Insulin Effects on Brain Energy Metabolism and the Related Hexokinase Distribution*

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SUMMARY

After 11 hours, male chicks (5 days old) injected intraperitoneally with 2 units of insulin, became hypoglycemic (2.51 ± 0.48 mm vs 14.42 ± 0.35 mm) and occasionally convulsed. The whole brain glucose, fructose, glucose 6-phosphate, lactate levels were diminished, respectively, from 15.6 ± 0.18 to 2.4 ± 0.04 mm, from 0.24 ± 0.16 to 0.02 ± 0.002 mm, from 0.139 ± 0.010 to 0.038 ± 0.003 mm, and from 0.18 to 1.74 ± 0.14 mm. Intraperitoneal injection of glucose resulted in recovery of all metabolites to near-normal levels within 10 min.

Hexokinase was found in both particulate and soluble fractions. After administration of insulin, hexokinase activity increased in the particulate fraction and decreased in the soluble fraction. After injection of glucose, hexokinase activity rapidly decreased in the particulate fraction and increased in the soluble fraction, indicating solubilization of the mitochondrially bound form of the enzyme. Thus, the following equilibrium was shown in vivo: mitochondrial hexokinase = soluble hexokinase. The percentage of hexokinase in the soluble fraction correlated directly with the intracellular glucose 6-phosphate level, suggesting that glucose 6-phosphate is an especially important metabolite in regulating the distribution of hexokinase.

Finally, the $K_i$ (glucose 6-phosphate competitive with ATP) was estimated to 6 μM for the soluble form and 30 μM for the bound form. Thus, the bound form of the enzyme is the more active form and under conditions of energy stress; e.g. insulin-induced hypoglycemia, the enzyme was converted to this form but reverted to the more inhibitable form (i.e. soluble) following glucose administration.

Hexokinase (EC 2.7.1.1) is found in mitochondrial and soluble fractions of rat brain homogenates (1-4). The distribution of hexokinase and the possibility of redistribution is important because the kinetic parameters of the bound and soluble forms of the enzyme have been found to vary such that the particulate enzyme is the more active form. The $K_m$ for ATP for the soluble bovine brain hexokinase has been reported as 1.7 mm (5), 2.83 mm (6), and 0.34 mm for the bound form (7). Whereas the brain form is type I (8, 9), muscle hexokinase, which is type II, has a $K_m$ for ATP of 1.33 mm in the bound form and 0.26 in the soluble form (10). The $K_i$ for glucose 6-phosphate for the muscle form of the enzyme also varies, 0.026 mm for the soluble form and 0.080 mm for the bound form (10). Similarly, although the $K_i$ was not determined, a 5-fold difference in $K_i$ was calculated for rat brain hexokinase (11). On the basis of the difference in kinetic parameters and the possibility of a hexokinase equilibrium between soluble and mitochondrial fractions, it has been suggested that such a mechanism may be important in controlling glucose phosphorylation (12). ATP (12-14) or glucose 6-phosphate (12, 13) applied singly can selectively solubilize hexokinase from mitochondria; Mg++ and inorganic phosphate (13) inhibit solubilization. Mitochondria partially freed of hexokinase will rebind hexokinase solubilized by glucose 6-phosphate (13). Following these reports, Purich and Fromm (15) incubated isolated rat brain mitochondria in 50 mM Tris and several metabolites at their in vivo levels; i.e. ATP, Mg++, ADP, GTP, UTP, and Pi and changed the glucose 6-phosphate and, in some experiments, the inorganic phosphate levels. Their studies revealed that the metabolites did not correlate well with respect to the solubilization or binding of hexokinase. Hence, they suggested that soluble-particulate distribution was relatively insensitive to changes in metabolite levels and therefore not likely to be important in regulation of hexokinase activity.

In recent experiments, we observed that hexokinase did redistribute in vivo and, in chick brain, varied according to the brain energy status (16). Under energy stress conditions imposed by galactose feeding or ischemia, the enzyme redistributed such that 80% of the enzyme was particulate and 20% was soluble, whereas approximately 50% was found in each fraction of cerebellar homogenates from control or untreated animals. The binding of hexokinase to mitochondria in vivo was very rapid; i.e. 2.5 min for changing from a 50:50 to an 80:20 mitochondrial-soluble distribution. The levels of numerous metabolites; e.g. glucose 6-phosphates, ATP, etc., associated with redistribution of hexokinase in vitro have been reported to change during ischemia (17, 18) and galactose feeding (19-22). Thus, the factor related to the binding could not be elucidated for the observed in vivo binding of hexokinase. In the earlier experiments (10), we did not report rapid solubilization of bound hexo-

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Kinase in vivo although such an observation is necessary for completion of the following equilibrium:

\[
\text{Soluble hexokinase} \rightleftharpoons \text{mitochondrial hexokinase}
\]

Excessive administration of insulin generally leads to prolonged and severe hypoglycemia followed by coma and ultimately convulsions. Following hyperinsulinemia, carbohydrate reserves are diminished (23) in mouse brain. Aves become moderately hypoglycemic after insulin treatment but are very resistant to insulin when compared with mammals (24–26). We have found, however, that with high doses and after several hours, the blood glucose level is decreased by 83% in young chicks. In the present investigation, we have examined aspects of brain energy metabolism in chicks rendered hypoglycemic by insulin and in chicks recovering from the effects of hyperinsulinemia by glucose administration. We report here a rapid solubilization of hexokinase in vivo in hypoglycemic animals ameliorated with injected glucose and the levels of selected metabolites that may be involved in the redistribution. Our interest in energy-deprived neural tissue stems from the observed brain energy deficiency in hypergalactosemic chicks where carbohydrate reserves are depleted and glycolytic intermediates are reduced (19–22). This report also shows a similarity in brain metabolism between hypergalactosemia and hyperinsulinemia in the chick.

**EXPERIMENTAL PROCEDURE**

**Animal Treatment**—Chicks were kindly provided by MacPherson Hatchery, Ionia, Mich., 1 day after hatching. They were housed in brooders at 32° and fed a commercial chick starter mash and water, ad libitum. For chicks treated with insulin, the diet was removed at 8:00 p.m. on the 5th day of age. Ten hours later, chicks weighing approximately 50 g each were injected with 2 units of Lente insulin zinc suspension (pur- chased locally). One milliliter of 1 mM glucose was administered intraperitoneally 11 hours after insulin treatment, and chicks were killed 2.5, 5, 10, and 20 min later. Metabolite Determinations—Chicks were decapitated directly into liquid nitrogen. The brains or brain regions were chipped out over Dry Ice, pulverized, and, where necessary, stored in a Revco freezer at −80°C. Metabolites were extracted as described by Lowry et al. (27) into perchloric acid, followed by potassium bichromate neutralization. Metabolites were assayed according to the procedure described by Lowry et al. (27) and as modified by Granett and Wells (18). Blood was collected in heparinized syringes, deproteinized according to the Somogyi method (28), and glucose was determined following addition to a hexokinase/glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (Sigma, St. Louis, Mo.) coupled assay (27).

**Tissue Treatment for Hexokinase Assay**—The chicks were decapitated, the cerebella removed and immediately submerged in cold 0.25 M sucrose, containing 1 mM mercaptoethanol, and homogenized by 10 complete strokes in a Potter-Elvejhem homogenizer. Fifteen to twenty seconds was the length of time between killing and initiation of homogenization. The homogenate was assayed, and a portion was centrifuged at 40,000 X g for 10 min to provide two further fractions to assay, the supernatant containing the soluble hexokinase and the pellet containing the particulate hexokinase.

**Hexokinase Assay**—Determination of hexokinase activity was a modification of the procedure described by Hernandez and Crane (29). Each assay cuvette contained 3.3 mM glucose, 6.7 mM ATP, 6.7 mM MgCl₂, 40 mM potassium Hepes, 1 mM 1-thioglycolate, 0.04 mM NADPH, and 1 unit of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (Sigma, St. Louis, Mo.) in a total volume of 1.0 ml (pH 7.5, 25°C). NADPH formation was followed at 340 nm, and unit of enzyme was defined as 1 μmole of NADPH formed per min.

**Kinetic Studies**—The affinity constants for hexokinase for glucose and mannose were obtained by determining the amount of hexose 6-phosphate formed by coupling with glucose 6-phosphate dehydrogenase. When using mannose, NADPH was added at the level of substrate, and mannose 6-phosphate formed in a 2-min incubation (terminated by boiling) at 25°C was determined. The enzyme was incubated in 40 mM Hepes, 1 mM hexose, 6.7 mM MgCl₂, and varying concentrations of ATP (pH 7.5, 25°C). The amount of inhibitor, glucose 6-phosphate, was estimated by addition of NADP⁺ and glucose 6-phosphate dehydrogenase. Fructose 6-phosphate was estimated by addition of phosphoglucoisomerase, and finally the product of the hexokinase reaction, mannose 6-phosphate, was quantified by adding phosphomannomutase.

**Preparation of Hexokinase for Kinetic Studies**—Particulate hexokinase was obtained as described in the section above. The 40,000 X g pellet was resuspended in 0.25 M sucrose containing 1 mM mercaptoethanol and reincubated at 40,000 X g, 10 min, before solubilization. The pellet was then resuspended in 0.25 M sucrose containing 1 mM mercaptoethanol utilized in the assays. Soluble hexokinase was prepared from the particulate fraction isolated above by adding 10 mM ATP and incubating 10 min at 25°C followed by centrifugation at 100,000 X g for 1 h. The procedure listed below was included to remove contaminating phosphoglucoisomerase and ATPase. The supernatant (10 ml) was added to DEAE-cellulose equilibrated with 0.01 M potassium phosphate, pH 7.0. The suspension was centrifuged and washed twice with 10 ml of 0.01 M potassium phosphate, pH 7.0, and then eluted following washes with 3 ml of 0.01 M potassium phosphate, pH 7.0, containing 0.2 mM KCl and 1 mM thioglycolate. The eluate containing the highest hexokinase activity and virtually free of the phosphoglucoisomerase and ATPase activities was used in measurements. In some experiments, the partially solubilized enzyme was re-added to the solubilized enzyme. In all instances, glucose 6-phosphate was a much more potent inhibitor of the solubilized hexokinase.

**RESULTS**

Insulin Effects on Blood Glucose Levels and Metabolites from Whole Brain—The 5- to 6-day-old chicks showed a decrease in plasma glucose from 14.4 ± 1.2 mM (n = 12) to 2.51 ± 0.48 mM (n = 14), less than 20% of the control level, 11 hours after administration of 2 units of insulin (Fig. 1A). Simultaneously, selected brain glycolytic intermediates all decreased from control levels (Table I). Glucose decreased from 1.6 ± 0.18 to 0.24 ± 0.04 mM following insulin treatment, glucose 6-phosphate, fructose diphosphate, and lactate decreased, respectively, from 0.044 ± 0.002 to 0.010 ± 0.002 mM, 0.139 ± 0.010 to 0.038 ± 0.003 mM, and 3.78 ± 0.18 to 1.74 ± 0.14 mM (mean ± S.E., n = 8). These changes are virtually identical to those observed following dietary galactosemia (19–22).

Intraperitoneal administration of glucose resulted in rapid (10 to 20 min) elevation in blood glucose (Fig. 1A) and in brain metabolites to levels equivalent to those observed for control animals.

The abbreviation used is: Hepes, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid.
Effect of insulin treatment followed by glucose injection on various metabolite levels of chick brain

Commercial diet was removed from male chicks 10 hours before insulin injection (2 units per animal). Experimental group and controls were decapitated into liquid nitrogen after 11 hours. Some animals were allowed to recover for 20 min following glucose administration (1 ml of 1 M). Data represent mean ± S.E.; n = 8 unless otherwise indicated by parentheses.

### Table I

<table>
<thead>
<tr>
<th>Brain metabolites</th>
<th>Control</th>
<th>11 hrs post-insulin treatment</th>
<th>11 hrs post-insulin treatment and 20 min following glucose injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose^a</td>
<td>1.35</td>
<td>0.20</td>
<td>1.70</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0.044</td>
<td>0.016</td>
<td>0.043</td>
</tr>
<tr>
<td>Fructose diphosphate</td>
<td>0.139</td>
<td>0.038</td>
<td>0.139</td>
</tr>
<tr>
<td>Lactate</td>
<td>3.78</td>
<td>1.74</td>
<td>3.50</td>
</tr>
<tr>
<td>Phosphocreatine</td>
<td>2.05</td>
<td>1.98</td>
<td>2.32</td>
</tr>
<tr>
<td>ATP</td>
<td>1.98</td>
<td>1.81</td>
<td>1.82</td>
</tr>
<tr>
<td>ADP</td>
<td>0.720</td>
<td>0.763</td>
<td>0.11</td>
</tr>
<tr>
<td>AMP</td>
<td>0.153</td>
<td>0.22</td>
<td>0.09</td>
</tr>
<tr>
<td>Energy charge</td>
<td>0.82</td>
<td>0.78</td>
<td>0.82</td>
</tr>
</tbody>
</table>

^a Corrected for contamination by plasma glucose as described in Ref. 22.

^p < 0.001, the Student t test, in comparison with either controls or 20-min recovery.

### DISCUSSION

The stress on energy and glycolytic metabolism following galactose-induced neurotoxicity in chicks has been well documented. Similar increases were also observed in galactosemic chicks injected with glucose (21, 22).

Insulin Effects on Cerebellar Metabolite Levels: Time Course during Recovery—The metabolite status of the cerebellum was quite similar to that of the whole brain (compare Table I with Fig. 1B). Glucose treatment resulted in rapid increases in the levels of many metabolites from their pretreatment nadirs. Glucose, glucose 6-phosphate, fructose diphosphate, and lactate rose almost in unison. Interestingly, glucose 6-phosphate levels stabilized before the other metabolites (e.g. fructose 1,6-diphosphate (F-1,6DP)) levels were plotted according to units on the right ordinate and glucose and lactate on the left ordinate.

The adenine nucleotides and phosphocreatine were measured at corresponding times after treatment with insulin (Fig. 2). Phosphocreatine was elevated significantly (p < 0.05) above the preinjection levels 5 min after glucose injection. Similarly, following glucose injection, AMP levels were decreased significantly (p < 0.05) at all time points when compared with either the control levels or those of insulin-treated animals.

Insulin Effects on Hexokinase Distribution in Cerebellum—In view of the known hexokinase distribution changes in chick cerebellum (16) and the ease of obtaining the cerebellum, this region was utilized for the hexokinase redistribution studies. Insulin treatment did not affect the total level of hexokinase activity but significantly altered the distribution from that observed in control animals. Control animals usually have from 45 to 50% of the activity in the soluble fraction (16), while the insulin-treated animals had only 23% of the activity in the soluble fraction (Fig. 3). Approximately 20% of the activity was soluble in chick cerebellum following galactose feeding or ischemia (16). Furthermore, following glucose administration, hexokinase rapidly disappeared from the particulate fraction and reappeared in the soluble fraction. The bulk of the redistribution occurred within a 5-min period.

**K**m for Hexose and **K**i for Glucose 6-Phosphate—The **K**m with respect to glucose has been observed as very similar for the soluble and bound forms of hexokinase (11). We have also observed that the affinity for glucose is 63 and 77 μM, respectively, for the bound and soluble forms for glucose, and 90 and 100 μM for mannose. In all cases, the **K**m value was near 10−4 M. The more important feature is related to the inhibition by glucose 6-phosphate (Figs. 4 and 5). The inhibition by glucose 6-phosphate is competitive with ATP, as reported earlier (7, 30, 31). Glucose 6-phosphate is clearly a more potent inhibitor of the hexokinase reaction compared to that of ATP.

**K**i for hexokinase was near 10− 4 M for glucose (30, 31). **K**i for hexokinase is competitive with AMP, as reported earlier (7, 30, 31). Glucose 6-phosphate is clearly a more potent inhibitor of the hexokinase reaction compared to that of ATP.


![Figure 1](https://example.com/fig1.png)
mented (16, 19-22). An interesting feature of that neurotoxicity rate is that the brain metabolite alterations are very similar to those that take place in chicks following insulin treatment, indicating that the requirement for glucose may be very important in either case. Hexokinase distribution has been implicated as an index of energy status in chick brain (33). It is of interest to note that both hypergalactosemia (21, 22) and hyperinsulinemia had identical effects on hexokinase distribution which were ameliorated by intraperitoneal injection of glucose.

It has been suggested that adenine nucleotide changes are quite dramatic during convulsive activity (34). However, the changes in the levels of the metabolites measured here corroborate an earlier observation with mice in which the levels of ATP and phosphocreatine were only slightly affected at the convulsive stage but the carbohydrate reserves were markedly depleted (24). The incidence of convulsions in the present study was relatively infrequent. Perhaps a longer hypoglycemic time period is required for both increased frequency of convulsions and alteration of the adenine nucleotide pool. Nevertheless, there were significant effects on both glycolytic metabolite levels and hexokinase distribution.

In order to provide evidence for the soluble hexokinase → mitochondrial hexokinase equilibrium, in vivo, the reverse reaction had to be shown. The ischemic study had earlier shown the...
or chick brain mitochondria. It is possible, however, that such an inhibitor of hexokinase. They indicated, however, that metabo-
to inhibit hexokinase to account for activities of glycolytic flux,
tween glucose 6-phosphate and hexokinase distribution is re-
but rather only in those samples obtained by decapitation di-
glucose 6-phosphate level was not estimated during this period
observed to prevent redistribution of hexokinase in vitro.2 The
conclusion may not be entirely valid since 20 s were required for
increase in hexokinase activity in the high speed supernatant frac-
tion.2 The data reported herein also corroborate other in vivo
based on results obtained with isolated mitochondrial prepara-
degradation in the other.
Thus, these data verify the suggestions
redistribution of hexokinase correlates best with the
increase in hexokinase activity in the high speed supernatant frac-
tions of the tissue (36). Secondly, a decrease in hexokinase activity in the high speed supernatant frac-
tion has been observed following isolation of the intestinal mu-
osa of fasted rats (36, 37) and rat epididymal fat pads (38). In
each of these cases, the enzyme appears to redistribute according
to the energy status of the tissue such that the more active form
predominates during energy-linked stress.

It would appear from the data obtained in the present study that
the redistribution of hexokinase correlates best with the
hexokinase levels (Fig. 6). It must be noted that ATP
levels did not change appreciably during the energy deprivation
period or following glucose administration. Therefore, it seems
less likely that under these conditions ATP levels can be related
to the observed redistribution although ATP is known to solu-
bilize hexokinase from isolated rat brain mitochondria (12–14)
or chick brain mitochondria.3 It is possible, however, that such a
conclusion may not be entirely valid since 20 s were required for
removal and cooling of the cerebellum to 0–1° which has been
observed to prevent redistribution of hexokinase in vitro.3 The
hexokinase 6-phosphate level was not estimated during this period
but rather only in those samples obtained by decapitation di-
rectly into liquid nitrogen. Nevertheless, the agreement be-
tween glucose 6-phosphate and hexokinase distribution is re-
markable (Fig. 6). Lowry and Passonneau (39) substantiated an
earlier observation (40) that glucose 6-phosphate is a potent
inhibitor of hexokinase. They indicated, however, that metabo-
lite regulation alone, in vivo, falls short of the control necessary
to inhibit hexokinase to account for activities of glycolytic flux,
in vivo; i.e., only 3% of the total enzyme activity is normally
expressed in mouse brain.

The following estimation of reaction velocity will indicate the
types of modifications that may be required for adjusting such
calculations. The values for $K_i$ of chicken brain hexokinase
were 6 μM (Fig. 4) for the soluble enzyme and 30 μM (Fig. 5) for
the bound form. The in vivo level of glucose 6-phosphate was
estimated to be 44 μM for control brains and 16 μM for energy-
depleted brains (Table I). One can calculate the effects of re-
distribution and changing glucose 6-phosphate levels on the
relative velocity employing the data plotted in Figs. 4B and 5F,
the in vivo ATP and glucose 6-phosphate levels expressed in
Table I, and the equation $v = 1 - \%$ inhibited activity in the
soluble Fraction $\times$ % soluble $+ 1 - \%$ inhibited activity in the
particulate Fraction $\times$ % particulate. A change from
50:50, bound-soluble distribution observed in controls, to an
80:20 distribution observed in energy-stressed animals results
in a 50% increase in the reaction velocity; i.e. $v$ (control) = 0.54; $v$ (stress) = 0.88. Because 20 s elapsed before cooling
the cerebella, during which time changes in metabolite levels
take place (17, 18, 20), the true in vitro bound to soluble hexo-
kinase ratio has not been established. Further calculations
presuming a distribution in control animals of 20:80 of bound-
soluble hexokinase, the velocity would decrease primarily due to
glucose 6-phosphate inhibition, by 18% from that observed when
the distribution is 50:50; i.e. $v$ = 0.46. Theoretically, the
maximum change in velocity due to the effects of changing
glucose 6-phosphate from 44 to 16 μM and redistributing hexo-
kine from all soluble to all bound would be 2.07-fold; i.e. $v$ for
100% soluble and glucose 6-phosphate of 44 μM would equal
0.42, $v$ for 100% bound and glucose 6-phosphate of 16 μM would
equal 0.87. In some limited experimentation, however, we have
been unable to shift the distribution of hexokinase to more than
50% in the soluble fraction even in chicks rendered unconscious
by elevated blood glucose.4 Thus, the initial calculations show-
ing a 1.5-fold increase appear to be more realistic for chick cere-
bellum.

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