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REGULATORY ASPECTS OF THE KINASES OF CARBOHYDRATE METABOLISM

ABSTRACTS

The adenine nucleotides in metabolic regulation
DANIEL E. ATKINSON.— Department of Chemistry, University of California, Los Angeles. USA.

The adenine nucleotides are the major energy transducing system linking catabolic and anabolic metabolic sequences. Thus we cannot realistically discuss regulation of the kinases of carbohydrate metabolism without considering broader aspects of energy metabolism. In regulating the rate of flow through glycolysis, the kinases are responding to the energy needs of the cell; thus their regulatory properties are not related to glycolysis only, but to the total metabolism of the cell. That is, since the function of glycolysis is to supply ATP and biosynthetic intermediates, glycolysis cannot be self-regulated, but must respond to changes in the amounts of ATP and intermediates required by the cell.

The energy status of the cell is expressed in the concentration ratio [ATP]/[ADP] or in the mole fractions of the adenine nucleotides. A quantitative measure of the energy status of the adenine nucleotide pool is given by the adenylate *energy charge*, the mole fraction of ATP plus half the mole fraction of ADP. This parameter is found by analysis to be maintained at a value near 0.9 for most or all normally metabolizing cells. This stabilization must depend on regulation of sequences that use ATP and of those that regenerate ATP (such as glycolysis) by the adenine nucleotides. Thus neither regulation of glycolysis and respiration nor regulation of biosynthesis could exist alone; they interact continuously and are in fact two parts of a single regulatory system.

Some aspects of regulation of enzymes by energy charge and of the stabilization of energy charge *in vivo* will be discussed as background for the more specific treatments of regulatory enzymes to be given by other speakers.

The significance of phosphofructokinase allostery

JORGE BABUL.—Departamento de Química, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile.

Escherichia coli contains two fructose-6-phosphate kinases. PFK I is the main activity in the wild type strain, a widely studied allosteric enzyme specified by the *pfkA* gene. PFK II, a non-allosteric enzyme, is present in strains carrying the *pfkB1* mutation, a suppressor of *pfkA* mutants, and very low levels of this enzyme have also been detected in strains not carrying the suppressor (*pfkB*$^+$). The non-allosteric protein has now been purified from three strains, one carrying *pfkB1* and *pfkA*$^+$, one carrying *pfkB1* and completely deleted for *pfkA*, and one carrying *pfkB*$^+$ and also deleted for *pfkA*. The enzyme is apparently the same (PFK II) in all three strains, indicating that *pfkB1* is a mutation affecting the amount of a normally minor enzyme. PFK II is a tetramer of slightly larger subunit molecular weight than PFK I (36,000 and 34,000 respectively). No inmunological cross reactivity was detected between PFK II and PFK I. Pure PFK I, as has been described frequently, shows a sigmoidal dependence of rate on fructose-6-phosphate concentration, inhibition by phosphoenolpyruvate, and activation to hyperbolic kinetics by ADP. Pure PFK II presents hyperbolic curves as a function of fructose-6-phosphate concentration, and is unaffected by ADP or phosphoenolpyruvate. ATP has only a slight effect on the velocity of either enzyme. Fructose-1,6-diphosphate partially inhibits the reaction of PFK II but was without such effect on PFK I. Glucose-6-phosphate and fructose-1-phosphate show marginal activity as substrates for PFK II. Tagatose-6-phosphate was a substrate for PFK II but not for PFK I. Tested as effectors of PFK II, AMP, citrate, and pyruvate were without effect. The normal function of the low level of PFK II in the wild type strain is not known.

Phosphofructokinase was also purified from *E. coli* grown in a glucose limited chemostat, both aerobically and anaerobically. The enzymes had the same electrophoretic mobility, the same subunit size, and the same kinetic characteristics of PFK I. This shows that there is no conversion of PFK I into PFK II under such conditions and that both enzymes are PFK I.

In an isogenic *E. coli* strain series with various combinations and amounts of PFK I and PFK II (J. P. Robinson and D. G. Fraenkel), there was no large difference in growth rates or yields in a minimal medium with glucose, glucose-6-phosphate, and glycerol, aerobically and anaerobically. This suggests that the allostery of PFK I is not an important factor in the regulation of the glycolytic pathway during growth in *E. coli*. When the velocity curve of PFK I as a function of fructose-6-phosphate concentration was carried in the presence of ADP and phosphoenolpyruvate concentrations close to those in growth on glucose, an hyperbolic curve was obtained like PFK II. This indicates that the characteristics of pure PFK I and PFK II *in vitro* and in the cell are alike.

Mitochondrial Hexokinase: Key to the High Aerobic Glycolysis of Tumor Cells.

ERNESTO BUSTAMANTE.—Universidad Peruana Cayetano Heredia. Apartado 5045. Lima, Perú.

A tumorigenic anchorage-dependent cell line (H-91) was established in culture from an azo-dye-induced rat ascites hepatoma. When grown in a glucose-containing medium the cells exhibit high rates of lactic acid production characteristic of rapidly growing tumor cells. However, when glucose is replaced with galactose the cells grow equally well but exhibit only moderately elevated rates of lactic acid production. The molecular basis for this observation cannot be attributed to differences in permeability because initial rates of glucose and galactose entry into hepatoma cells are identical. Rather, the activity of hexokinase is found to be high in hepatoma cells, about 20-fold higher than that of control and regenerating rat liver. Moreover, tumor hexokinase activity is not inhibited by low concentrations of the reaction product glucose-6-*P*. Additionally, 50% of the hexokinase activity of hepatoma cells is found associated with the mitochondrial fraction. This fraction is 3-fold enriched in hexokinase activity relative to the homogenate and 4-fold enriched relative to the nuclear and post-mitochondrial fractions. Tumor mitochondrial hexokinase appears to be coupled directly to oxidative phosphorylation, because addition of glucose to respiring hepatoma mitochondria (after a burst of ATP synthesis) results in stimulation of respiration. In contrast, glucose has no effect on the respiration of mitochondria from control and regenerating liver. These results suggest that the high glycolytic capacity of H-91 hepatoma cells is due, at least in part, to an elevated form of hexokinase concentrated in the mitochondrial fraction of the cell.

Other rapidly growing, highly glycolytic, tumors (but not low glycolytic tumors) were found to have a significant portion of the total cell hexokinase bound to the mitochondrial fraction. It seems, therefore, that the expression of mitochondrial hexokinase in these hepatoma cells (that originate from hepatocytes wich do not have hexokinase but glucokinase) is an apparent function of the growth rate of the tumors and correlates well with their glycolytic activity.

Free ATP and glucose-6-*P* are specific agents for releasing hexokinase from the outer mitochondrial membrane. The non-hydrolyzable ATP analog AMP-PNP is also effective although not as much as ATP. ITP can partially substitute for ATP, but ADP, AMP, EDTA, and 0.1 M NaCl are ineffective as releasing agents. Addition of Mg^{++} can prevent release of hexokinase by ATP. In addition, inhibitor studies show that energization of mitochondria (either through ATPase action, ATP translocation, or a proton gradient) is not required for the solubilizing effect of ATP.

Kinetic studies indicate that the true substrate for hexokinase activity is MgATP and that free ATP is a rather potent competitive inhibitor of the reaction in addition to being a specific agent for releasing the mitochondrially-bound enzyme. The Km (MgATP) is 5-fold lower for both the mitochondrially-bound form

and the cytosolic hexokinase relative to the ATP-solubilized enzyme. However, the Km (glucose) of the three forms of the enzyme are almost identical.

The release and kinetic data are consistent with the view that there are two different kinds of hexokinase in H-91 hepatoma cells: a) *A mitochondrial form,* which may bind to or be released from the mitochondria depending on metabolic conditions. When bound to the outer membrane, this enzyme has a higher apparent affinity for MgATP than when solubilized. b) *A cytosolic form,* which has a high apparent affinity for MgATP.

Kinetic cooperativity of glucokinase with glucose.
MARIA LUZ CARDENAS.— Departamento de Biología, Facultad de Ciencias, Universidad de Chile.

Hepatic glucokinase present in several species of vertebrates shows cooperativity with respect to glucose and mannose as substrates, with a Hill coefficient (n_H) of 1. 5-1. 6. However, no cooperativity is observed when 2-deoxyglucose is used as the substrate.

Several attempts to modify cooperativity have failed. Thus, photooxidation with methylene blue or heating of the enzyme for variable periods before the assay, as well as the addition of Triton X-100 (0.1%) or urea (0.2 and 1.0 M) to the assay medium, while decrease V_{max} and increase $K_{0.5}$ do not alter significantly the n_H values. Likewise, by lowering the pH of the assay medium down to 6.5, V_{max} decreases to 47% and $K_{0.5}$ increases to 18.5 mM glucose, while n_H does not change. On the other hand, an increase of the ionic strength by KCl or NaCl (up to 0.4 M) does not modify n_H, even though V_{max} and $K_{0.5}$ decrease.

However, the degree of cooperativity for glucose is dependent on the concentration of Mg-ATP. As the concentration of this substrate decreases below 1 mM, $K_{0.5}$ and n_H values diminish. The function becomes hyperbolic at 0.2 mM Mg-ATP (2.5 times lower than K_m). On the other hand, increasing Mg-ATP concentrations up to 20 mM do not change significantly the n_H values, which remain about 1.6 The alternative substrates, mannose and 2-deoxyglucose, and the analog N-acetylglucosamine (GlcNAc), act as competitive inhibitors for glucose, and as $K_{0.5}$ increases, the cooperativity with glucose decreases. For example, increasing mannose concentration up to 20 mM (approximately 2 times its $K_{0.5}$) $K_{0.5Glc}$ varies from 5 to 32.5 mM and n_H from 1.6 to 1.0. In the presence of 10 mM GlcNAc (K_i *ca.* 0.4 mM) $K_{0.5Glc}$ increases to 208 mM, and n_H decreases to 1.0.

Rat glucokinase is a monomeric protein and does not polymerize under conditions similar to those of the assay. Thus, during gel filtration at 30°C in the presence of substrates and products, singly and in combination, only one symmetrical peak of activity is observed. The apparent molecular weight (54,000) agrees with the values reported for glucokinase in the presence of denaturating agents. GlcNAc does not modify the monomeric nature of the enzyme. The persistent monomeric form of glucokinase excludes the possibility that the cooperativity is the result either from the interaction of subunits or from an association-dissociation equilibrium in which the kinetic properties of the enzyme depend on the particular molecular weight species.

The observations are compatible with a steady-state model which consider at least two different pathways to convert the substrates into the products, probably derived either from the kinetic mechanism itself or from the existence of more than one conformational isomer of glucokinase with different reactivity.

Mammalian hexokinases: A system for the study of co-operativity in monomeric enzymes.
ATHEL CORNISH-BOWDEN, BERNARD A. CONNOLLY, MARY GREGORIOU, MICHAEL J. HOLROYDE, ANDREW C. STORER AND IAN P. TRAYER.— Department of Biochemistry, University of Birmingham, P. O. Box 363, Birmingham B15 2TT.

Glucokinase and hexokinase type II from the rat are both monomeric enzymes, but glucokinase (mol. wt. 48 000) is only half the size of hexokinase type II (mol. wt. 96 000). The amino acid compositions are strikingly similar, and statistical analysis indicates sequence identity of the order of 85%. Thus the sequence of hexokinase type II may consist of two similar halves, each of which resembles the sequence of glucokinase. We have tried to relate these presumed structural similarities to the substantial differences between the catalytic properties of the two enzymes.

Hexokinase type II is relatively unspecific for the hexose substrate; it is very sensitive to product inhibition by glucose 6-phosphate (K_i = 0.08μM); the K_m for glucose is low (0.2mM); it displays no co-operativity with either glucose or $MgATP^{2-}$. Glucokinase is relatively specific for glucose; it is insensitive to glucose 6-phosphate (K_i = 60mM); the half-saturation point for glucose is in the physiological range (about 5mM); the dependence of the rate on the glucose concentration is co-operative, with a Hill coefficient that rises from about 1.2 to about 1.6 as the $MgATP^{2-}$ concentration is raised from low levels to saturation, even though there is no $MgATP^{2-}$ co-operativity.

These observations suggest that the co-operativity of glucokinase is kinetic in origin, with only one binding site for glucose. At low $MgATP^{2-}$ concentrations the binding of glucose to the enzyme can equilibrate and there is no co-operativity, but at high $MgATP^{2-}$ concentrations co-operativity occurs because the enzyme-glucose complex reacts too fast for equilibration. In principle the results might be explained by the existence of two interacting glucose-binding sites, but the structural similarity to hexokinase type II makes this unlikely. Two glucose sites on glucokinase would imply four similar sites on the single chain of hexokinase type II. This is both implausible and in conflict with affinity labelling studies: inactivation of hexokinase type II by N-bromoacetyl-2-amino-2-deoxy-D-glucopyranose is competitively inhibited by glucose, and is complete when 1 mol of reagent is incorporated into 1 mol of enzyme.

The model for glucokinase co-operativity predicts that a less efficient substrate than $MgATP^{2-}$ would also be less efficient than $MgATP^{2-}$ in promoting glucose co-operativity, but the limiting Hill coefficient

would be the same. We are currently testing this prediction with $MgITP^{2-}$ and other poor substrates. Preliminary results are in accordance with those predicted.

Studies on bacterial pyruvate kinase: properties of the enzyme from Pseudomonas aeruginosa and Thermus thermophilus.

JAIME EYZAGUIRRE PH.— Instituto de Ciencias Biológicas. Pontificia Universidad Católica de Chile, Santiago.

Pyruvate kinase is a regulatory enzyme which responds to different kinds of modifiers, depending on its origin. Considerable differences are observed in this respect among bacterial enzymes, the nature of the effectors depending probably on the metabolic properties of a particular species. Although bacterial pyruvate kinases are known from several species, few detailed studies have been performed on their regulatory properties using purified enzymes.

For this work, pyruvate kinase from *Pseudomonas aeruginosa* (aerobic bacterium using the Entner-Doudoroff pathway) and from *Thermus thermophilus* (an extreme thermophile, whose metabolism is little known) have been selected.

Both enzymes have been partially purified, obtaining preparations with over 50% purity, as established by polyacrylamide gel electrophoresis. Kinetic studies show that both enzymes present homotropic cooperativity towards phosphoenolpyruvate while the ADP saturation curve is hyperbolic (in agreements with most other known pyruvate kinases). Glucose-6-phosphate and ribose-5-phosphate are heterotropic activators of both enzymes, while AMP and fructose 1,6 diphosphate (known activators of other bacterial pyruvate kinases) show no effect. Inorganic phosphate inhibits, in agreement with findings in other species. Chemical modification of the active site, using pyridoxal phosphate, suggests that lysine residues participate in their catalytic mechanism.

Despite of their very different origins, both enyzmes show great similarity in the properties studied so far. The main difference found is the thermostability presented by the enzyme from *Thermus thermophilus* The regulatory properties described for the *Pseudomonas* enzyme are compatible with a feed-forward effect by glucose-6-phosphate, but no conclusions can be drawn at present the metabolic significance of the regulatory findings observed with the *Thermus thermophilus* enzyme.

Kinetic mechanism of glucokinase. Order of addition of the substrates using 2-deoxiglucose as the sugar substrate.

OCTAVIO MONASTERIO.— Departamento de Biología, Facultad de Ciencias y Departamento de Bioquímica, Facultad de Medicina Norte, Universidad de Chile, Santiago.

Glucokinase is a monomeric enzyme exhibiting a sigmoidal function for glucose and mannose. The sigmoidicity disappears at low concentrations of MgATP. However, 2-deoxyglucose (2-dGlc) shows hyperbolic kinetics throughout a wide range of concentrations of both 2-dGlc and MgATP. The Km values for 2-dGlc and MgATP were 21 and 0.46 mM respectively. In view of the non-sigmoidal dependence of velocity upon 2-dGlc concentration, an attempt was made to learn about the mechanism of the reaction using this substrate.

Glucokinase was purified from rat liver. The kinetic studies were carried with an enzyme having a specific activity of about 1 unit/mg of protein. Test for contaminating enzymes showed that no hexokinase, N-acetyl-glucosamine kinase and adenosine triphosphatase activities were present in the preparation. Samples of partially purified glucokinase with different specific activities showed identical initial-rate behavior. The assay was performed in 0.5 ml total volume by measuring the ADP production through changes in absorbance at 340 nm at 30°. The assay mixture included: 80 mM Tris-HCl, pH 8.0; 12 mM $MgCl_2$; 100 mM KCl; 2.5 mM DTT; 1 mM EDTA; 0.3 mM NADH; 2.5 mM PEP; 1 unit of pyruvate kinase; 1 unit of lactic dehydrogenase and variable concentrations of MgATP, 2-dGlc, AMP and N-acetylglucosamine (GlcNAc).

The kinetic data were plotted graphically to determine the patterns. The kinetic constants were calculated by the least-squares method using an Altair 8800 b computer.

Initial velocity double reciprocal plots for both substrates showed straight lines intersecting to the left of the vertical axis, indicating a sequential mechanism. When a constant ratio of concentrations of both substrates was used a parabolic double reciprocal plot gave further support to this view.

Inhibiton product studies were not possible, owing to the very low affinity of 2-dGlc-6-phosphate. The dead-end inhibition protocols were used to know the order of addition of substrates in the enzyme reaction. GlcNAc, a competitive inhibitor for 2-dGlc, was a non-competitive inhibitor relative to MgATP at wide range of concentrations of 2-dGlc. On the other hand, AMP was competitive inhibitor for MgATP and uncompetitive inhibitor with respect to 2-dGlc at 0.41 mM MgATP. These observations were compatible with an ordered mechanism in wich 2-dGlc is the first substrate. However, the inhibition of AMP against 2-dGlc changed in character as the MgATP concentration increased over twice its Km. Thus, at 1.2 and 3.6 mM MgATP, the inhibition was clearly non-competitive. These results suggest that when the concentration of the nucleotide substrate is relatively high a random mechanism operates, i. e., the reaction flux going through the path in which MgATP is added before 2-dGlc becomes recognizable.

Effect of phenylalanine and alanine on the pyruvate kinase from Concholepas concholepas *muscle.*

A. MORAN, R. GONZALEZ y S. MUÑOZ.— Departamento de Bioquímica, Instituto de Ciencias Médico Biológicas, Universidad de Concepción, Chile.

Pyruvate kinase (ATP: pyruvate phosphotransferase, E. C. 2. 7. 1. 40) catalyzes an essentially unidirectional step in the glycolytic pathway. The enzyme occurs in at least three noninterconvertible forms in mammalian tissues, two of which exhibit a sigmoidal velocity response with respect to one of its substrates (phosphoenolpyruvate), suggesting multimolecular kinetics. In the presence of fructose 1-6 bis phosphate, the response is transformed into a classical hyperbolic relationship and the apparent affinity of the enzyme for the substrate increases.

The enzyme was purified from the muscle of the mollusc *Concholepas concholepas*. The tissue was homogenized with two volumes of ice-cold 10 mM Tris. HCl buffer, pH 7.4 containing 1 mM EDTA and 1 mM mercaptoethanol. To the supernatant liquid solid $(NH_4)_2 SO_4$ to 35-55% saturation was added. After centrifugation, the pellet was resuspended in buffer and further purified by gel filtration on Sephadex G100 and DEAE-cellulose chromatography. Pyruvate kinase activity was measured according to the method of Bücher and Pfleiderer. The rate of NADH oxidation at 340 nm, in a coupled reaction with excess lactic dehydrogenase, was used as an estimate of pyruvate formation.

The results show that pyruvate kinase has allosteric properties and that it is activated by K^+ and Mg^{++} ions. The $K_{0.5}$ values for increasing concentrations of PEP (at a constant level of ADP) at pH values of 6.5, 7.0, 7.4, 8.0 and 9.0 were 0.15, 0.14, 0.13, 0.13 and 0.14 mM respectivaly. The addition of 1 mM phenylalanine produced a marked effect on the substrate cooperative interactions and the $K_{0.5}$ values were raised to 0.40, 0 37, 0.40, 0.50 and 0.47 mM respectively. The Hill coefficient values were 1.2, 1.4, 1.59, 1.75, and 1.70 and were increased to 2.2, 1.80, 2.00, 2.37, and 2.30 in the presence of phenylalanine. Alanine had a similar effect, but to a lesser extent than phenylalanine. 0.1 mM Fructose 1-6 bis phosphate counteracted the inhibition of both aminoacids and hyperbolic kinetics were obtained in its presence.

Adaptive properties of liver glucokinase.

HERMANN NIEMEYER.— Departamento de Biología, Facultad de Ciencias, Universidad de Chile Casilla 653, Santiago, Chile.

Glucokinase is one of four glucose phosphorylating enzymes present in the liver of most mammals, amphibians and lower reptiles. The isozymes differ in kinetic properties, electric charge, molecular weight and immunological reactivity. A very distinctive feature of glucokinase is the sigmoidal saturation function for glucose, characterized by a Hill coefficient (n_H) about 1.6 and a rather high $K_{0.5}$ (concentration for half saturation), varying between 1.5 and 8 mM in the different species so far studied.

In order to explain the cooperativity with glucose it is important to consider that glucokinase is a monomeric enzyme under reacting conditions, and thus any mechanism implying an oligomeric structure must be disregarded.

The levels of glucokinase in rat liver depend strictly on the supply of carbohydrate in the diet. Thus glucokinase decays with a half-life of 33 hr when rats are starved or fed a carbohydrate-free diet, and is induced by the administration of glucose. The adaptive character is not exhibited by all mammals, indicating evolutionary discrimination probably related to feeding habits. The endocrine system plays an important role in glucokinase adaptation. Insulin is essential for glucose-dependent glucokinase induction and, in contradistinction, glucagon and catecholamines prevent the induction. Glucocorticoids and some pituitary hormones modulate the rate of induction. The variations in liver glucokinase correspond to changes in the amount of enzyme protein as assessed by immunochemical titration.

The kinetic parameters would permit an increased efficiency of the liver uptake of glucose at the changeable concentrations in the portal blood resulting from variations in the amount of dietary glucose. Considering the variable levels and the kinetic properties of glucokinase and glucose 6-phosphatase it is possible to estimate the relative contribution of the two enzymes to the balance of glucose uptake and release by the liver under different physiological conditions.

Factors regulating the appearance of glucokinase in neonatal rat liver.

NICOLA C. PARTRIDGE.— Departament of Biochemistry, University of Western Australia, Nedlands, Western Australia. Present address: Department of Medicine, University of Melbourne, Repatriation General Hospital, Heidelberg, Victoria, Australia.

The liver, in overall glucose homeostasis, has several unique features compared to other tissues. One of these is the presence of glucokinase (EC 2. 7. 1. 2). The kinetic properties of glucokinase allow the liver to control instantly the uptake of glucose in relation to the portal blood glucose concentration. This ability is lacking in the neonatal rat since glucokinase appears late in development; at weaning in the rat which is 21 days after birth. Once glucokinase activity appears, the liver can carry out blood glucose regulation with a capability equivalent to that found in adult tissue.

Although glucose and insulin regulate glucokinase activities in the adult animal, these factors do not seem to be responsible for the initial appearance of glucokinase in the weanling animal. The involvement of glucocorticoids and thyroid hormones in controlling the development of other parameters at this time has suggested a similar role for either of these hormones in the development of glucokinase. The appearance of glucokinase around weaning has been shown to coincide with high circulating thyroid hormone concentrations. The role of thyroid hormones, glucocorticoids, growth hormone and corticotropin in controlling the appearance of glucokinase has been examined. Only thyroid hormones are capable of prematurely inducing glucokinase although administration of glucose is always required. A normally functional thyroid gland is necessary for development of glucokinase and,

as well, for premature induction of glucokinase by glucose alone.

Since glucose is an absolute requirement for premature induction of glucokinase, its role has been investigated in relation to insulin, glucagon, adrenalin and glucocorticoids. Following the primary inductive event caused by administration of thyroid hormones, glucose together with insulin in responsible for the appearance of glucokinase while the gluconeogenic hormones are inhibitory in action.

It is concluded that increased circulating thyroid hormones in the late-suckling period cause the initial appearance of glucokinase. The ingestion of increased carbohydrate and decreased fat associated with weaning to an adult diet appears to modulate the rate of accumulation of the enzyme via the diet-related hormones insulin and glucagon.

The role of phosphofructokinase in muscle contraction

GOPI A. TEJWANI.— Departments of Pharmacology and Radiology, College of Medicine. The Ohio State University, Columbus, Ohio, 43210.

Skeletal muscle contraction results from interactions between troponin, tropomyosin and actin in the thin filament and myosin in the thick filament, in the presence of Ca^{2+} and energy. The energy for this process is provided by hydrolysis of ATP. The ATP used must be replenished for continued muscle contraction. There are four different sources for the availability of ATP. 1. *ATP Reserves:* The normal ATP concentration in muscle is about 7 μmoles/g wet weight. However, this concentration of ATP is not sufficient for even one second of rigorous muscle contraction; 2. *Phosphocreatine:* Its high energy phosphate group is very rapidly transfered to ADP, by the action of creatine kinase present in sarcoplasm. Even though the concentration of phosphocreatine in muscle is about five times that of ATP, it is sufficient to produce energy for only a few seconds; 3. *Oxidative phosphorylation:* ATP is synthesized by this process in the mitochondria, which are sparse and present in a small number in skeletal muscle. This system is too slow for the needs of muscle cells which consume energy at a high rate. Also, the oxygen supply for this process becomes limiting to the muscle contracting for prolonged periods; 4. *Anaerobic glycolysis:* The last and most important method by which sarcoplasmic reticulum synthesizes ATP is by means of anaerobic glycolysis, the process that generates energy already stored in glycogen.

The glycolytic rate in the skeletal muscle stimulated at different frequencies, may be increased 10 to 100 fold over the rate observed in resting muscle. This increase is correlated with the activation of phosphorylase *a*, and phosphofructokinase (PFK) which is the rate-limiting enzyme in the glycolytic pathway. It is inhibited by a high concentration of ATP, and this inhibition is reversed by fructose 6-P, AMP, ADP and P_i. Karpatkin *et al.* (J. Biol. Chem. *239:* 3139, 1964) concluded that the observed alterations in the concentrations of these effectors in stimulated muscle, are not sufficient to activate PFK by 10 to 100 fold, on the basis of the individual effect these effectors on the enzyme, as observed under *in vitro*. They therefore suggested that the activation of PFK is geared to the contractile process itself.

However, we (Tejwani *et al.*, Arch. Biochem. Biophys. *158:* 195, 1973) observed that the decrease in the "energy charge", and the increase in fructose 6-P, P_i and especially NH_4^+ concentrations observed in stimulated muscle act *synergistically* to increase the activity of muscle PFK 300-fold over its activity observed at the concentrations of above effectors and substrates found in the muscle at rest. An increase in the concentration of Mg^{2+}, which may occur in the sarcoplasm during muscle contraction, is also associated with an increase in the activity of PFK. It is concluded that contraction of the muscle is associated with a favorable ratio of positive to negative effectors of PFK. Positive effectors increase the activity of PFK in a synergistic manner, leading to the activation of enzyme by several hundred fold in contracting muscle, and that this activation of enzyme may not necessarily be geared to the contractile process itself. (Supported in part by EPA Grant Nº R804201-01-0).

Phylogenetic and ontogenetic studies of glucose phosphorylating isozymes.

T. URETA.— Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile.

ATP: Hexose 6-phosphotransferases (isozymes A, B, C, and D, EC 2. 7. 1. 1.; trivial name: hexokinases) have been separated by DEAE-cellulose column chromatography from a variety of vertebrates. Marked differences, both qualitative and quantitative, have been observed when individuals of different taxa are compared. Hexokinase A is present in all mammals and turtles studied, bufonid toads and some leptodactylid frogs. Hexokinase B has been found in some mammals, all turtles and amphibians analyzed. Hexokinase C, the substrate-inhibited isozyme, is present in some mammals and amphibians but not in birds, reptiles and fishes. Isozyme D, the so-called glucokinase, has so far been found in most mammals, turtles and amphibians. It is conspicuously absent in the liver of birds, higher reptiles and most fishes.

Hexokinases from birds and higher reptiles, although similar in general properties to isozymes A and B from other Vertebrates, differ nevertheless in their Michaelis constants, substrate specificities and chromatographic mobilities. Nomenclatural designations for the two or three sauropsid hexokinase isozymes is pending until further studies on purer preparations are performed.

The hexokinase pattern from mammalian skeletal muscle (isozymes A and B only), characterized by a marked preponderance of isozyme B, is very different to the liver pattern. A similar situation occurs in amphibian muscle. On the other hand, the hexokinase profiles from sauropsid muscle are undistinguishable from those of liver.

Quantitative analyses of the developmental patterns

of rat liver hexokinases show that hexokinase D is absent before and around birth appearing rather abruptly at weaning. The levels of hexokinases A, B, and C on the other hand reach maximal values (4-fold higher than in adults) at days +1, +3 and +7, respectively. Subsequently, the activity levels of these isozymes decrease sequentially to the low adult values. In chicken liver, the hexokinase profiles change very little during development. In frog liver, however, hexokinase D is very high at the tadpole stages to decrease gradually after the final step to the adult frog has been completed. Frog hexokinase C levels are very low at the tadpole stages to rise gradually to the high adult values (Supported by Servicio de Desarrollo Científico y Creación Artística, Universidad de Chile, PNUD-UNESCO RLA 76/006 and the Organization of American States).

Structural and developmental aspects of hepatic glucokinase

D. G. WALKER, M. B. ALLEN, M. J. O. WAKELAM, C. GIMENEZ AND C. ARAGON.— Department of Biochemistry, University of Birmingham, P. O. Box 363, Birmingham B15 2TT, UK.

An improved procedure for the purification of hepatic glucokinase in high yield resulted in a preparation that appeared homogenous by sedimentation-equilibrium ultracentrifugation and by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and having a mol. wt. of 48,000. Further studies have been made on preparations of glucokinase purified by procedures designed to achieve maximum recovery of activity. Electrophoresis on agarose gels, inmunodiffusion against an antibody raised to the purified enzime, shallow salt-concentration gradients on DEAE-Sephadex, inmunotitrations and inmunoelectrophoresis have revealed heterogeneity of kinase activity in these preparations. The several forms appear to be glucokinase in that their substrate specificities are identical. An electrophoretically-slower and quantitatively minor form, tentatively designated GKa, has a higher mol. wt. (~ 90,000), is inmunologically different, appears to be non-adaptive physiologicalli yet also has similar substrate specificity and a high Km.

The availability of an improved electrophoretic technique for detecting low glucokinase activity has facilitated a reappraisal of factors affecting glucokinase development in the neonatal rat. Following a reported suggestion that the thyroid hormones may be a natural trigger of normal glucokinase development, we have reached three conclusions:

a) While treatment with tri-iodothyronine does enhance the precocious development of hepatic glucokinase by glucose in the neonatal rat, glucokinase does appear and substantial activities can be induced in grossly hypothyroid animals. Increased circulating thyroid hormone concentrations are not therefore an essential requirement for development.

b) Even if the lactose, and hence carbohydrate, content of rat milk was substantially higher than it is, an earlier appearance of glucokinase is prevented by an inhibitory effect of galactose upon the "inducing" potential of glucose.

c) Precocious development of glucokinase is also prevented by the known neonatal hormonal status controlling in some way the synthesis of glucokinase and experiments suggest this operates via cyclic AMP.