Adenosine Triphosphate-Hexose Phosphotransferase in Developing Chick Embryo Heart.* (30842)

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In the course of studies on the regulation of glucose uptake by chick embryo hearts, evidence was given that, under conditions in which glucose transport across the cell membrane is not limiting, the maximal rate of intracellular phosphorylation of the hexose decreases sharply between the 5th and 10th day of embryologic development(1,2). This result could be explained either by a progressive reduction in the absolute amount of the intracellular enzymes responsible for glucose phosphorylation or by a decrease in their efficiency, which is known to vary under different circumstances(3). Therefore, it seemed worthwhile to study these enzymes in detail, to follow changes in their activity during development and to explore the factors which might regulate their efficiency.

The present work deals with measurements of total ATP-hexose phosphotransferase activity in hearts during embryologic and postnatal development and describes some properties of this enzyme system.

Materials and methods. Tissue preparation. Hearts from chick embryos, chicks and adult chickens were dissected rapidly as described before(1), washed in Krebs-Henseleit bicarbonate buffer, trimmed and minced with scissors when necessary, blotted on acid-hardened Whatman filter paper and weighed as quickly as possible. Tissue samples were homogenized in chilled glass tubes (Teflon pestle) with 19 volumes (w/v) of ice cold 10 mM Tris buffer containing 5 mM EDTA and 10 mM 2-mercaptoethanol, pH 7.4. For kinetic studies, studies of enzyme distribution in subcellular fractions and studies of pH optima, 0.3 M sucrose was used instead of Tris buffer and the homogenates were centrifuged for 5 minutes at 600 $\times$ *g*, for 15 minutes at 9000 $\times$ *g*, and for 60 minutes at 105,000 $\times$ *g*, at 2°C. The concentration of the initial homogenate (Tris buffer) was increased 7-fold when studies on enzyme inhibition by glucose-6-phosphate were performed. Protein in tissue extracts was estimated by a biuret procedure(4). Dry weight was determined after drying the tissues at 100°C for 24 hours.

Enzyme assay. The activities of ATP-hexose phosphotransferases (EC 2.7.1.1./2) were assayed by coupling the production of glucose-6-phosphate from ATP and glucose to the reduction of NADP, in the presence of added glucose-6-phosphate dehydrogenase(5). The cuvettes contained 45 mM Tris buffer adjusted to pH 7.4, 0.5 mM EDTA, 10 mM $MgCl_2$, 15 mM ATP (pH 7.4), 1 mM NADP and 0.5 international unit of glucose-6-phosphate dehydrogenase. In addition, cuvette A contained 50 mM acetylglucosamine(6); cuvettes B, C and D contained 0.5, 2 and 100 mM glucose, respectively. The reaction was started by adding 0.1 ml of tissue extract (crude homogenate or supernatant) to 2.9 ml of the above mixtures previously heated to 25°C. Absorbance was measured at 1-minute intervals for 20 minutes at 340 mμ and 25°C. When pH optima were studied, 50 mM sodium glycyl-glycinate was used instead of Tris buffer in the mixture; in kinetic experiments, glucose concentration ranged between 1.5 $\times$ 10^{-5} and 0.1 M. In this assay system, no reduction of NADP by endogenous substrates occurs either in the absence or in the presence of acetylglucosamine and no direct oxidation of glucose takes place in the absence of ATP, even at the highest sugar concentrations (0.1 M) tested(6). Using 6-phosphogluconate as substrate, no appreciable activity of endogenous 6-phosphogluconate dehydrogenase (decarboxylating; EC

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TABLE I. Activity of ATP-hexose Phosphotransferase in Crude Homogenates and Cell Fractions of 10-Day Old Chick Embryo Hearts. Glucose concentration 10^{-2} M. Assay system described in text. Values are mean of 4 experiments.

Cell fractions	Activity, % of whole homogenate	Specific activity (μmoles glucose phosphorylated/ 100 mg protein/min)
Whole homogenate	—	1.1
600 × *g* supernatant	—	2.1
Cell debris (unwashed)	19	—
900 × *g* supernatant	—	2.2
Mitochondria (unwashed)	5	—
Microsomes	3	—
105,000 × *g* supernatant	73	2.5

1.1.1.44) was detected in the homogenates (1:19); therefore, the ratio of μmoles NADP reduced to μmoles of glucose phosphorylated approached unity. Since this ratio may increase above unity as a result of a differential concentration of endogenous 6-phosphogluconate dehydrogenase in various subcellular fractions, an excess of the latter enzyme (0.04 international unit) was added to the assay system. In this manner, approximately 2 moles of NADP were reduced per mole of glucose phosphorylated. In experiments on enzyme inhibition by glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADP were omitted and glucose disappearance was followed by a glucose oxidase-peroxidase method(7), using Somogyi filtrates after treatment with Dowex 50 ion-exchange resin.†

Results and discussion. The total glucose-phosphorylating activity of heart homogenates from chick embryos, chicks and adult chickens was not influenced significantly by the concentration of the substrate. When expressed as μmoles of glucose phosphorylated/ g wet tissue per minute, phosphorylation varied between 0.8 ± 0.04 and 2.2 ± 0.12 when the glucose concentration was 0.5 mM; between 0.8 ± 0.05 and 2.5 ± 0.09 when the glucose concentration was 2.0 mM and between 0.8 ± 0.08 and 2.5 ± 0.12 when the glucose concentration was 100 mM. These results indicate the presence of a low K_m phosphotransferase (hexokinase, EC 2.7.1.1) and the absence of appreciable quantities of high K_m glucokinase (EC 2.7.1.2), confirming previous observations in extra-hepatic tissues(5) and in fetal liver(8). After differential centrifugation, the ATP-hexose phosphotransferase activity of the embryonic heart was recovered almost completely in the 100,000 × *g* supernatant fraction (Table I). Maximum activity was found at pH 8.0-8.5, with little sensitivity to pH changes between 7.4 and 9.0 (Fig. 1). The K_m for glucose was found to be 6.1 ± 0.8 × 10^{-5} M at pH 8.0 (mean of 4 experiments; Fig. 2). The enzyme was inhibited by glucose-6-phosphate (Fig. 3) and in this respect, behaved in a manner similar to mammalian hexokinase(9, 10). Results presented in Fig. 4, where total enzyme activity is referred to the dry weight and to the protein content of the tissue, indicate that no changes occurred between the 5th and 7th day of embryologic development. After the 7th day of prenatal life, enzyme activity decreased moderately, approaching minimal values between the 12th and 15th day and fluctuating somewhat thereafter, until a few days after hatching, when the values were still somewhat lower than those observed in adult chickens.

It has been reported previously(2) that, under conditions in which glucose transport across the cell membrane is not limiting, the maximal rate of intracellular glucose phosphorylation in chick embryo heart decreases from 50-60 μmoles/g wet tissue/hr to 20-30 μmoles/g wet tissue/hr, between the 5th and the 10th day of development. The data reported here show that the reduction in phosphotransferase activity during the same period is not sufficient to account for the de-

† This step eliminates the barium ions remaining in solution after deproteinization and is necessary to prevent precipitation of the barium salt of ATP which otherwise would cloud the solution during the development of color.

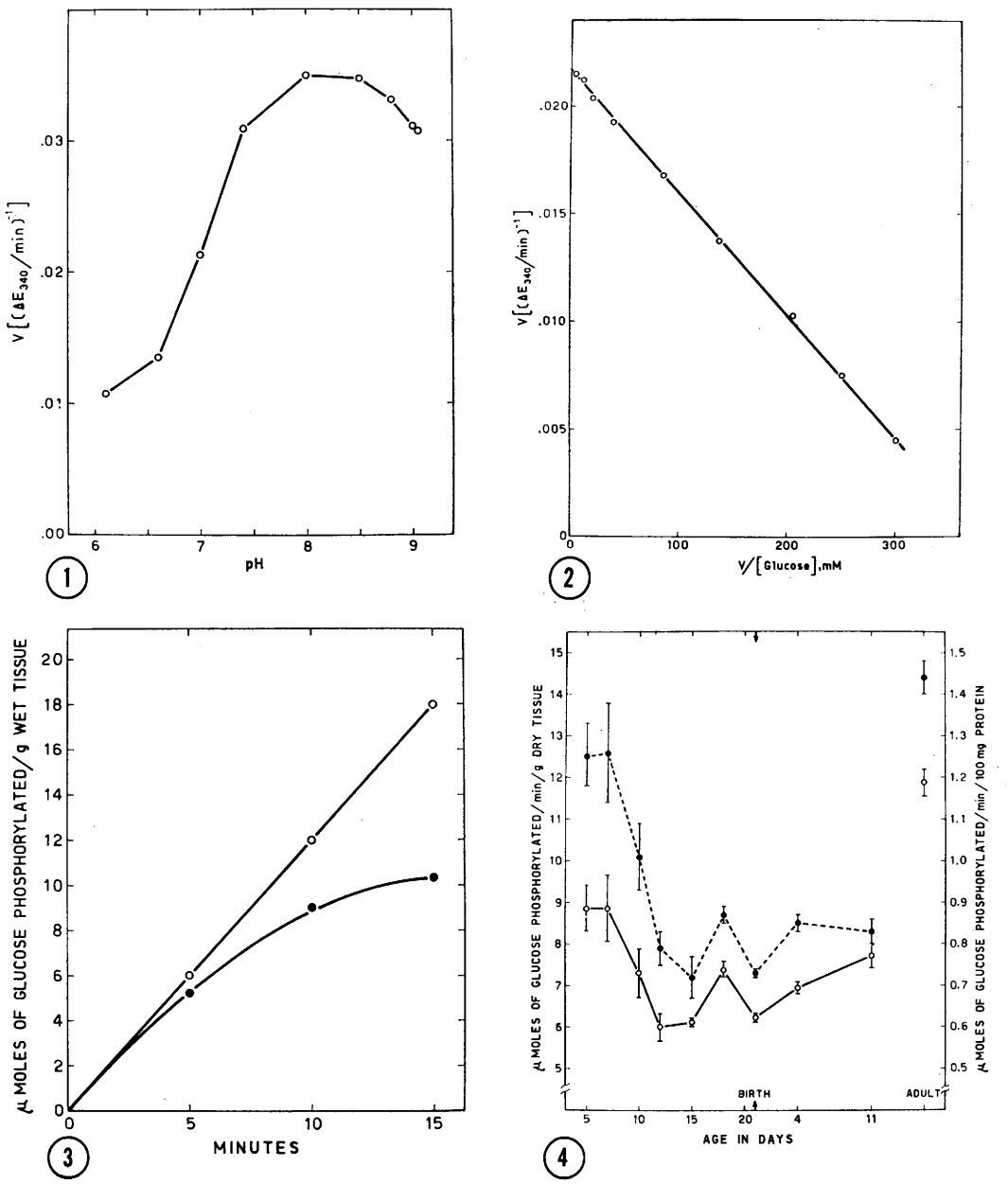

FIG. 1. Glucose phosphorylation as a function of pH in 100,000 × *g* supernatants of homogenates from 10-day old chick embryo hearts. Buffer: Na glycyl-glycinate. Glucose concentration, 2 x 10^{-2}M. Temperature, 25°C. Assay system described in text.

FIG. 2. Glucose phosphorylation as a function of glucose concentration in 100,000 × *g* supernatants of homogenates from 10-day old chick embryo hearts. Substrate concentration ranged from 1.5 x 10^{-5}M to 5 x 10^{-3}M. Assay system described in text. Curve derived using method of least squares. This experiment gives a K_m of 5.7 x 10^{-5}M glucose (pH 8.0; 25°C).

FIG. 3. Inhibition of ATP-hexose phosphotransferase by glucose-6-phosphate in homogenates from 10-day old chick embryo hearts. In control tubes (○——○), glucose-6-P was removed during incubation by the action of glucose-6-phosphate dehydrogenase and NADP. In the other tubes (●——●) glucose-6-P was allowed to accumulate. Incubation was carried out at pH 7.4 and at 30°C. Glucose (initial concentration, 1.5 x 10^{-3}M) was estimated by an enzymatic procedure described in text. Points are means of 3 determinations.

FIG. 4. ATP-hexose phosphotransferase activity in hearts from developing chick embryos, chicks and adult chickens. Assay conducted on whole homogenates at pH 7.4 and at 25°C, as described in text. Glucose concentration, 2 x 10^{-3}M. Values referred to dry weight (○——○) or to tissue protein (●——●) are means of 8 experiments ± S.E.

crease in the maximal rate of glucose phosphorylation. Hence, it must be concluded that other factors probably are involved in the regulation of rate of intracellular glucose phosphorylation. Among these possible factors are: changes in phosphofructokinase activity(3) and in concentration of hexose phosphates(11), nucleotides(12), or citrate (13,14) and, after the 7th day of embryologic development, the appearance of a transport system capable of regulating glucose entry into the cardiac cell(15). At physiologic plasma glucose concentrations (approximately 8 mM), this transport system limits glucose uptake by the 10-day-old chick embryo heart to values which do not allow accumulation of free intracellular glucose(2,16). The latter may be detected only when glucose entry is accelerated by abnormally high concentrations of glucose in the medium (24 mM or more) and by insulin stimulation of glucose transport. These findings suggest that, under normal conditions, the glucose phosphorylating system of the cardiac cell is far from being saturated. For this reason, it is concluded that the observed changes in ATP-hexose phosphotransferase activity after the 7th day of development are not likely to be a very important factor in the control of glucose utilization by the chick embryo heart.

Summary. Determinations of ATP-hexose phosphotransferase activity in chick embryo hearts demonstrated the presence of a soluble hexokinase. The enzyme has a low K_m for glucose, a maximum activity at pH 8.0-8.5 and is inhibited by glucose-6-phosphate. Total enzyme activity does not change between the 5th and 7th day of development, decreases moderately thereafter and increases after hatching. The slight reduction of total enzyme activity observed between the 5th and 10th day of embryologic development is inadequate to account for the previously reported decrease in the maximal rate of intracellular glucose phosphorylation occurring in intact hearts during the same period.

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