Quantitative Correlation of Glucose Uptake and Phosphorylation with the Activities of Glucose-phosphorylating Enzymes in Perfused Livers of Fasted and Fed Rats*

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The purposes of these studies were (a) to examine the kinetics of glucose uptake by isolated perfused livers over a wide range of glucose concentrations (i.e. 2 to 80 mM), and (b) to compare observed rates of net hepatic glucose uptake and hepatic glucose phosphorylation calculated therefrom with levels of the liver glucose-phosphorylating enzymes glucokinase and hexokinase which were measured in biopsy samples from perfused livers. Levels of glucose-6-phosphate phosphohydrolase and carbamyl phosphate-glucose phosphotransferase activity also were assayed in these biopsy samples. Preparations from 48-h fasted rats were compared with those from normal fed rats. Observed rates of net glucose uptake by perfused livers from either fasted or fed rats were nonlinear functions of varying perfusate glucose loads. Further, rates of glucose utilization by livers of fasted rats were essentially equal to rates with those of fed rats, despite glucokinase being lower by about 60% in the former. At higher tested perfusate glucose loads (i.e. >11 mM with fasted rats; >30 mM with fed rats), rates of net glucose utilization were greater than could be accounted for on the basis of observed, combined glucokinase plus hexokinase activities. Estimated levels of carbamyl phosphate-glucose phosphotransferase activity were relatively small with fed animals, somewhat higher with livers from fasted rats with higher glucose levels, but in no situation sufficient to rectify the observed discrepancies between net glucose uptake rates and those of glucokinase plus hexokinase. Net glucose uptake rates were inhibited by 30 mM L-ornithine and by 30 mM 3-O-methyl-D-glucose, but not by 30 mM L-glucose. It is suggested that glucose phosphorylation mechanisms in addition to glucokinase and hexokinase, possibly certain phosphotransferase activities of glucose-6-phosphatase or other, uncharacterized high $K_m$ kinases, might be functional in rat livers when glucose loads are high, as in refeeding after a fast.

Hepatic glucose phosphorylation is currently considered to be catalyzed by (a) a less specific constitutive hexokinase (ATP:α-hexose 6-phosphotransferase; EC 2.7.1.1) (3-8), and (b) a relatively more specific, insulin-dependent glucokinase (ATP:D-glucose 6-phosphotransferase; EC 2.7.1.2) (5-9). Hepatic hexokinase is present in relatively low levels and has a very small $K_m$ for glucose of approximately $10^{-5}$ M (3, 4). In contrast, glucokinase, with a $K_m$ for glucose in the millimolar range, appears (when present, see Ref. 10) to be ideally suited for substrate-dependent glucose phosphorylative activity in the range of physiological concentrations (5, 8, 9).

A number of recent studies, reviewed in detail elsewhere by Nordlie (10), have raised questions as to an exclusive role for glucokinase and hexokinase in hepatic glucose phosphorylation in vivo. These observations seem to predicate a careful, quantitative re-examination and redetermination of the extent to which hexokinase and glucokinase contribute to in vivo hepatic glucose phosphorylation under various conditions. We have approached this problem by studying, as a function of glucose concentration, the net uptake of unlabeled D-glucose by isolated perfused livers from fed and 48-h fasted rats, a relatively simple, direct technique which obviates certain potential complications inherent in the use of "C- or 3H-labeled glucose (see "Experimental Procedures"). Levels of various glucose-metabolizing enzymes were concurrently measured in biopsy samples from these perfused livers. Direct comparisons were made between glucose uptake rates in perfused livers, estimated rates of total glucose phosphorylation in such preparations, and levels of various hepatic glucose-phosphorylating enzymes (singly and in combination) functional under presumed cellular conditions at a number of perfusate glucose levels.

From these studies it appears that glucokinase (plus hexokinase) activity may be insufficient to account for noted rates of net glucose uptake in liver preparations from fasted rats or, possibly, from fed rats perfused with high levels of this hexose.
**EXPERIMENTAL PROCEDURES**

**Animals** — Male Sprague-Dawley rats were obtained from ARS-Sprague-Dawley, Madison, Wis. Liver donors were 45 to 55 days old and weighed 180 to 210 g; blood donors were retired breeders. Except when food was withheld, rats were maintained on Purina Laboratory chow ad libitum. Tap water was routinely available to all animals.

**Technique of Liver Perfusion** — Livers, isolated from rats either fed or fasted for 48 h, were perfused by recycling 100 ml of perfusate, pH 7.4 to 7.6, consisting of rat erythrocytes suspended in Krebs-Ringer bicarbonate buffer (hematocrit 20 to 22%) containing 3% bovine serum albumin. Rats were pretreated with glucagon (300 μg/kg body weight) by intraperitoneal injection in order to deplete extant hepatic glycogen; surgery was initiated 3 h later. Other details have been described by Alvares and Ray (11). All perfusions were at 37 ± 0.5°C.

**Experimental Protocol for Liver Perfusion** — A representative experiment is depicted in Fig. 1. The procedure referred to above (i.e., Ref. 11) was adhered to except for certain specific modifications, following: After a 15 to 20 min "preperfusion" period an aliquot of perfusate (0.2 ml) was removed for determination of glucose ("0 min" in Fig. 1). Perfusate was sampled (0.2-ml aliquots) for glucose disappearance (from endogenous sources) at 8- or 10-min intervals thereafter for the duration of the experiment. Glucose production (from endogenous sources) by the perfused liver generally reached a plateau at 30 to 40 min after withdrawal of the first aliquot of perfusate; at this time a load of n-glucose (of predetermined concentration which varied with each perfusion experiment) was added (O in Fig. 1). This glucose was added as 1 ml of hypertonic stock solution in Krebs-Ringer bicarbonate, ranging in concentration from 1 to 1.7 M. After a 5- to 10-min equilibration period, glucose disappearance from the perfusate was monitored at 8- to 10-min intervals for at least 60 min following introduction of the initial load. Glucose uptake by red cells in the absence of perfusing livers, determined from separate experiments to be 0.58 μmol/min/100 ml of perfusate, regardless of glucose load, was accounted for in computations of glucose disappearance attributable to hepatic uptake. At this time (that is, after 110 min of intermittent perfusate sampling) two samples (pooled from segments of the left and median lobes) were taken from the liver for the assay, respectively, of glucokinase and hexokinase, and of glucose-6-phosphatase (phosphohydrolase and phosphotransferase). Subsequently the experiment was terminated, the liver removed, blotted dry and weighed.

In one initial study (see Table I) d-[U-14C]glucose (25 μCi/100 ml of perfusate) was included in the perfusates along with the desired concentration of cold n-glucose, and time-dependent disappearance of this added glucose was measured by scintillation spectrometry (Ref. 11; Packard model 2425 scintillation spectrometer). In all other studies, net glucose disappearance was monitored by chemical assay (see below). By using this latter approach, in which net glucose uptake is measured directly, we have obviated possible experimental complications which might exist with either d-[14C]glucose disappearance (12, 13) or release of H from d-[2-3H]glucose (14) utilized in attempts to quantify total glucose phosphorylation rates in perfused livers. A wide range of glucose concentrations, extending up to quite high levels, were employed in the present studies in which we were considering possible involvement of glucose kinases with high Km values for glucose. Under such circumstances, were isotopically labeled glucose employed, glucose-6-P-glucose phosphorylase activity of glucose-6-phosphatase (Equation 1; 15-17) might complicate accurate quantitation. For example, transphosphorylation from cold, endogenous glucose-6-P would serve to remove

\[
\text{Glucose-6-P} + \text{glucose} \Rightarrow \text{glucose} + \text{glucose-6-P}
\]

14C-glucose from perfusate without, however, bringing about any net glucose uptake. Likewise, transphosphorylation from endogenous glucose-6-P to [2-3H]glucose would produce 3H-labeled glucose-6-P which in turn would quantitatively lose H to medium water (14), without, however, providing for any net removal of glucose from perfusate (1 mol of cold glucose is produced for each mole of 3H-labeled glucose phosphorylated).

**Analytical Assays** — Liver samples were homogenized as rapidly as possible in 9 volumes of a buffer solution (pH 7.3) consisting of 150 mM KCl, 5 mM sodium EDTA, 5 mM MgCl₂, and 10 mM 2-mercaptoethanol. The homogenate was assayed immediately for glucokinase by a procedure modified by us for discontinuous assay from that of Sharma et al. (7). Reaction mixtures, pH 7.4, contained in 15 ml of 20 mM sodium cacodylate, 20 mM sodium salt of Hepes, 7.5 mM MgCl₂, 100 mM glucose, and 5 mM sodium ATP. The reaction was initiated by addition of 0.2 ml of the aforementioned homogenate, and terminated after a 10-min incubation period (without shaking), by the addition of 0.5 ml of perchloric acid (12%, v/v). Hexokinase was measured identically, except that concentrations of glucose (0.2 mM) were employed. These latter values were subtracted from those determined with 100 mM glucose to give glucokinase activity values. Glucose-6-P produced was assayed as previously described (18). Under our conditions, involving discontinuous assay and highly purified glucose-6-P dehydrogenase from Boehringer, it was established that a 1:1 stoichiometric relation

1 Perfusion glucose levels at the initiation of experiments with fasted rat livers were quite low (30 to 40 mg/100 ml); with livers from fed rats such initial levels were higher (200 to 300 mg/100 ml). Depletion of extant hepatic glycogen in the fed rat by glucagon administration 3 h prior to liver perfusion resulted in a decrease of initial, endogenous perfusate glucose levels to 30 to 40 mg/100 ml (thus approximating the level found in the fasted rat liver). Such low initial levels were necessary in order to permit achievement of desired perfusable glucose loads. Rates of hepatic uptake of a given glucose load and the assayable activities of the enzymes in perfused livers from fed or fasted rats were unaffected by pretreatment with glucagon (supplementary studies).

2 Assayable activities of glucokinase, hexokinase, carbamyl-P dehydrogenase and glucose-6-P phosphatase, and aldolase B were not affected by perfusion of the isolated liver with glucose loads of varied concentrations. This was established from the results of preliminary perfusion experiments in which such activities were compared in biopsy samples taken immediately before and 60 or 90 min after the addition of the glucose load. It was further determined that the net rate of hepatic uptake of glucose, adjusted for liver weight, was not significantly affected even after biopsy samples were removed in sequential fashion from the same liver.
Hepatic Glucose Uptake and Glucose Phosphorylation

...microsomes of glucose-6-P present and micromoles of NADP reduced. Inhibition by accumulated glucose-6-P (3), in the combined glucokinase plus hexokinase assay was calculated to be, maximally, less than 2.2% of total hexokinase activity and was disregarded.

Hydrolytic and synthetic activities of glucose-6-phosphatase were assayed in liver samples homogenized in 9 volumes of sucrose (0.25 M). Sodium deoxycholate solution was added to sucrase homogenates to a final concentration of 0.2%, w/v. Carbamyl-P-glucose phosphotransferase was determined as previously outlined (19); glucose-6-P phosphohydrolase activity was measured on the basis of P_i liberation as described by Nordlie and Arion (18). Assays for these latter two activities were at pH 7.0.

Supplemental, preliminary studies established that all enzymic activities, as assayed, were linear functions of both incubation time and protein concentrations within the ranges employed.

Methods for calculation of hepatic glucose phosphorylation rates and rates of the several individual enzymic activities at 37° and under various presumed physiological conditions are described under "Appendix." In certain selected experiments, samples of hepatic tissue were taken from perfusing livers immediately prior to, and 60 min following, the introduction of a glucose load. Each sample was halved. One of these halves was rapidly homogenized in 6 N perchloric acid, and the glycogen content of this homogenate was assayed using amyloglucosidase (20). Total lipid content of perfused livers was assayed in the other half of each biopsy sample using the chloroform-methanol-water extraction procedure described by Bligh and Dyer (21).

Materials—Trizma base (2-amino-2-hydroxymethyl-1,3-propanediol), NADP, Hepes, L-carnitine hydrochloride, glucagon [crystalline], dilithium salt of carbamyl-P, and the sodium salts of glucose-6-P, ATP, deoxycholic acid, and heparin (Grade I, 158 USP units/mg) were obtained from Sigma Chemical Co.; bovine serum albumin (fatty acid poor) was from Miles Laboratories; glucose oxidase-peroxidase reagent (Glucostat X 4) from Worthington Biochemical Corp.; D-glucose from Mallinckrodt; glucose-6-P dehydrogenase, carbamyl-P, due to its lability in solution, was introduced prior to adjustment to final volume and stored at 4° or -20° as required. Carbamyl-P, due to its lability in solution, was introduced into reaction mixtures just prior to assay (19).

RESULTS

Rates of Uptake of D-[14C]Glucose and Unlabeled Glucose—In initial studies with fed rats, the rates of uptake of [14C]-labeled and unlabeled n-glucose in livers perfused with various levels of this hexose were compared (Table I). In general, the rate of disappearance of [14C]glucose from perfusate somewhat exceeded the rate of net uptake of unlabeled glucose, a result anticipated since the rate of glucose phosphorylation (i.e., [14C]glucose utilization) should exceed the net disappearance rate of glucose. However, as pointed out in detail under "Experimental Procedures," above, systematic errors may possibly be introduced in the attempted measurement of glucose uptake with the use of [14C]glucose and unlabeled, endogenously generated glucose-6-P catalyzed by glucose-6-phosphatase (15-17). And, due to the relatively high K_m for glucose with this system (10, 15-17), such possible errors might become progressively more pronounced with the high levels of glucose selected for many of the studies presented. The use of D-[2-3H]glucose to assess glucose phosphorylation rates (14) may be similarly complicated by glucose-6-P:[2-3H]glucose phosphotransferase activity and is further contraindicated in the present context on economic grounds. Accordingly, it was decided to avoid such potential complications by measuring directly net glucose uptake chemically in the studies following.

It is worthy of note that, since net rates of glucose uptake are always less than total rates of hepatic glucose phosphorylation, this approach constitutes a highly conservative basis for comparison with hepatic glucokinase and hexokinase levels.

Rates of Net, Concentration-dependent Glucose Uptake by Perfused Livers from Fed and Fasted Rats—Rates of net glucose uptake during the first 40- to 50-min period following introduction of a load of this hexose into the perfusate system were assessed as a function of varied initial glucose loads. Each individual perfusion experiment (see Fig. 1 for an example) was subjected to linear regression analysis by the method of least squares (22) based on glucose concentrations determined in the several perfusate samples removed at successive 8- to 10-min intervals. Rates of glucose uptake in each individual experiment, ± 1 S.D. for these values, are presented as closed circles and vertical bars, respectively, in Fig. 2, A and B. With liver preparations from both fasted (Fig. 2A) and fed rats (Fig. 2B), a progressive, concentration-dependent, nonlinear rate of uptake was noted with increasing levels of glucose over the entire range studied. Further, the rates of glucose uptake with livers from fasted rats approximate those from fed rats.

To assess further the statistical significance of progressive increases in rates of uptake observed with increasing levels of perfusate glucose, data from Fig. 2, A and B, were grouped, according to perfusate glucose concentrations. Although the exact replication of perfusate glucose concentration from experiment to experiment was not feasible, it was possible to place data into several groups corresponding to relatively narrow ranges of glucose levels varying from low to quite high. Data thus grouped were subjected to analysis by the Student t test (23). Mean values for both perfusate glucose levels and rates of glucose uptake for each such group, along with standard deviations and p values based on comparisons of each group with successively higher perfusate glucose levels, are included in Table II where data for both fed and fasted rat preparations are presented. It is apparent from these data that, with one exception, each successive increment in glucose uptake noted with progressive increases in perfusate glucose level is statistically highly significant (i.e. p < 0.02), compared with the rate noted with the immediately preceding (and all lower) levels of glucose.

Formation of Glycogen and Lipid from Perfused Glucose—With the higher levels of glucose employed in the present studies, rates of hepatic glucose utilization observed appear to be the greatest which have yet been described in the literature. To gain some insight regarding the metabolic fate...
of this utilized glucose, we perfused livers of fasted rats, in the usual way, either with 74.7 mm glucose or without added glucose (controls), and determined the rate of glucose uptake in a period of 1 h. Additionally, biopsy samples of perfused livers were taken immediately prior to the introduction of glucose into the perfusion system and 1 h later. These samples were analyzed for glycogen (20) and total lipid content (21).

Based on these estimates and considerations, it is calculated that, of the 176 pmol of glucose taken up during the 1-h perfusion, 143 μmol, i.e. 81%, may be accounted for as synthesized glycogen and lipid.

The noted rate of glycogen formation is within the limits of hepatic UDP-glucose synthetase and total glycogen synthetase activity levels (see Ref. 24). However, triglyceride synthesis of 0.1 μmol/min/g of liver (i.e. 6.08 μmol/h/g of liver) requires a minimum formation of 2.4 μmol of acetyl-CoA/min/g of liver. This value exceeds either that of active pyruvate dehydrogenase observed in fasted rat livers by Wieland et al. (25) or total pyruvate dehydrogenase (active plus inactive; 1.7 μmol/min/g of liver, calculated by us for 37°C) noted by these same workers. However, in a very recent paper, Batenburg and Olson (26) report levels of rat liver total pyruvate dehydrogenase equivalent to as high as 2.3 μmol/min/g of liver (again calculated by us to 37°C). In fasted, refed rats the proportion of pyruvate dehydrogenase in the active form increases appreciably (25). The possibility is thus suggested that in our preparations of livers from fasted rats in which extremely high levels of glucose are perfused, either the glucose itself or some metabolite thereof might serve to activate the great proportion of existing pyruvate dehydrogenase. This idea, strictly speculative at present, deserves further detailed experimental examination as does the whole question of the metabolic fate of utilized glucose under our extreme conditions. Our present values on lipid formation are presented here because of their particular interest in the present context; we would emphasize their preliminary nature.

**Levels of Various Glucose- and Glucose-6-P-metabolizing Enzymes in Perfused Livers—Levels of glucokinase, hexokinase, glucose-6-P phosphohydrolase, and carbamyl-P:glucose phosphotransferase measured at 30°C in biopsy specimens obtained from perfused livers (see "Experimental Procedures") are presented in Table III. Under the conditions of assay employed, with relatively high substrate levels, the last mentioned activity exceeded that of glucokinase by about 14-fold in normal animals. This difference is exaggerated further...
(to about 53-fold) in fasting where insulin-dependent glucokinase decreases nearly 60% while synthetic and hydrolytic activities of glucose-6-phosphatase-phosphotransferase increase significantly. These changes in levels of glucokinase (6, 27) and synthetic and hydrolytic activities of glucose-6-phosphatase (28) in fasting are consistent with earlier observations, as is the absence of response of hexokinase (6, 7, 27).

Kinetics and Stability of Glucokinase——To ascertain whether or not a dependence of apparent Michaelis constant values on concentrations of "second substrate" might be involved (and to determine $K_m$ values), now-classical "five-hy-five" kinetic studies (see, for example Lueck et al. (29)) of glucokinase were carried out. Assays were performed by continuous recording at 340 nm generally as outlined by Sharma et al. (7). All assay mixtures, pH 7.4, contained in 3 ml of 40 mM Hepes buffer, 7.5 mM MgCl$_2$, 1.4 IU of glucose-6-P dehydrogenase, 0.75 mM NADP, varying amounts of ATP (0.10, 0.13, 0.20, 0.40, 1.0 mM) and glucose (6.7, 10, 20, 35, 50 mM), and 1.35 mg of liver cytosol protein. Activity with 0.5 mM glucose present also was measured at all ATP concentrations, and such values were subtracted to adjust for hexokinase activity (7). Data, plotted according to the method of Lineweaver and Burk (30), consisted of families of straight lines converging, in each instance, at points on the axes of abscissa (from which $K_m$ for MgATP and for $n$-glucose were calculated as negative reciprocal values). It is thus established that $K_m$, MgATP, 0.38 mM, and $K_m$, glucose, 23 mM, for both fed and fasted rats are independent of concentrations of glucose and ATP, respectively, and that $K_m$, MgATP-Glc = $8.7 \times 10^{-5}$ m$^2$ (i.e., $K_m$, Glc $\times$ $K_m$, MgATP). These parameters, which are in good agreement with values determined with the 870-fold purified rat liver enzyme (31), are utilized in later calculations, below.

Glucokinase and hexokinase levels were assayed, as described under "Experimental Procedures," in fresh preparations and at 36-min and later 60-min intervals with preparations kept on ice. No significant change in level of either activity was noted over the 5-h duration of the study. Stability of the enzyme at 0° previously reported by others (32) is thus confirmed.

Comparison of Net Glucose Uptake Rates with Levels of Glucokinase plus Hexokinase——With the use of enzymic activity levels in Table III and kinetic parameters from the above studies, levels of glucokinase with presumed physiological levels of MgATP (see "Appendix") and varying concentrations of glucose were calculated and adjusted to 37°. To these values, hexokinase levels (invariant over the glucose concentration range studied) were added (see dashed lines, Fig. 2, A and B). With liver preparations from fed rats (Fig. 2B), levels of such combined, traditional glucose-phosphorylating enzymes approximate observed rates of net glucose uptake with 10 to 30 mM glucose, but fall short as glucose concentrations are increased above 30 mM. With preparations from fasted rats (Fig. 2A), where glucokinase levels are decreased (see Table III), the discrepancy at higher glucose levels is considerably more pronounced, and correspondence of summation of

<table>
<thead>
<tr>
<th>Condition of animal</th>
<th>Glucokinase</th>
<th>Hexokinase</th>
<th>Carbamyl-P-glucose phosphotransferase</th>
<th>Glucose-6-P phosphohydrolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>0.93 ± 0.15</td>
<td>0.12 ± 0.01</td>
<td>12.9 ± 1.8</td>
<td>13.4 ± 0.8</td>
</tr>
<tr>
<td>Fasted</td>
<td>0.38 ± 0.17</td>
<td>0.12 ± 0.01</td>
<td>20.3 ± 4.5</td>
<td>19.3 ± 3.8</td>
</tr>
</tbody>
</table>

* Enzymic activity = micromoles of glucose-6-P produced (kinases and phosphotransferase) or hydrolyzed (phosphohydrolase) per min per g of wet liver at 30°. Mean values ± 1 S.D. are presented. 
* $p < 0.005$ for fasted compared with fed rats (23).

Inhibitory Effects of Ornithine on Glucose Uptake Rates——The possibility that synthetic activity of glucose-6-phosphatase might be involved in hepatic glucose phosphorylation has previously been suggested (10). The most potent of these activities at physiological pH is carbamyl-P-glucose phosphotransferase (10, 19) (Equation 2). A major potential source of rat liver

$$\text{Carbamyl-P + } n\text{-glucose} \rightarrow \text{n-glucose-6-P} + \text{CO}_2 + \text{NH}_3$$

orcellular carbamyl-P is mitochondrial, N-acetylglutamate-dependent carbamyl-P synthetase (CPS I) (see Refs. 33 and 34). The possibility that carbamyl-P synthesized by this enzyme may be employed as a substrate for glucose phosphorylation (35) and pyrimidine biosynthesis (36-38) as well as for urea formation (33, 34) has previously been suggested. The first step in the urea-biosynthetic cycle is the carbamylation of ornithine (see 33, 34). If, indeed, carbamyl-P produced by CPS I does serve as a substrate for glucose phosphorylation and urea biosynthesis, then ornithine might compete with perfusate glucose for this common substrate. To explore this possibility, ornithine was added to perfusates after an initial 50-min period of perfusion with a defined glucose load to assess uninhibited rate of glucose uptake. Perfusion with ornithine was continued for 60 min. With liver preparations from both fasted and fed rats, inhibitions of glucose uptake were noted at all of several perfusate glucose levels studied (Table IV). Control experiments indicated that the effects of ornithine could not be explained on the basis of the very small amounts of gluconeogenesis arising from the added ornithine itself (see Table IV, also see Ref. 39), or from a time-dependent falling off in rate of glucose uptake (supplemental control experiments were performed in which perfusions were carried out for 110 min with the various glucose loads without ornithine addition). Ornithine, at the level employed, was without effect on the glucose assay. The possible inhibitory actions of ornithine directly on hexokinase, glucokinase, glucose-6-P phosphohydrolase, and carbamyl-P-glucose phosphotransferase also were checked and found minimal or absent. Rat liver cytosol was employed as enzyme source for the former two enzymes while microsomal preparations were the source of the latter two activities. Reaction mixtures were generally as described under "Experimental Procedures," except that glucokinase was assayed with 20
In duplicate studies, livers from fasted animals were perfused for 55 min with 30 mM D-glucose and rates of D-glucose uptake monitored. Then, osmolarity was further increased by addition of 30 mM L-ornithine; perfusion with intermittent monitoring of D-glucose levels was continued for another hour. No significant differences in rates of D-glucose uptake (0.75 ± 0.10 and 0.71 ± 0.13 μmol/min/g of liver before and after addition of L-glucose, respectively; p > 0.5) were noted. In contrast, had the effect on D-glucose concentration in perfusate been simply an osmotic one, a significant increase in apparent D-glucose uptake rate comparable with that obtained in going from a concentration of 30 mM D-glucose to 60 mM D-glucose, an approximately 2.5-fold increase (see Fig. 24), should have been observed.

In a second set of duplicate studies, the effects of 3-O-methyl-D-glucose on rates of D-glucose uptake were similarly studied. This compound is without effect on hexokinase or glucokinase, but is a potent inhibitor of D-glucose phosphorylation by transferase activities of glucose-6-phosphatase-phosphotransferase (43) (its K<sub>i</sub> value of 110 mM for rat microsomes approximates the K<sub>i</sub> for D-glucose). In the present studies, the rate of uptake of 30 mM D-glucose, 0.74 ± 0.13 μmol/min/g of wet liver, was significantly reduced (to 0.36 ± 0.07 μmol/min/g of wet liver) by the addition of 30 mM 3-O-methyl-D-glucose to perfusates. (Neither 3-O-methyl-D-glucose nor L-glucose affected the analytical method employed for D-glucose.)

**DISCUSSION**

Other Glucose Uptake Studies—Although isolated perfused liver has been a preparation of choice for the study of glucose production (gluconeogenesis) in recent years, only a relatively few studies have been carried out in which glucose uptake and utilization have been investigated with such preparations. Miller (44) demonstrated glucose uptake by perfused livers from normal fed and diabetic rats. McCraw et al. (12, 45) carried out interesting studies in which both glucose utilization and gluconeogenesis from added lactate were measured concurrently by isotopic labeling techniques. Liver preparations from normal fed, fasted, diabetic, and adrenalectomized rats were employed. It was concluded that hepatic glucose-phosphorylating mechanisms auxiliary to glucokinase, perhaps P<sub>1</sub>,glucose phosphotransferase activity of glucose-6-phosphatase, were operative. (Friedmann et al. (46) have made a similar speculation based on results of studies of gluconeogenesis in vivo following glucagon-induced depletion of hepatic glycogen in fed and fasted normal and variously hormonally manipulated rats.) Brunnengraber et al. (47) measured the rate of conversion of label from D-[14C]glucose to fatty acids and 3-β-hydroxyester in perfused rat livers and concluded that their results would not be expected simply on the basis of glucokinase kinetic characteristics. In a recent study, Clark et al. (48) looked at glucose uptake as part of their study of futile cycles in isolated perfused livers, employing isotopic techniques, and concluded that "...there is an extensive futile cycle between glucose and glucose-6-P."

In studies most directly comparable with the present, Gor-

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**Table IV**

<table>
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<tr>
<th>Initial perfusate glucose load</th>
<th>Observed rate of net glucose uptake&lt;sup&gt;a&lt;/sup&gt; in fasted Glucose phosphorylative capacity in...</th>
<th>Glucose phosphotransferase assay. Incubations were carried...</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>μmol/min/g wet liver</td>
<td>Substrates were 5 mM. Glucose was 100 mM in the...</td>
</tr>
<tr>
<td>29.2</td>
<td>0.94 ± 0.07</td>
<td>Carbamyl-30 mM ornithine in reaction mixtures. Hexokinase...</td>
</tr>
<tr>
<td>41.3</td>
<td>1.55 ± 0.11</td>
<td>40 mM, as well as 100 mM, glucose. All phosphor...</td>
</tr>
<tr>
<td>54.1</td>
<td>1.79 ± 0.25</td>
<td>Glucose was 100 mM in the car...</td>
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*<sup>a</sup> Rates of net glucose uptake are presented as mean values ± 1 S.D. Rates of uptake of glucose in the absence of ornithine represent values obtained in perfused livers prior to the addition of ornithine to perfusates. Rates of uptake in the presence of ornithine were measured in the 60-min period following addition of ornithine to perfusates. These values have been adjusted for the low rate of net glucose production (0.18 ± 0.02 μmol/min/g of wet liver) from 30 mM L-ornithine noted in the absence of added glucose.

<sup>b</sup> Calculated as the numerical difference between values in the second and third vertical columns.

and 40 mM, as well as 100 mM, glucose. All phosphorylating substrates were 5 mM. Glucose was 100 mM in the carbamyl-P-glucose phosphotransferase assay. Incubations were carried out for 10 min, in duplicate, in the absence and presence of neutral 30 mM ornithine in reaction mixtures. Hexokinase was unaffected, glucokinase was increased an average of 3%, and hydrolytic and synthetic activities of glucose-6-phosphatase were inhibited approximately 5%

Although consistent with the involvement of carbamyl-P-glucose phosphotransferase in hepatic glucose uptake as outlined above, observed inhibition by ornithine is not unequivocal proof of this idea. For example, Stubbs and Krebs (40) have observed that ornithine, by mechanisms as yet not understood, inhibits gluconeogenesis from lactate in isolated liver cells from fed rats. If the effect proves to be one of the inhibition of a unidirectional, specific gluconeogenic enzyme, then our contention stated here would be strengthened, while if the effects involve one of the enzymes common to gluconeogenesis and glycolysis, then both glucose production and uptake might be impeded by added ornithine.

Effects of L-Glucose and 3-O-Methyl-D-glucose on D-Glucose Uptake—The 5- to 10-min interval between addition of the glucose load to perfusates and the first subsequent sampling appears more than adequate to allow for an equilibration of glucose between perfusate and liver cells, based on our quantitative considerations of rates of facilitated transport of glucose through such cellular membranes, using kinetic parameters recently described by Baur and Heldt (41). A continuous, prolonged, relatively constant rate of "pulling out" of water from liver cells due to osmotic differences between liver cells and the perfusate resulting from addition of glucose to such perfusates (which would result in a continuous dilution of perfusate glucose), thus appears remote. However, to further rule out any possibility that our observed apparent rates of D-glucose uptake might result artifactually from a continuous such dehydration of liver cells over a period of 2 h induced by differential osmolar conditions as a result of addition of sugar to perfusate, the following experiments were performed.

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<sup>1</sup> R. C. Nordlie, unpublished observations. Since K<sub>i</sub> for 3-O-methyl-D-glucose = K<sub>i</sub> for glucose, the former equimolar with the latter in perfusates (i.e. 30 mM as in the present experiments) should inhibit 50% any net glucose uptake dependent on phosphorylation via transferase activities of glucose-6-phosphatase with phosphoryl donors other than glucose-6-P.
Hepatic Glucose Uptake and Glucose Phosphorylation

Gordon (49) looked at glucose consumption by perfused, isolated livers from rats fasted for 18 to 20 h. In contrast with the broad range of concentrations of perfused glucose employed in our work, Gordon limited her studies to four concentrations of this compound between 100 and 400 mg/100 ml. Net glucose uptake values at these perfusate levels were very comparable to those reported here. For example, Gordon's values, calculated from data in Fig. 5 of Ref. 49, with 100, 200, 300, and 400 mg of glucose/100 ml of perfusate, respectively, were 0.23, 0.44, 0.74, and 1.30 μmol/min/g of wet liver. These values agree well with our corresponding values for fasted rats, given in Fig. 2A.

Original Experimental Features – The present studies are unique in that they involve preparations from both fed and fasted rats and extend to very high (approximately 80 mM) maximal perfusate glucose concentrations, both manipulations purposefully introduced to assess the possible involvement of an insulin-dependent, high Kₘ glucose-phosphorylating activity. Unique attempts to correlate hepatic glucose uptake with hepatic enzyme levels are described. Additionally, treatment of experimental animals with glucagon to minimize experimental complications due to hepatic glycogenolysis is included. A preliminary effort to correlate disappearance of glucose at high perfusate levels with its conversion to glycogen and total lipid is provided. Inhibitions by ornithine and 3-O-methyl-D-glucose of D-glucose uptake in perfused livers are reported for the first time.

Some Unique Observations – One of the most striking observations made is that the rates of glucose uptake in perfused liver preparations from fasted rats, where glucokinase is significantly diminished, approximate the rates observed with preparations from normal, fed rats (compare Fig. 2, A and B). Further, with preparations from fasted rats, rates of glucose uptake with perfusate glucose levels > 11 mm exceed combined rates of glucose phosphorylation via glucokinase plus hexokinase. Observed glucose uptake rates with levels of perfusate glucose > 30 mm also exceed such combined kinase activity in livers of fed animals.

Validity of Glucokinase and Hexokinase Values – It is always possible, of course, that due to instability some of the hepatic glucokinase might routinely be lost before assay. We would point out, however, that (a) our assays were carried out quickly with fresh preparations under conditions designed to stabilize the enzyme and to optimize assays (7), and (b) demonstrably, in our hands (see "Results") glucokinase was stable at 0°C for at least 5 h. Results reported here are consistent with values obtained in a variety of hands in our laboratory over a period of 5 years with livers of young adult male albino rats from Sprague-Dawley. Our glucokinase and hexokinase levels in livers of both fasted and fed rats compare well with values calculated from data of Sharma et al. (7). Values obtained by them and recalculated by us, for comparative purposes, on the basis of expression of enzymic activity employed in Table III (i.e. 30°C) are, for glucokinase 1.02 (fed) and 0.52 (fasted), and for hexokinase 0.13 (fed) and 0.2 (fasted) μmol of glucose phosphorylated/min/g of wet liver.

We have further compared our values with literature values from the review by Scrutton and Utter (24) and elsewhere.

In their review, Scrutton and Utter (24) indicate "best" fed rat liver glucokinase and hexokinase values, respectively of 4.3 and 0.7 μmol/min/g of liver at 37°C (i.e. 2.68 and 0.44 μmol/min/g of liver at 30°C), but give no primary references. We have obtained from ADI Auxiliary Publications Project, Library of Congress, materials supplementary to this review, and have examined primary references.

We have taken the liberty of recalculating all activity values to 30°C and expressing them as micromoles of glucose-6-P formed per min per g of liver. Glucose and MgATP concentrations were generally as in the present study. Thus expressed, glucokinase activity values for fed rats of various strains were 1.02 (7), 1.35 (51), 1.46 (52), 1.50 (8), 1.58 (32), 1.88 (53), and 2.06 (6). Comparable values for livers of fasted rats were <0.07 (8), 0.13 to 0.47 (53), 0.31 (6), 0.52 (7), and 0.58 (32). Hexokinase values, generally little affected by fasting, ranged from 0.12 to about 0.5 (7, 8, 51, 52, 54), with an isolated report of a value of 0.85 (6).

It would thus appear that our noted differences between net glucose uptake rates at higher glucose levels and combined glucokinase plus hexokinase activities would persist with fasted animals (Fig. 2A) regardless of whether our own or other enzyme level data are used as the basis of calculations. However, this would not necessarily be the case with preparations from fed animals. (We would emphasize two points here. First, net glucose uptake values, as presented in Figs. 2, A and B, are less than hepatic glucose phosphorylation rates by an amount equal to the rate of glucose-6-P hydrolysis (see "Appendix"). Second, it seems to us eminently reasonable that enzyme activity levels assayed in the same livers which had been perfused provide the most meaningful basis for comparisons.)

Some Alternative Explanations – Some possible alternative explanations for the present observations, and some comments thereon, follow: (a) as with any enzyme, glucokinase kinetics in situ might differ from those in vitro. Our own (see "Results") and Perry and Walker's (31) kinetic studies of this enzyme do not indicate deviations from classical Michaelis-Menten kinetics within the ranges of substrate concentrations dealt with in the present studies. (b) Glucose concentrations in the vicinity of glucokinase molecules might be higher than in the liver generally (which in turn reflects glucose levels in perfusate or blood (55, 56)). This is strictly conjecture; the same speculation might be made, e.g., with regard to synthetic activities of glucose-6-phosphatase. (c) Removal of glucose from perfusates metabolically by nonphosphorylative processes could conceivably serve to complicate the presently employed approach. However, quantitative considerations of glucose dehydrogenase (57), the only known such liver activity, seem to eliminate this possible complication. For example, using rat liver glucose dehydrogenase values and kinetic considerations of Horne and Nordlie (37) (Kₘ, glucose = 0.5 mM), we have calculated that with saturating pyridine nucleotide levels and physiological concentrations of the activators listed therein. Many of these references were of little or no value for our present purpose, including their Ref. 30, from which the "best" glucokinase value appears to have originated. Often, no indication is given of whether or not reduction of a second NADP by endogenous 6-phosphogluconate dehydrogenase was considered. A glucose concentration of 2.8 mm was used to measure "glucokinase" in one instance; in another pH 8.0 was employed for assay; in yet others "glucokinase" was used to describe a combination of glucokinase plus hexokinase, no attempt being made to measure and subtract the latter. Data were expressed as units per 100 g of body weight in several studies, and as units per mg of protein in another, although whether per mg of homogenate protein or per mg of high speed supernatant protein is not indicated. Three references were to reviews; we have included primary references involving the work of two of these groups (Sols and co-workers 6, 8, 27 and Sharma et al. (7)), and have extended our own search (including references listed by Weinhouse (50)) to include more recent studies on levels of these two enzymes in livers of both fed and fasted rats. Values in the text are from these sources which include the leading workers in the glucokinase field.
The possibility that apparent glucose disappearance resulted from higher glucose levels and certainly in fasted preparations. Bars 1) and combined glucokinase plus hexokinase (cross-hatching, vertical bars 2) appears capable of making a lesser but still real contribution, relative to glucokinase plus hexokinase; and its potential contribution becomes progressively larger as glucose levels increase, consistent with its $K_{m,Glc}$ value of 125 mM (29). The relative, potential contribution by this transferase activity is comparatively small in fed animals (Fig. 3B). With neither the fasted nor fed preparations can this activity by itself serve to fill wholly the void existing between total hepatic glucose phosphorylation and calculated levels of hexokinase plus glucokinase. Possibly, such activity of glucose-6-phosphatase might make a partial contribution in this regard, dependent upon both enzyme levels and availability of carbamyl-P to the cytosol.

Although demonstrated levels of hepatic cytosolic carbamyl-P synthetase II are very small (32, 34), we have calculated from the data of Schimke (58) that mitochondrial carbamyl-P synthetase I is capable of generating 5.5 $\mu$mol of carbamyl-P/min/g of liver at 37$^\circ$C with livers from fed rats and 14 $\mu$mol/min/g of liver with livers of fasted rats, values more than ample to accommodate rates of calculated carbamyl-P-glucose phosphorylating enzymes (Figs. 3, A and B). We must emphasize that because actual levels of carbamyl-P in cytosol of liver have not been measured, our calculations and reflections based thereon are speculative. In this regard, we would point out however that Tremblay and co-workers (36-38) in recent years have presented an imposing body of evidence supporting the availability, in ureotelic livers, of mitochondrially generated carbamyl-P for pyrimidine biosynthesis in hepatic cytosol, and Lueck and Nordlie (35) have demonstrated the utilization of mitochondrially generated carbamyl-P for microsomal glucose phosphorylation in a reconstituted system. Also, the $K_e$ for carbamyl-P with rat liver ornithine transcarbamylase, 3 mm (59), is quite similar to that for carbamyl-P-glucose phosphorylase (2.5 mm; Ref. 29).

Fig. 3. Comparison of rates of glucose utilization and estimated total glucose phosphorylation in isolated perfused livers (Columns 1) with rates of hepatic glucose-phosphorylating enzymes calculated for various glucose levels and otherwise presumed physiological conditions (Columns 2).
phosphoryl donors other than glucose-6-P.$^8$ This compound is neither substrate nor inhibitor with glucokinase (42) or hexokinase,$^6$ but is both an effective inhibitor$^7$ (43) and substrate (60) with synthetic activities of glucose-6-phosphatase ($K_i = K_m = 110 \text{ mM at pH 7.4 (60).}$)$^7$

**Conclusions**—Those studies were designed to bring to light possible "high $K_{m,\text{glc}}$" enzyme(s) of liver which might work supplemental to glucokinase. The ideal experimental model, i.e. a small, perfusable liver naturally devoid of glucokinase, was not at hand. Accordingly, glucokinase was minimized by fasting of rats, and, at extremely high, quite nonphysiological levels of glucose were perfused to accentuate any such activities which might be present. Our major concern here has been, not to investigate normal physiological function, but to show that rat livers might be capable of manifesting appreciable glucose uptake by some mechanism under these extreme experimental conditions. The intriguing idea, raised by the present studies, that one or several phosphotransferase activities of glucose-6-phosphatase, or some other as yet undiscovered "high $K_{m,\text{glc}}$" enzyme(s), might play hepatic glucose phosphorylative roles under certain conditions (e.g. in the refed, fasted rat, or in the diabetic) is under further study in this laboratory.

**Acknowledgments**—We are indebted to R. L. Bearman, S. Bagan, R. A. Jorgenson, W. T. Johnson, and Dr. J. L. Herrman for valuable assistance in certain early phases of this work.

**APPENDIX**

Calculations of data for presumed physiological conditions were carried out generally as outlined in detail on pp. 105-110 of Nordlie (10) where applicable rate equations are presented.$^1$ The basis of such calculations for the four enzymic activities glucokinase, hexokinase, glucose-6-P phosphohydrolase, and carbamyl-P-glucose phosphotransferase was the assayed activity of each as determined in *vitro* at fixed pH with constant, relatively high levels of substrates (Table III). Activity levels so obtained were extrapolated mathematically to infinite concentrations of substrates (i.e. $V_{\text{max}}$ values, see Table V). Such values for $V_{\text{max}}$ were then used to calculate the extant hepatic activity of each enzyme taking into consideration experimentally employed values for perfusate glucose load and presumed "physiological levels" of phosphoryl donor compounds. Finally, data so obtained were adjusted for inhibition by various metabolites at concentrations at which the latter are thought to exist in hepatic tissues. Glucose-6-P phosphohydrolase also was adjusted for inhibition by the various, relevant concentrations of glucose. All such activities were finally adjusted to $37^\circ$ with the use of the Arhenius equation and $E_0$ values of 12.5 kcal/mol for glucokinase (50) and activities of glucose-6-phosphatase-phosphotransferase (61, 62). In the absence of information, $E_0$ for liver hexokinase was also presumed to be 12.5 kcal/mol. Presumed physiological metabolite levels, Michaels constant values, and inhibitor constant values were as recorded in Table V, along with literature references. Glucose-6-P phosphohydrolase values

$^8$ Acidic pH optima have been a major obstacle to the acceptance of physiological relevance of this phosphotransferase with PP, or ATP as phosphoryl donor (see Ref. 10). Glucose-6-P-glucose phosphotransferase would produce no net glucose uptake in our system.

$^9$ Please note that due to a typographical error the term "+11" was omitted from the general denominator term on the right side of Equation 17, p. 109, Ref. 10.

### Table V

<table>
<thead>
<tr>
<th>Glucokinase</th>
<th>Hexokinase</th>
</tr>
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<tbody>
<tr>
<td>$K_{m,\text{Glc}}$, 0.38 mm$^a$</td>
<td>$K_{m,\text{Glc}}$, 0.15 mm$^a$</td>
</tr>
<tr>
<td>$K_{m,\text{Glc-6-P}}$, 23 mm$^b$</td>
<td>$K_{m,\text{Glc-6-P}}$, 0.01 mm (64)</td>
</tr>
<tr>
<td>$K_{m,\text{Glc-6-P}}$, 8.7 x $10^{-4}$ M$^c$</td>
<td>$K_{m,\text{Hex-6-P}}$, 1.5 x $10^{-4}$ M$^c$</td>
</tr>
<tr>
<td>$V_{\text{max, fasted}}$, 0.50 mmol/min/g wet liver</td>
<td>$V_{\text{max, fasted or fed}}$, 0.126 mmol/min/g wet liver</td>
</tr>
<tr>
<td>$V_{\text{max, fed}}$, 1.23 mmol/min/g wet liver</td>
<td>$V_{\text{max, fed}}$, 65.4 μmol/min/g wet liver</td>
</tr>
<tr>
<td>$V_{\text{max, fed}}$, 41.5 μmol/min/g wet liver</td>
<td>Inhibition by glucose: 35% at all concentrations of glucose studied; see Fig. 10 of Ref. 3</td>
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</tbody>
</table>

**Glucose-6-P phosphohydrolase**

<table>
<thead>
<tr>
<th>Carbamyl-P:glucose phosphotransferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{i,\text{carbamyl-P}}$, 2.3 mm (29)</td>
</tr>
<tr>
<td>$K_{i,\text{Glc-6-P}}$, 1.8 mm (29)</td>
</tr>
<tr>
<td>$K_{i,\text{ATP}}$, 11 mM$^b$</td>
</tr>
<tr>
<td>$K_{i,\text{ADP}}$, 11 mM$^c$</td>
</tr>
<tr>
<td>$K_{i,\text{Glc}}$, 70 mM (66)</td>
</tr>
</tbody>
</table>

**Presumed metabolite levels**

| Glc-6-P, 0.133 mm, fed (67); 0.092 HCO$_3^-$, 20 mM, fed and fasted$^d$ |
| ATP, 4.8 mM, fed (67); 4.67 mM, P$_i$, 10 mM, fed and fasted$^d$ (70) |
| ADP, 2.05 mM, fed (47); 1.43 mM, C$^14$, 43 mM fed and fasted$^d$ (71) |
| Carbamyl P, 0.20 mM, fed and fasted$^d$ (68) |

$^a$ This paper; $K_{m,\text{Glc-ATP}} = K_{m,\text{Glc}} \times K_{m,\text{ATP}}$.

$^b$ J. L. Herrman and R. C. Nordlie, unpublished data cited in Ref. 10.

$^c$ $K_{m,\text{Glc-6-P}} = K_{m,\text{Glc}} \times K_{m,\text{Glc-6-P}}$ (see Ref. 10).

$^d$ All $V_{\text{max}}$ values for all activities apply to 30$^\circ$.

$^e$ W. Colilla and R. C. Nordlie, unpublished observation cited in Ref. 10.

$^f$ $K_{i,\text{Glc-6-P}} = K_{i,\text{Glc-6-P}}$ in Glc-6-P phosphohydrolase reaction.

$^g$ R. C. Nordlie, unpublished observation.

$^h$ Primary literature references are given in parentheses. It has been presumed that metabolites are distributed uniformly throughout liver cell water, and that such water constitutes 60% of total weight of the liver. See also Ref. 10 for additional details and references.

$^i$ Possibly, carbamyl-P$^e$ levels may be higher in livers of fasted animals where gluconeogenesis is accelerated; levels of hepatic carbamyl-P synthetase I increase in such situations (58).

$^j$ Values for fasted animals are considered equal to those reported for fed animals.
were adjusted for inhibitions by glucose, ATP, ADP, P, \( \text{HCO}_3^- \), Cl\(^-\), and carbamyl-P. Carbamyl-P;glucose phosphotransferase values were adjusted for inhibitions by ATP, ADP, P, \( \text{HCO}_3^- \), Cl\(^-\), and glucose-6-P. Hexokinase values were adjusted for inhibitions by glucose and glucose-6-P (3).

Further specific details of calculation are as in Ref 10. The kinetic parameters and activity level values used for calculation of activities of glucose-6-phosphatase-phosphotransferase are those obtained with detergent-treated preparations. With microsomal preparations, but not with isolated intact nuclei, those noted with either detergent-dispersed microsomal preparations or with intact nuclear membrane preparations (63). Further, kinetic parameters assessed with such preparations agree well with recent, ongoing work in this laboratory indicates that 60 to 80% of such activities are manifest rather than latent when assayed in permeabilized, intact hepatocytes in which the structural integrity of the endoplasmic reticulum as well as nuclear membrane is maintained (60). Further, kinetic parameters assessed with such preparations agree well with those noted with either detergent-dispersed microsomal preparations or with intact nuclear membrane preparations (63). Thus, our choice of parameters seem most appropriate in the present context.

Total hepatic glucose phosphorylation rates (total vertical bars 1 in Fig. 3, A and B) were calculated from the relationship

\[
\text{observed net rate of glucose uptake (or production) } = \frac{\text{rate of glucose-6-P hydrolysis}}{(3)}
\]

Glucose dehydrogenase activity was assessed with the aid of rate equations and kinetic parameters described previously by Horne and Nordlie (57).

**REFERENCES**


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15. This equation makes the hepatocyte is freely permeable to glucose and is impermeable to glucose-6-P (55, 56). Hepatic glucose phosphorylation effectively 'captures' glucose as glucose-6-P. Before free glucose can be released by the hepatocyte, glucose-6-P must first be hydrolyzed by glucose-6-P phosphohydrolase (56).
Quantitative correlation of glucose uptake and phosphorylation with the activities of glucose-phosphorylating enzymes in perfused livers of fasted and fed rats.

F L Alvares and R C Nordlie


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