equivalent to either rat hexokinase C or hexokinase D. The latter is a high K_m isozyme presenting sigmoidal kinetics for glucose and a molecular weight of about 50,000 (8-10, 51). With respect to hexokinase C, it has been characterized as an enzyme which is markedly inhibited by glucose levels above 0.2 mM (6,7,26). Thus, the new chick hexokinases 1 and 4, and the "adult" isozymes 2 and 3 as well, seem to be quite different to rat hexokinases C and D. It is also clear that the identification of any of the chick isozymes with rat hexokinases A or B is dangerous since the similarities (chromatographic mobilities, sugar specificities and molecular weights) are not in any way diagnostic of the isozyme types involved.

The subcellular distribution of the hexokinase isozymes in embryonic chick liver also differs from that observed in the developing rat liver. As seen in Fig. 5, hexokinase activity of chick liver is practically absent from mitochondria whereas a significant portion of the enzyme in neonatal (but not in adult) rat liver is present in that subcellular fraction. It has been shown that glucose 6-P or ATP readily solubilize neonatal rat liver mitochondrial hexokinase*. On the other hand, chick liver "particulate" hexokinase activity was entirely refractory to that treatment.

The chromatographic patterns of hexokinase activity shown in Fig. 3 do not readily lend themselves to quantitative analyses of the temporal behavior of each isozyme. Nevertheless, it seems clear that hexokinases 2 and 3 are present in the liver even before the 10th day of egg incubation and that their levels increase at least four-fold after hatching. Thus, these isozymes should be included in enzyme cluster IV as defined by Greengard and Thorndike (52). No cluster was defined by these authors for enzymes (isozymes) which disappear during development as it is the case of chick liver

* T. Ureta, J. Radojković and E. Bustamante, submitted for publication.

hexokinases 1 and 4 (this paper), chick skeletal muscle aldolase C (44), and one of the two isozymes of chick muscle AMP deaminase (42).

It is pertinent to ask for the functional significance of the fact that two hexokinases are present in chick liver during the embrionic period only. As shown in Table 1, $K_{m\ glucose}$ values or substrate specificities of the four hexokinases are almost identical and thus, those features do not help for speculations about the specific involvement of each isozyme in the metabolism or its regulation in the chick embryo. We have put forward a hypothesis for the presence of isozymes which postulates that each component of an isozymic system participates in one and only one specific metabolic pathway (47). Therefore, we would like to suggest that the embryonic hexokinases 1 and 4 are involved in pathways of glucose utilization which are necessary only before hatching. However, at the present time we cannot offer any suggestion concerning those putative pathways.

Finally, we would like to stress the fact that the ontogenetic changes of the hexokinases from chick liver add another parameter to the list of peculiarities of the avian isozymic system for glucose phosphorylation (5). It is possible that those differences underlie the uniqueness of the regulation of carbohydrate metabolism in avian liver (53,54).

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