myo-Insitol Uptake and Metabolism in Isolated Rat Liver Cells*

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The uptake of myo-inositol into isolated rat liver parenchymal cells and its incorporation into phosphatidylinositol were studied by isotopic and chemical means. The uptake was concentration- and time-dependent, but a maximal rate could not be defined. The intracellular concentration of myo-inositol never exceeded the extracellular concentration, and the uptake was not affected by inhibitors of mitochondrial ATP synthesis. High concentrations of D-glucose (10-100 mM) and of scyllo-inositol (0.4-4 mM) inhibited the uptake of 0.1 mM myo-inositol and its incorporation into phospholipid, as did cytochalasin B (10-100 µM) and phloretin (0.1-4 mM), cytochalasin D was without effect. Phloretin and cytochalasin B were also shown to inhibit myo-inositol efflux.

[2-3H]myo-Insitol was incorporated into phosphoinositides (95% into phosphatidylinositol and 4% into phosphatidylinositol-4-P plus phosphatidylinositol-4,5-P) in a concentration- and time-dependent manner. The medium [3H]myo-inositol concentration for half-maximum synthesis of phosphatidylinositol was about 0.4 mM. All subcellular fractions showed incorporation of isotope into phosphatidylinositol, but this was highest in the smooth endoplasmic reticulum. Uncouplers of oxidative phosphorylation caused only a slight decrease in phospholipid labeling, whereas Mn2+ (0.1-10 mM) and ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (5 mM) increased it significantly.

Epinephrine (1-10 µM), vasopressin (0.1 µM), angiotensin II (0.1 µM), and glucagon (0.1 µM) significantly stimulated (by 14-32%) the incorporation of 0.1 mM [3H]myo-inositol into phospholipid and reduced the intracellular concentration of [3H]myo-inositol. The ionophores A23187 (1 µM) and valinomycin (10 µM) were without effect. The effects of epinephrine were not evident before 30 min and were blocked by the α-adrenergic antagonist prazosin.

It is concluded that myo-inositol uptake by liver cells is nonactive and occurs by a carrier-mediated process which is different from that for glucose. [3H]myo-Inositol taken up by liver cells is incorporated into phosphatidylinositol by a process chiefly involving the phosphatidylinositol:myo-inositol exchange enzyme of the endoplasmic reticulum. Hormones which act on the liver mainly by mobilizing intracellular Ca2+ cause a stimulation of phosphatidylinositol synthesis which develops too slowly to play a primary role in the Ca2+ changes.

Changes in phosphatidylinositol metabolism have been proposed to play a primary role in the actions of α-adrenergic agonists, vasopressin, and angiotensin II on liver metabolism (1-5). Most of the studies carried out in support of this proposal have involved measurements of changes in the labeling of phosphatidylinositol in isolated liver preparations incubated with inorganic [32P]phosphate, [3H]oleate, or myo-inositol labeled with 3H or 14C (1-4, 6). Although the hormones cause marked increases in the incorporation of 32P, and [3H]fatty acid into phosphatidylinositol consistent with a stimulation of its synthesis (1-4, 6), there is disagreement with respect to the effect of hormones on the incorporation of labeled myo-inositol into this phospholipid (Ref. 3, cf. Ref. 6). For example, DeTorrontegui and Berthet (6) reported that epinephrine caused an almost 2-fold stimulation of the incorporation of 1.3 mM [3H]myo-inositol into phospholipids in rat liver slices during 30 min, whereas Toberl et al. (3) could find no effect of epinephrine or vasopressin on the synthesis of labeled phosphatidylinositol from 0.4 µM [3H]myo-inositol in isolated hepatocytes incubated for 1 h.

The apparent discrepancy between the effects of hormones on the incorporation of labeled precursors into phosphatidylinositol led us to explore in greater detail the uptake and metabolism of [3H]myo-inositol in hepatocytes. To our surprise, we could find only one previous study (7) of the hepatic uptake process for myo-inositol using an isolated liver preparation. The results of this study indicated that the uptake was passive, but no evidence for a carrier-mediated process was obtained. These results were surprising since in vivo experiments have suggested that the liver actively accumulates myo-inositol (8) and several studies have shown that uptake occurs by an active, carrier-mediated system in other tissues such as central nervous system (9, 10), kidney (11-13), lens (14-16), and small intestine (17).

The present communication indicates that hepatic myo-inositol uptake is nonactive, but can be inhibited by phloretin and cytochalasin B suggesting that it is carrier-mediated. It also shows that epinephrine, vasopressin, and angiotensin II are without effect on myo-inositol uptake, but stimulate the incorporation of [3H]myo-inositol into phosphatidylinositol.

EXPERIMENTAL PROCEDURES

Hepatocyte Incubation—Hepatocytes were isolated from 200-250-g Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) which were fed ad libitum on Purina Lab Chow unless otherwise noted. The procedure of hepatocyte isolation was that described previously (18). Cells (36-50 mg/ml) were preincubated with shaking (60 rpm) for 10 min at 37 °C in Krebs-Henseleit bicarbonate buffer (pH 7.4) gassed with O2:CO2 (95:5) before addition, in 2-ml aliquots, to 25-ml
polypropylene Erlenmeyer flasks containing various concentrations of 2-[14C]myo-inositol. Except in time course experiments, the period of incubation with [14C]myo-inositol was 60 min.

**Hepatocyte Incubates**—Hepatocyte cultures were routinely separated from medium by layering 1 ml aliquots of the cell suspension on 12 ml of ice-cold 0.9% (w/v) NaCl containing 10% (w/v) sucrose, and rapidly centrifuging at 1500 g for 5 min in a conical tube. The supernatant was then withdrawn by aspiration and the sides of the inverted tubes washed with distilled water. After washing the sides of the tubes dry with facial tissues, the pellet was resuspended in 2 ml of distilled water with vortexing, and 0.2 ml was transferred to 5 ml of ACS scintillation fluid (Amersham Corp.) for determination of total cell radioactivity. Tests of this procedure using [14C]linulin in the incubation medium indicated negligible (3.9 ± 0.06%) recovery. This was determined by adding to the trichloroacetic acid extracts of cells obtained as described under "Hepatocyte Analyses." The nonaqueous phases were reduced to dryness in borosilicate tubes and the lipid residues were dissolved in 100 μl of chloroform. Aliquots (40 μl) were then dried in scintillation vials and counted in ACS scintillation fluid. Other portions (3 ± 2 μl) were processed for lipid phosphorus (24) using asin in borosilicate tubes at 195 °C and the phosphate assay of Baginski et al. (25). Another portion (40 μl) was subjected to two-dimensional thin layer chromatography on silica gel as described by Abdel-Latif et al. (26). Lipids were identified using iodine vapor and were then scraped off into scintillation vials and counted in 10 ml of ACS scintillant fluid. In some experiments, the lipid extraction procedure for whole cells and subcellular fractions was modified in order to preserve phosphatidylinositol-4-P and phosphatidylinositol-4,5-P2, which were separated by two-dimensional thin layer chromatography (25). Lipids were identified and counted for radioactivity as described above.

**myo-Inositol Determination**—Gas-liquid chromatography was employed in some experiments to determine the intracellular and extracellular concentrations of inositol (27). Ether-extracted trichloroacetic acid extracts of cells obtained as described under "Hepatocyte Analyses" were lyophilized to dryness (VirTis Freezemobile II). Samples of medium obtained in the same experiments by centrifuging incubations were similarly treated with trichloroacetic acid and the lipid residues were dissolved in 0.2 ml of diethyl ether and lyophilized. The dry residues were dissolved in 0.2 ml of 1.5 M N-trimethylsilyl-1-

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**Table I**

**Intracellular distribution of labeled phosphatidylinositol**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>N'-Nucleotidase</th>
<th>Glucose-6-phosphatase</th>
<th>Cytochrome c oxidase</th>
<th>Radioactivity in phospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/h-mg protein</td>
<td>pmol/min-mg protein</td>
<td>cmol/min-mg protein</td>
<td>cmol/min-mg protein</td>
</tr>
<tr>
<td>Total homogenate</td>
<td>1.13 ± 0.05</td>
<td>1.05 ± 0.14</td>
<td>63.5 ± 13.2</td>
<td>2208 ± 306</td>
</tr>
<tr>
<td>Light microsomes</td>
<td>2.18 ± 0.20</td>
<td>3.06 ± 0.50</td>
<td>2.8 ± 0.2</td>
<td>5000 ± 971</td>
</tr>
<tr>
<td>Heavy microsomes</td>
<td>2.26 ± 0.17</td>
<td>5.18 ± 0.61</td>
<td>14.7 ± 2.5</td>
<td>4263 ± 820</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.87 ± 0.06</td>
<td>0.29 ± 0.06</td>
<td>295 ± 41.0</td>
<td>2382 ± 347</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>10.8 ± 0.8</td>
<td>7.82 ± 0.12</td>
<td>ND</td>
<td>2414 ± 541</td>
</tr>
</tbody>
</table>

ND, not determined.

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*S. N. N' N'-tetraacetic acid.*
Inositol Uptake and Metabolism in Rat Hepatocytes

**RESULTS**

**Time Course of myo-Inositol Uptake and Incorporation into Phosphatidylinositol**

Fig. 1 shows the uptake of a wide range of concentrations of \([^{3}H]myo\)-inositol (0.01–100 mM) into hepatocytes as a function of time of incubation. It shows that uptake was time- and concentration-dependent. At 10 and 100 mM, the accumulation reached a plateau at about 90 min. In some experiments, more extensive measurements were made at early time points, and uptake was found to be linear up to 30 min with 0.01 to 10 mM myo-inositol. Table II shows these initial rates as a function of the medium myo-inositol concentration. Surprisingly, uptake was almost linearly related to the myo-inositol concentration up to 10 mM and it was not possible to define a maximal uptake rate and hence a concentration giving the half-maximal uptake rate.

Fig. 1 also shows the incorporation of isotope into cell lipid in these experiments. Although it was reasonable to expect that the label would be almost exclusively in phosphoinositides, the lipid extracts were subjected to two-dimensional thin layer chromatography as described under "Experimental Procedures." This showed that 95 ± 2% \((n = 4)\) of the total radioactivity in the lipids was attributable to phosphatidylinositol, with 4 ± 1% co-migrating with phosphatidylinositol-4-P and phosphatidylinositol-4,5-P<sub>2</sub>. Similar to the situation regarding whole cell inositol uptake, the incorporation of \([^{3}H]myo\)-inositol into the cell lipid was time- and concentration-dependent. However, in contrast to the uptake data the incorporation was saturable. When the initial rates of phosphoinositide synthesis, i.e. those during the first 30 min, were plotted as a function of the medium myo-inositol concentration (data in Table II), a maximum rate of myo-inositol incorporation (~125 pmol/mg of cells·30 min) and myo-inositol concentration for half-maximum incorporation (~0.4 mM) could be calculated (data expressed in terms of medium myo-inositol). It is also evident from Fig. 1 and Table II that with increasing medium myo-inositol, the fraction of total cell radioactivity in the lipid fraction decreased, i.e. it was ~50% at 0.01 mM myo-inositol and <1% at 100 mM myo-inositol. Trichloroacetic acid-soluble radioactivity in the cells was also measured in these experiments. The data are shown as the open squares in Fig. 1 and illustrate the fact that, at all time points and at all medium \([^{3}H]myo\)-inositol concentrations, total cell radioactivity could be fully accounted for as the sum of trichloroacetic acid-soluble and lipid radioactivities. Although it seemed very likely that the acid-soluble radioactivity represented mainly unmetabolized \([^{3}H]myo\)-inositol, cell extracts were treated with a mixed-bed ion exchange resin (Amberlite MB-3) to remove charged molecules. The recovery of radioactivity in the extracts was 96 ± 2% \((n = 14)\) indicating the presence of only negligible amounts of myo-inositol phosphates.

**Relationship between Intracellular and Extracellular myo-Inositol Concentrations**

The experiments of Fig. 1 showed a tendency for the cell-associated radioactivity to reach a maximum at 90 to 120 min in the presence of high (>1 mM) medium myo-inositol, i.e. when most of the cell radioactivity was in the form of free myo-inositol. To prove that this was because extracellular and intracellular myositol concentrations were approaching equilibrium, estimates were made of the intracellular \([^{3}H]myo\)-inositol concentration. Measurements of the water content of packed cell pellets from experiments determined by weighing them before and after desiccation at 105 °C gave values of 473 ± 7 µl/g of cells \((n = 10)\) and measurements of entrapped extracellular medium determined using \([^{3}H]linulin\) gave values of 3.9 ± 0.2 µl/g of cells. Intracellular water was therefore taken to be 0.47 µl/mg, wet weight. Based on the assumption (see above) that the acid-soluble radioactivity in the cells was

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**Table II**

<table>
<thead>
<tr>
<th>Medium myo-inositol concentration (mM)</th>
<th>myo-Inositol uptake (pmol/mg cells·30 min)</th>
<th>Phosphatidylinositol synthesis (pmol/mg cells·30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>5.7 ± 0.5</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>0.1</td>
<td>45 ± 4</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>1.0</td>
<td>273 ± 17</td>
<td>81 ± 19</td>
</tr>
<tr>
<td>10.0</td>
<td>3200 ± 370</td>
<td>106 ± 17</td>
</tr>
</tbody>
</table>

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<sup>2</sup> Since solid myo-inositol had to be added to the incubation flasks to achieve 100 mM concentration, initial rate of uptake was not examined with this concentration. Solubility limitations also precluded accurate measurements of myo-inositol uptake at concentrations higher than 100 mM.
due almost entirely to \([^{3}H]m[yo-inositol, the data of Table III indicate that, at the end of the incubations (120 min), intracellular and extracellular myo-inositol concentrations became almost equal.

Conclusive support for the view that myo-inositol uptake is nonconcentrative in hepatocytes came from chemical measurements of extracellular and intracellular myo-inositol performed by gas-liquid chromatography. As also shown in Table III, the concentrations of myo-inositol across the plasma membrane at 120 min were not significantly different at 10 and 100 mM extracellular myo-inositol.

Labeling of Phosphatidylinositol in Subcellular Fractions—As described under "Experimental Procedures," in some experiments, plasma membranes, mitochondria, and heavy and light microsomes were isolated from hepatocytes incubated with \([^{3}H]m[yo-inositol. Fig. 2 shows that the incorporation of isotope into the phospholipids of the subcellular fractions followed the general time course for the labeling of total cell phospholipids. On the basis of total phospholipid content of each fraction (measured as phospholipid-P), the incorporation was highest into the microsomal fractions (Table I) as expected since this is the principal intracellular location of CDP-diacylglycerol:inositol transferase (28, 29) and of the enzyme exchanging \([^{3}H]m[yo-inositol into phosphatidylinositol (30, 31). Analysis by two-dimensional thin layer chromatography (see "Experimental Procedures") showed that 94% or more of the label was in phosphatidylinositol in all the subcellular fractions. No radioactivity was detected in the region of phosphatidylinositol-4-P or phosphatidylinositol-4,5-P\(_{2}\), due presumably to their breakdown during the fractionation procedures.

Effects of Glucose Transport Inhibitors and myo-Inositol Analogues on myo-Inositol Uptake and Incorporation into Lipid in Hepatocytes—Chen and Vu (7) reported that phlorizin did not inhibit \([^{3}H]m[yo-inositol uptake by hepatocytes and concluded that it therefore was not carrier-mediated. Fig. 3 (left) shows that phloretin, the aglycone of phlorizin, inhibited the uptake of 0.1 mM \([^{3}H]m[yo-inositol in a dose-dependent manner and caused a corresponding inhibition of phosphatidylinositol labeling. With 10 mM \([^{3}H]m[yo-inositol, in which the intracellular label was mainly acid-soluble, i.e. not metabolized, phloretin was also markedly inhibitory (Fig. 3, right). Cytochalasin B another inhibitor of glucose transport was also tested. Fig. 4 shows that this compound inhibited the uptake of 10 mM \([^{3}H]m[yo-inositol, whereas cytochalasin D was noninhibitory. Similar findings were obtained with 0.1 mM \([^{3}H]m[yo-inositol (data not shown). Mercuric chloride (0.1 mM) markedly suppressed \([^{3}H]m[yo-inositol uptake and incorporation into lipid (data not shown).

Several structural analogues of myo-inositol were also tested. As illustrated in Table IV, d-glucose caused a concentration-dependent inhibition of the uptake and incorporation

<table>
<thead>
<tr>
<th>Initial medium myo-inositol mM</th>
<th>Intracellular myo-inositol nmoI/mg</th>
<th>Intracellular myo-inositol%</th>
<th>Extracellular myo-inositol mM</th>
<th>Intracellular myo-inositol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.0068</td>
<td>9.14</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.1</td>
<td>0.049</td>
<td>10.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1.0</td>
<td>0.31</td>
<td>10.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>4.1</td>
<td>8.8</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>100</td>
<td>47.5</td>
<td>101</td>
<td>101</td>
<td>94</td>
</tr>
</tbody>
</table>

\(^{a}\) Calculated from the trichloroacetic acid-soluble radioactivity measured in cells at 120 min and from the initial specific radioactivity of \([^{3}H]m[yo-inositol.

\(^{b}\) Calculated on the basis of 0.47 µl of intracellular water/mg of cells.

\(^{c}\) Determined by gas-liquid chromatography at 120 min.

\(^{d}\) ND, not determined.

Fig. 2. Time courses of the incorporation of radioactivity into the lipids of subcellular fractions of hepatocytes incubated with \([^{3}H]m[yo-inositol. Hepatocytes were incubated with 0.01 mM \([^{3}H]m[yo-inositol (0.5 µC/ml) for 90 min and samples were withdrawn at 30, 60, and 90 min for subcellular fractionation and lipid analysis as described under "Experimental Procedures." A representative experiment of two is shown.

Fig. 3. Inhibition by phloretin of \([^{3}H]m[yo-inositol uptake by hepatocytes. Hepatocytes were incubated for 60 min with 0.1 mM (left) or 10 mM (right) \([^{3}H]m[yo-inositol and phloretin at the concentrations shown. Total and lipid radioactivities in the cells were measured as described under "Experimental Procedures." Phloretin was added in ethanol. A similar volume of ethanol was added to the control. As shown in Table IV, this had no effect by itself. A representative experiment of two is shown. TCA-Sol, trichloroacetic acid-soluble.
cytochalasin myo-inositol exchange enzyme requires Mn²⁺ for activity (28, 29) and CDP-diacylglycerolinositol transferase is markedly stimulated by low concentrations of Mn²⁺ (28, 30, 31). Both activities are inhibited by Ca²⁺ (32). Furthermore, Tolbert et al. (3) have reported very large effects of Mn²⁺ on the incorporation of [³H]myo-inositol into lipids in hepatocytes, i.e. a 20-fold stimulation of 0.4 μM inositol incorporation with 1 mM

of 0.1 mM [³H]myo-inositol into lipid. L-Glucose was also inhibitory at high concentrations (100 mM) and appeared to be about 10-fold less potent than D-glucose. Scylo-Inositol, a stereoisomer of myo-inositol, also inhibited the uptake of [³H]myo-inositol and was at least an order of magnitude more potent than D-glucose. The uptake of 0.1 mM [³H]myo-inositol was similar in hepatocytes from fed and 24-h fasting rats and was at least an order of magnitude more potent than D-glucose, L-glucose, and scylo-inositol compared with cells from rats fed ad libitum (data not shown). Effects of Inhibitors of Mitochondrial ATP Synthesis—KCN, an inhibitor of cytochrome c oxidase, and valinomycin and A23187 which are cationophores which discharge the mitochondrial proton gradient were without significant effect on the uptake or incorporation of 0.1 mM [³H]myo-inositol into cell lipid (Table IV) although they were tested at concentrations known to be effective on mitochondrial functions in hepatocytes. Two other uncouplers of oxidative phosphorylation, 2,4-dinitrophenol and carbonyl cyanide m-chlorophenylhydrazone caused a small (~15%) inhibition of 0.1 mM [³H]myo-inositol uptake. Similar results were obtained when all these agents were tested on the uptake of 0.1 mM [³H]myo-inositol (data not shown). These findings support the view that the uptake of inositol is nonactive.

Effects of Mn²⁺ and EGTA—The phosphatidylinositol: myo-inositol exchange enzyme exchange enzyme requires Mn²⁺ for activity (28, 29) and CDP-diacylglycerolinositol transferase is markedly stimulated by low concentrations of Mn²⁺ (28, 30, 31). Both activities are inhibited by Ca²⁺ (32). Furthermore, Tolbert et al. (3) have reported very large effects of Mn²⁺ on the incorporation of [³H]myo-inositol into lipids in hepatocytes, i.e. a 20-fold stimulation of 0.4 μM inositol incorporation with 1 mM

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>Total [³H]myo-inositol uptake</th>
<th>Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>78 ± 2</td>
<td>35 ± 4</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>10</td>
<td>71 ± 4</td>
<td>36 ± 1</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>100</td>
<td>52 ± 4</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>L-Glucose</td>
<td>10</td>
<td>76 ± 1</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>L-Glucose</td>
<td>100</td>
<td>71 ± 5</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>scylo-Insol</td>
<td>0.4</td>
<td>64 ± 3</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>scylo-Insol</td>
<td>4</td>
<td>58 ± 3</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>KCN</td>
<td>0.01</td>
<td>80 ± 3</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>2,4-Dinitrophenol</td>
<td>0.1</td>
<td>68 ± 4</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>Carbonyl cyanide m-chlorophenylhydrazone</td>
<td>0.02</td>
<td>64 ± 8</td>
<td>28 ± 3</td>
</tr>
</tbody>
</table>

* p < 0.05 compared with control.
* p < 0.005 compared with control.
Mn$^{2+}$, although they erroneously attributed this to stimulation of the reversal of the CDP-diacylglycerol:inositol transferase reaction (33).

Table IV shows that Mn$^{2+}$ stimulated the incorporation of 0.1 mM $[^{3}H]$myo-inositol into the hepatocyte lipid fraction in a concentration-dependent manner. However, there was no significant increase in inositol uptake into the cells and, correspondingly, the trichloroacetic acid-soluble radioactivity (chiefly unmetabolized $[^{3}H]$myo-inositol) was decreased (Table IV). Addition of 5 mM EGTA produced effects similar to Mn$^{2+}$ (Table IV). MnCl$_2$ and EGTA caused changes in 10 mM $[^{3}H]$myo-inositol metabolism similar to those seen with 0.1 mM $[^{3}H]$myo-inositol (data not shown).

**Effects of Hormones**—No hormones tested caused any change in the cellular uptake of 0.1 or 10 mM $[^{3}H]$myo-inositol (Fig. 5 and data not shown). However, maximally effective (2, 4, 34) concentrations of epinephrine (10 $\mu$M), glucagon (0.1 $\mu$M), vasopressin (0.1 $\mu$M), and angiotensin II (0.1 $\mu$M) all caused small, but significant, increases in the labeling of cell lipid (Fig. 5). The effects of epinephrine, glucagon, and angiotensin II were accompanied by significant decreases in the cellular trichloroacetic acid-soluble radioactivity, confirming the conclusion that the hormones stimulated the incorporation of $[^{3}H]$myo-inositol into phospholipid and not the uptake of $[^{3}H]$myo-inositol by the cells. Similar changes were produced by epinephrine (the only hormone tested) in hepatocytes incubated with 0.01 and 1 mM $[^{3}H]$myo-inositol (Fig. 6 and data not shown).

Subcellular fractionation studies and two-dimensional thin layer chromatography indicated that the increased incorporation of $[^{3}H]$myo-inositol was into phosphatidylinositol and was evident in all the subcellular fractions analyzed (data not shown). The effects of epinephrine to increase the synthesis of labeled phosphatidylinositol and to decrease the radioactivity in the trichloroacetic acid-soluble fraction were not consistently observed before 30 min of incubation and tended to increase up to 90 min (Fig. 6). They were observed with 1 $\mu$M epinephrine and were inhibited more potently by the $\alpha$,-adrenergic antagonist prazosin than by the $\alpha_2$-blocker yohimbine and the $\beta$-blocker propranolol (data not shown). The effects of EGTA on the hormone stimulation of phosphatidylinositol labeling were not studied since EGTA added alone produced a marked stimulation, as noted already (Table IV).

**Studies of $[^{3}H]$myo-Inositol Efflux from Hepatocytes**—Efflux of $[^{3}H]$myo-inositol was also examined in hepatocytes incubated for 1 h with 10 mM $[^{3}H]$myo-inositol. The major fraction (97%) of the radioactivity accumulated by the cells under these conditions is in the form of free $[^{3}H]$myo-inositol (Fig. 1 and Table II). After washing the cells twice in myo-inositol-free Krebs-Henseleit buffer, they were resuspended and distributed in 10-ml aliquots into 250-ml flasks containing various additions at 37°C. The rise in radioactivity in the incubation medium was measured by withdrawing 0.5-ml samples at intervals, centrifuging them, and counting the supernatant. The data showed that phloroerin (1 $\mu$M) and cytochalasin B (0.1 mM) inhibited the efflux of $[^{3}H]$myo-inositol during 40 min by 75% and 68%, respectively, whereas glucose (0.1 $\mu$M), epinephrine (10 $\mu$M), vasopressin (0.1 $\mu$M), A23187 (1 $\mu$M), and carbonyl cyanide m-chlorophenylhydrazone (20 $\mu$M) were without effect. These findings thus corroborated those on $[^{3}H]$myo-inositol uptake.

**DISCUSSION**

**Characteristics of myo-Inositol Transport**—The present study shows that myo-inositol uptake by hepatocytes is non-active. The intracellular/extracellular concentration ratio rose throughout 120 min (Fig. 1), but never exceeded 1 (Table II), and uptake was not decreased by KCN or uncouplers of oxidative phosphorylation (Table IV). In contrast to the conclusion of Chen and Vu (7), we believe that myo-inositol uptake is carrier-mediated. The most striking evidence is the dose-dependent inhibition of myo-inositol uptake and efflux by phloretin and cytochalasin B which are known to inhibit glucose transport in a variety of cells (35). Additional support comes from the findings with D-glucose and scylllo-inositol which inhibited myo-inositol uptake in a competitive manner (Table IV, and data not shown). The negative findings of Chen and Vu (7) may relate to the use of phlorizin instead of its aglycone, phloretin, and the addition of D-glucose at only one concentration (5 mM) which would produce little inhibition of 0.1 mM myo-inositol uptake (Table IV). Alternatively, as suggested below, the permeability characteristics of their hepatocytes might have been abnormal.

Despite the evidence presented above in support of myo-inositol uptake occurring by facilitated diffusion, it was not possible to clearly demonstrate that the uptake process was saturable (Fig. 1 and Table II). A possible explanation is that the transport system has a very high $K_m$ for myo-inositol, i.e. $>$100 mM, but this would be at least 3 orders of magnitude higher than the serum level (0.08 mM) of myo-inositol (36). It does not seem likely that the myo-inositol is being transported by the glucose carrier system. This is because $K_m$ for D-glucose of this system determined using the perfused rat liver is 17 mM (37), and yet 100 mM D-glucose inhibited myo-inositol uptake.
The rates of myo-inositol uptake observed in the present study are about 1 order of magnitude less than those given in the paper of Chen and Vu (7). However, it should be noted that in their study intracellular/extracellular concentration ratios close to 1 were achieved after only 5 min of incubation with 0.1-5 mM myo-inositol (values of earlier times were not measured). These findings plus the failure of these workers to see inhibition of myo-inositol uptake by any agent raises the suspicion that the plasma membranes of their hepatocytes may have been "leaky." As presented elsewhere (38), the hepatocytes used in the present study maintain normal levels of intracellular K+ (103 mM) and Na+ (10 mM) which are rigorous indices of plasma membrane intactness.

Characteristics of Phosphatidylinositol Synthesis—The data for Fig. 1 and Table II illustrated that myo-inositol uptake was not limiting for phosphatidylinositol synthesis at any concentration examined. Expressed in terms of extracellular myo-inositol, the apparent $K_m$ for synthesis of this lipid was 0.4 mM. This would correspond to an intracellular myo-inositol concentration of 0.2 mM or less during the initial 30 min when phosphatidylinositol synthesis was measured (Fig. 1). Comparison of this value with the $K_m$ values of liver CDP-diacylglycerol:myo-inositol transferase and the phosphatidylinositol:inositol exchange enzyme for myo-inositol (2.5 and 0.04 mM, respectively) (29, 31) suggests that the labeling of the phospholipid in the hepatocytes was due mainly to the operation of the exchange enzyme. This conclusion is supported by the fact that inhibitors of mitochondrial ATP formation had little effect on the labeling of phosphatidylinositol (Table IV). Reduced ATP production would be expected to lead to a decrease in CTP and hence CDP-diacylglycerol, thus reducing the rate of the CDP-diacylglycerol:inositol transferase reaction.

As predicted from the subcellular localization of the two enzymes potentially responsible for phosphatidylinositol labeling (28-31), the specific radioactivity of the synthesized phosphatidylinositol was highest in the microsomes. The labeled phosphatidylinositol found in the mitochondria and plasma membranes was probably transferred there from the endoplasmic reticulum by means of phospholipid transfer proteins (39, 40).

In agreement with Tolbert et al. (3), MnCl$_2$ and EGTA stimulated the incorporation of $[^3H]$myo-inositol into phosphatidylinositol in hepatocytes. However, the magnitudes of the effects were much less than those reported by these workers and no effect of MnCl$_2$ on myo-inositol uptake was observed. These differences may relate to the fact that Tolbert et al. (3) used a medium myo-inositol concentration of 4 mM, whereas we used 0.1 mM, a physiological concentration. As noted above, both the liver enzymes forming labeled phosphatidylinositol can be stimulated by Mn$_2^+$ and inhibited by Ca$_2^+$ (28-32). Thus, the data with these agents cannot be used to indicate which enzyme is mainly responsible for the labeling of phosphatidylinositol.

The present results do not support the claim by Tolbert et al. (3) that labeled myo-inositol is a poor indicator of phosphatidylinositol synthesis in hepatocytes. This claim was based on differences between the effects of hormones on the incorporation of $[^3H]$myo-inositol and $[^3P]$P into phosphatidylinositol. It was supposed that myo-inositol was incorporated into phosphatidylinositol by an exchange reaction catalyzed by CDP-diacylglycerol:myo-inositol transferase, and that the pool of myo-inositol involved did not exchange with myo-inositol in the medium (3). However, as pointed out by Eisenberg and Hasegawa (33), the data of Tolbert et al. (3) can be readily explained by the operation of the phosphatidylinositol:inositol exchange enzyme.

Hormone Effects on Phosphatidylinositol Synthesis—The changes in radioactivity in the lipid and trichloroacetic acid-soluble fractions of the hepatocytes exerted by the Ca$^{2+}$-dependent hormones, epinephrine, vasopressin, and angiotensin II are consistent with a stimulation of phosphatidylinositol synthesis. An alternative explanation based on an increase in the specific radioactivity of intracellular myo-inositol seems unlikely in view of the decrease in label in this pool and also because the stimulation of phosphatidylinositol synthesis was observed with 0.01, 0.1, and 1 mM extracellular myo-inositol which would produce greatly different intracellular myo-inositol concentrations (see also Footnote 5).

The effect of epinephrine on phosphatidylinositol synthesis from myo-inositol was shown by blocker studies to be mediated by adrenergic receptors of the $\alpha_1$ type, in agreement with Tolbert et al. (3). It was apparently of slower onset (Fig. 6) than seen in studies with $^{32}$P (1, 2, 4). However, even the effects seen in the latter studies are still too slow to be of causal significance in the early metabolic effects mediated by this receptor, which can be observed at 2 s (42). The stimulation of phosphatidylinositol synthesis induced by vasopressin, epinephrine, or angiotensin II has been postulated to be secondary to receptor-induced breakdown of plasma membrane phosphatidylinositol (43, 44) catalyzed by a phosphatidylinositol-specific phospholipase C (or phosphodiesterase) which produces diacylglycerol and a mixture of myo-inositol 1,2-cyclic P and myo-inositol-1-P (45, 46). This hypothesis is described and discussed in more detail in the following paper (19), but suffice it to say that it is hypothesized that the rise in diacylglycerol leads to increased synthesis of phosphatidate and hence CDP-diacylglycerol and hence phosphatidylinositol by the consecutive actions of diacylglycerol kinase, phosphatidate cytidylyltransferase, and CDP-diacylglycerol:inositol transferase, as first proposed by Hokin and Hokin (47). This would explain the increased incorporation of $^{32}$P; into phosphatidylinositol observed in hepatocytes incubated with vasopressin, epinephrine, and angiotensin II (1-4).

Since $^{32}$P entering phosphatidylinositol via $[\gamma-^{32}P]CTP$ would be expected to be accompanied by an equimolar amount of myo-inositol, explanations have been sought for why the effects of hormones on $[^3H]$inositol incorporation into phosphatidylinositol are much less than those on $^{32}$P incorporation into this phospholipid. The present findings provide a possible explanation, viz. that the majority of the incorporation of $[^3H]$myo-inositol is catalyzed by the phosphatidylinositol:myo-inositol exchange enzyme, i.e. does not involve CDP-diacylglycerol.

The mechanism(s) by which vasopressin, epinephrine, and angiotensin II stimulate phosphatidylinositol synthesis was not explored in the present study. As stated above, it has been

Additional support for the conclusion that myo-inositol is not transported by the glucose carrier in liver comes from studies of 3-O-['$^3$H]methylglucose uptake by hepatocytes. Whereas glucose competitively inhibited the initial rate of uptake of this hexose, myo-inositol was without effect (J. H. Exton and V. Prip, unpublished observations).

An additional problem with the studies of Tolbert et al. (3) is that they did not consider possible changes in the specific radioactivity of $[^3H]$myo-inositol. As shown in the following paper (19), intracellular myo-inositol in rat hepatocytes is 0.33 mM under basal conditions and can increase to over 1 mM with hormone stimulation.

$^{3}$This effect was also seen with $^{10}$M glucagon which induced consistent ($p < 0.005$) changes in lipid radioactivity (+14%) and in acid-soluble radioactivity (−30%) in hepatocytes incubated with 0.1 mM $[^3H]$myo-inositol (Fig. 5). It is possible that the effect was due to changes in cell Ca$^{2+}$ induced by the high concentration of glucagon used (32, 34, 41). Lower concentrations were not tested.
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postulated to be secondary to the breakdown of the phospholipid (2-4) which is examined in detail in the following paper (19). Coupling of the breakdown with the synthesis of phosphatidylinositol would require special considerations. First, the diacylglycerol resulting from the breakdown of phosphatidylinositol would need to be specifically directed to the diacylglycerol resulting from the breakdown of phosphatidylinositol rather than to the synthesis of triacylglycerol or other phospholipids. This could arise from compartmentation other than a much higher rate of phosphatidylinositol synthesis compared with the other potential products. However, studies of phospholipid synthesis in hepatocytes incubated with $^{32}P$ or $[^4C]glycerol do not support the latter explanation, i.e. the basal rate of incorporation of isotope into phosphatidylinositol was lower than that into phosphatidylethanolamine or phosphatidylcholine (3, 4). An alternative is that phosphatidylinositol synthesis per se is stimulated by the hormones, but this could be a "late" effect, i.e. one seen after the initial changes in cell Ca$^{2+}$ have subsided (42).

It has been proposed that cell Ca$^{2+}$ changes are not responsible for the hormonal stimulation of phosphatidylinositol turnover in hepatocytes (2, 4). This proposal is based on the following published observations. 1) An elevation in cytosolic Ca$^{2+}$ induced by A23187 does not promote phosphatidylinositol synthesis or breakdown (2, 3, 19); 2) treatment with EGTA, which should decrease cytosolic Ca$^{2+}$, causes a stimulation of phosphatidylinositol synthesis from $[^3H]$inositol (3); 3) EGTA treatment reduces, but does not abolish, the effects of hormones (2-4). However, as discussed in Footnote 7 and 8, the published data on this point are equivocal. Furthermore, as discussed in the following paper (19), our data indicate that the breakdown of phosphatidylinositol induced by hormones in hepatocytes is the result rather than the cause of the Ca$^{2+}$ changes, and a role for Ca$^{2+}$ in the actions of hormones on phosphatidylinositol metabolism in liver is still very possible. Cockcroft (48) has recently presented evidence that the "phosphatidylinositol response" in many other tissues is also Ca$^{2+}$-dependent.

REFERENCES

7. It should be noted that, in the studies of Tolbert et al. (3), although EGTA markedly stimulated the incorporation of $[^3H]$inositol, it simultaneously markedly decreased the incorporation of $[^3P]$ into this phospholipid. One explanation for this discrepancy would be that the Ca$^{2+}$ depletion induced a reaction in the CDP-diacylglycerol-dependent pathway of phosphatidylinositol synthesis, but stimulated synthesis catalyzed by the phosphatidylinositolinositol exchange enzyme.
8. Since no measurements of cell Ca$^{2+}$ were made in the experiments reported in Refs. 2-4, no definite conclusions regarding a role for Ca$^{2+}$ in the actions of hormones on phosphatidylinositol metabolism can be drawn. In many of the experiments in Ref. 3, the situation was obfuscated by the fact that EGTA exerted a large inhibition (>80%) of basal $[^3P]$ incorporation into phosphatidylinositol. In the experiments of Refs. 2 and 4, no index of Ca$^{2+}$-depletion was presented.