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Research Article

The cellular uptake of nonphosphorylated *myo*-inositol (MI) and its incorporation into phosphoinositide in the rat epitrochlearis muscle was measured. Cellular uptake of [2-^{3}H]MI was determined by the difference between total uptake and [2-^{3}H]MI present in the extracellular fluid determined with [1-^{14}C]mannitol. Cellular uptake was parabolic and directly proportional to medium MI concentrations between 25 and 3,200 μM. Saturation of a MI carrier was not evident. Moreover, uptake was not inhibited by 2 mM ouabain, 0.3 mM 2,4-dinitrophenol, or 22 mM glucose. Insulin, 100 mU/ml, was without effect on either cellular uptake of [2-^{3}H]MI or its incorporation into phosphoinositides. In muscles that were preloaded with [2-^{3}H]MI and then incubated in media that contained a constant amount of MI but no [2^{3}H]MI, 44.3% of the [2-^{3}H]MI was released after 10 min increasing to 62.5% by 120 min. Cellular MI concentrations were 0.18 μmol/g wet tissue (four times plasma levels) in rapidly isolated and frozen epitrochlearis muscle. When muscle was incubated without MI, 48% of endogenous MI was lost rapidly. Restoration of cellular MI in 50 μM MI media occurred in two phases, a rapid uptake phase lasting 10 min and a subsequent slow phase of MI uptake.

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Concentration of *Myo*-inositol in Skeletal Muscle of the Rat Occurs without Active Transport

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ABSTRACT The cellular uptake of nonphosphorylated *myo*-inositol (MI) and its incorporation into phosphoinositide in the rat epitrochlearis muscle was measured. Cellular uptake of [2-^{3}H]MI was determined by the difference between total uptake and [2-^{3}H]MI present in the extracellular fluid determined with [1-^{14}C]mannitol. Cellular uptake was parabolic and directly proportional to medium MI concentrations between 25 and 3,200 μM. Saturation of a MI carrier was not evident. Moreover, uptake was not inhibited by 2 mM ouabain, 0.3 mM 2,4-dinitrophenol, or 22 mM glucose. Insulin, 100 mU/ml, was without effect on either cellular uptake of [2-^{3}H]MI or its incorporation into phosphoinositides. In muscles that were preloaded with [2-^{3}H]MI and then incubated in media that contained a constant amount of MI but no [2-^{3}H]MI, 44.3% of the [2-^{3}H]MI was released after 10 min increasing to 62.5% by 120 min. Cellular MI concentrations were 0.18 μmol/g wet tissue (four times plasma levels) in rapidly isolated and frozen epitrochlearis muscle. When muscle was incubated without MI, 48% of endogenous MI was lost rapidly. Restoration of cellular MI in 50 μM MI media occurred in two phases, a rapid uptake phase lasting 10 min and a subsequent slow phase of MI uptake.

It is concluded that MI enters and leaves skeletal muscle cells freely by a process that does not involve active transport. Neither insulin nor hyperglycemia affected MI transport nor its incorporation into phosphoinositides. The intracellular to medium concentration gradient may be dependent on reversible binding to tubulin and possibly to other intracellular components.

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INTRODUCTION

Myo-inositol (MI)[1] is a naturally occurring cyclic hexitol maintained at a concentration of 40–80 μM in plasma. Tissue levels of nonphosphorylated MI are 4–80 times plasma levels (1–3). The mechanism by which intracellular:plasma gradient is maintained has not been clarified but it has been widely assumed that the cellular concentration depends on a mechanism of active transport. Active MI transport occurs in kidney slices (4–6), kidney particulates (7), and isolated brush border vesicles (8). This transport is inhibited by glucose, which accounts for the inosituria that occurs in diabetes mellitus (8, 9). Active transport of MI occurs also in the small intestine (10). The brain contains the highest known concentration of nonphosphorylated MI of any tissue in the body but its mechanism of concentration remains in dispute. The blood brain barrier prevents free access of plasma MI into brain but Spector and Lorenzo (11) and Spector (12) have clearly demonstrated that both MI and *scyllo*-inositol are accumulated within the choroid plexus by a single, specific, energy-requiring transport process that depends on intracellular reduced sulfhydryl bonds. After its concentration in the choroid plexus, MI apparently enters cerebral spinal fluid by simple diffusion. The final concentration in cerebrospinal fluid is five times greater than that of plasma. Brain levels of MI, however, are more than 20 times that of cerebrospinal fluid. Nevertheless, active transport has not been demonstrable with brain slices (11) and isolated brain synaptosomes (13). The uptake of MI by synaptosomes was not saturable and was not inhibited by dinitrophenol or ouabain. A reported depletion of peripheral nerve MI in diabetes mellitus has been attributed to inhibition of MI transport without clear evidence that such a process exists (14).

In this paper, we examine the mechanism of uptake and release of MI by skeletal muscle. Although the

[1] *Abbreviation used in this paper*: MI, *myo*-inositol.

concentration of MI in skeletal muscle is relatively low, only about five times that of plasma, it accounts for ~50% of total body MI. For these studies we have selected the rat epitrochlearis muscle because of previous work from this division, which established that its thinness permits rapid access of oxygen and substrates and that the absence of cut fibers makes it appropriate for transport studies. This muscle maintains normal carbohydrate, amino acid, and energy metabolism for at least 4–6 h in incubation (15).

METHODS

Materials and animals. MI, dibutyryl cyclic AMP, ouabain, and 2,4-dinitrophenol were purchased from Sigma Chemical Co., St. Louis, Mo. [2-^{3}H]MI, [U-^{14}C]MI, and D-[1-^{14}C]mannitol were purchased from New England Nuclear, Boston, Mass. Chromatography on Whatman No. 1 paper (Whatman, Inc., Clifton, N. J.) in acetone water (4:1 vol/vol) and phenol water (4:1 vol/vol) established that the labeled MI contained <3% contamination. Bovine parathyroid hormone, 1–84 (900–1,500 U/mg by rat bioassay), was purchased from Inolex Corp., Biochemical Div., Glenwood, Ill. Male Sprague-Dawley rats weighing between 80 and 90 g were purchased from Eldridge Laboratory Animals, Barnhart, Mo. The rats were fed Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) ad libitum and housed in the animal quarters for 1 wk before experiments.

Experimental procedures. Rats that weighed 110–120 g were killed by a blow to the back of the head and the epitrochlearis muscle, a strap-like forearm muscle, was carefully dissected bilaterally and removed as described elsewhere (15). Muscles were individually incubated at 37°C in 0.5 ml of medium in 17 × 100-mm stoppered polystyrene culture tubes in a shaking incubator. Total time elapsing between killing the rat and initiating incubation was <1 min.

The incubation medium consisted of Krebs-Henseleit buffer modified to contain half the original calcium concentration, and the buffering strength was increased by addition of 5 mM Hepes buffer pH 7.4. The medium contained 5.5 mM glucose except when 22.2 mM was substituted and a variable concentration of MI. In certain experiments the following additives were present in the incubation medium: insulin, 100 mU/ml; 2,4-dinitrophenol, 0.3 mM; ouabain, 2 mM; cyclic AMP, 0.1 mM; and parathyroid hormone, 2.5 μg/ml. The media were gassed with 95% O_2:5% CO_2.

After 15 or 30 min of preincubation the muscles were transferred into fresh media and the incubation periods of 1–120 min were initiated. The media contained MI as indicated, [2-^{3}H]MI, 0.6–1.5 × 10^6 dpm, and [1-^{14}C]mannitol, 0.2–0.5 × 10^5 dpm.

Cellular release of [2-^{3}H]MI was studied by first preloading muscles for 60 min in medium that contained [2-^{3}H]MI, 25 μM MI, and [1-^{14}C]mannitol. Four muscles were fast frozen for determination of initial [2-^{3}H]MI pools and four were transferred to medium that contained 25 μM MI and no radioactive compounds. 5 μl of medium were sampled at intervals for measurement of radioactivity.

After incubation, the muscles were removed, blotted on filter paper, and rapidly frozen in liquid freon previously cooled in liquid nitrogen. The frozen muscles were added to 1 ml of 0.9 M perchloric acid and homogenized in a Polytron (Brinkmann Instruments, Inc., Westbury, N. Y.) with a PT10ST blade at a setting of 8 for 45 s. The resulting homogenate was centrifuged at 2,500 *g* for 15 min and 750 μl of supernate were neutralized with KOH-imidazole KCl buffer (16) and recentrifuged. Recovery of [2-^{3}H]MI added after homogenization and neutralization was 85.6±4% SE. Results were appropriately corrected for this loss. Rat plasma was deproteinized with perchloric acid and neutralized with KOH-imidazole KCl buffer, but the incubation medium was analyzed directly.

Radioactivity of 100-μl aliquots of the deproteinized muscle extract or medium was determined after the addition of Instagel (Packard Instrument Co., Inc., Downers Grove, Ill.). Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. Automatic external standard ratios were used to correct for quenching. Crossover of ^{3}H and ^{14}C counts at varying automatic external standard ratios was determined in a series of vials containing 100 μl of H_2O and variable amounts of acetone, which served as the quenching agent.

The extracellular space [2-^{3}H]MI was calculated by the following formula:

$$\text{medium [2-}^3\text{H]MI, dpm/ml} \times \frac{\text{muscle [1-}^{14}\text{C]mannitol, dpm/g}}{\text{medium [1-}^{14}\text{C]mannitol, dpm/ml}}.$$

Cellular [2-^{3}H]MI was determined as the difference between total [2-^{3}H]MI and extracellular fluid [2-^{3}H]MI.

The cellular uptake of MI was calculated from the uptake of [2-^{3}H]MI using the following equation: cellular [2-^{3}H]MI (dpm/mg wet weight)/MI specific activity (dpm/nmol).

Metabolism of [2-^{3}H]MI to ^3H_2O was investigated by measuring the radioactivity of 100-μl aliquots of medium before and after lyophilization.

Determination of [2-^{3}H]MI incorporated into lipid inositides was carried out as detailed elsewhere (1) except hydrolysis and isolation of MI were not required because all tritium counts were contained in the inositol portion of the lipid inositide (6). Briefly, the perchloric acid precipitate from the muscle homogenate was washed with 1 ml 0.69 M perchloric acid and recentrifuged. After discarding the supernate, the precipitate was extracted with 4 ml of chloroform/methanol/HCl (500:500:3) by vortexing vigorously. 800 μl of a solution containing 1 N HCl and 30 mM $CaCl_2$ was then added; this solution was vortexed and allowed to separate. The lower phase was collected and the upper phase reextracted. The two lower phases were combined, dried under N_2, resuspended in 200 μl chloroform/methanol (1:1), and counted in 10 ml of Instagel.

For measurement of nonlabeled MI, 400-μl aliquots of the deproteinized muscle extracts were lyophilized to dryness and then heated to 100°C for 1 h under vacuum to remove residual water. The residue was dissolved in 100 μl of silylation reagent, prepared by adding one part of a mixture of 90% *N,O*-bis(trimethylsilyl)-trifluoroacetamide and 10% trimethylchlorosilane to one part pyridine. Mass spectrometry of MI was performed as described by Sherman et al. (17, 18), except that a 5-ft glass U tube that contained 3% SE-30 at column temperatures of 230°C with a helium flow rate of 30 on a Finnigan 3,200 spectrometer (Finnigan Corp., Sunnyvale, Calif.) was used. Samples were quantitated by comparison of peak heights produced by silylated inositol standards using ions 305.2 and 318.2.

ATP and phosphocreatine were measured by the method of Lowry and Passonneau (16).

RESULTS

Because the weight of the epitrochlearis muscle represented only 5% of the incubation medium, there was

TABLE I

Changes in Incubation Media [2-^{3}H]MI, MI, and ^3H_2O during Incubation

Time, (*min*)	0	5	10	30	60	120
MI, μM	54±0.05	56±0.04	52±0.06	57±0.04	53±0.5	53±0.04
[2-^{3}H]MI, *dpm/ml* × 10^{-3}	605±14	580±16	601±20	590±29	610±9	600±24
^3H_2O, *dpm/ml*	3±1	5±1	4±1	6±1	2±1	3±0.05

Muscles were preincubated in medium without MI or [2-^{3}H]MI. After 30 min, muscles were transferred to a medium that contained [2-^{3}H]MI. Values are mean±SE, n = 3.

no significant change in medium [2-^{3}H]MI and MI concentrations during the course of these incubations (Table I). Also, there was no evidence of metabolism of [2-^{3}H]MI to volatile metabolites (i.e., ^3H_2O) as determined by radioactivity measurements of medium before and after lyophilization.

The measured [1-^{14}C]mannitol space as a function of time is shown in Fig. 1. Equilibration occurred rapidly so that at 3 min the apparent space was already 70.3% of the space at 10 min. The slow rise subsequent to 10 min may represent some tissue swelling.

The total and intracellular [2-^{3}H]MI during 20 min of incubation is plotted in Fig. 2. Both total and extracellular fluid [2-^{3}H]MI increased rapidly during the first 10 min in a parabolic pattern. Thereafter the rate of uptake was greatly reduced. The difference between total and extracellular fluid [2-^{3}H]MI represents intracellular MI, which reached 18% at 10 min. For greater clarity intracellular [2-^{3}H]MI is replotted on a different scale in Fig. 3. The pattern of uptake of [2-^{3}H]MI also had a parabolic pattern. For most subsequent experiments a 10-min period of incubation was selected.

Media concentrations of MI from 25 to 3,200 μM were examined at glucose concentrations of either 5.5 or 22.2 mM (Fig. 4). Cellular uptake was linear throughout the concentration range of medium MI providing evidence that even at concentrations greatly exceeding the extracellular fluid MI concentration, there was no evidence that uptake was mediated by a saturable carrier. Uptake of MI was not significantly different at the two glucose concentrations.

MI uptake by epitrochlearis muscle was not inhibited by 2 mM ouabain, an inhibitor of Na^+ linked active transport processes in cells.

To determine whether MI uptake was dependent on high energy phosphate levels, we preincubated the epitrochlearis muscle for 30 min with 0.3 mM 2,4-dinitrophenol. At this time, muscle ATP had fallen from 3.7±0.9 to 0.9±0.2 μmol/g and phosphocreatine had fallen from 13.0±1.7 to 1.11±0.26 μmol/g. During the subsequent 10 min when [2-^{3}H]MI uptake was measured, the decrease in ATP and phosphocreatine did not inhibit MI uptake. In fact, mean uptake was actually higher (3.98±0.54 vs. 3.00±0.24 nmol/g) but this increase was not significant.

Because of a possible connection between MI metabolism and diabetes mellitus, we tested the effect of insulin, 100 mU/ml on the 10 min MI uptake with negative results. Although we had previously reported an effect of parathyroid hormone on renal MI excretion (19, 20), we observed no effect of parathyroid hormone or dibutyryl cyclic AMP on MI uptake in skeletal muscle.

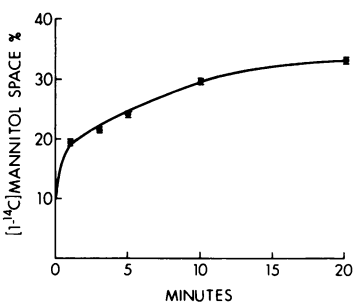

FIGURE 1 Epitrochlearis muscles were incubated with [1-^{14}C]mannitol for the indicated times. The apparent volume of distribution of [1-^{14}C]mannitol was calculated from the concentration of the isotope in muscle and medium. Mean±SE are shown, n = 3.

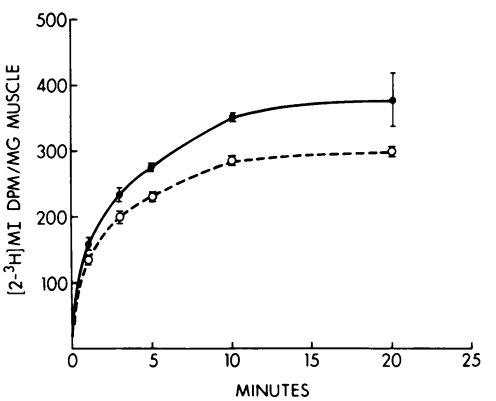

FIGURE 2 Total uptake of [2-^{3}H]MI by rat epitrochlearis muscle is plotted as a function of duration of (● —— ●). (The increased variance observed at 20 min was due to one value that was much lower than the other two muscles.) The [2-^{3}H]MI in the muscle interstitial fluid as determined by the [2-^{3}H]mannitol space is shown (○ - - - ○). Mean±SE are shown, n = 3.

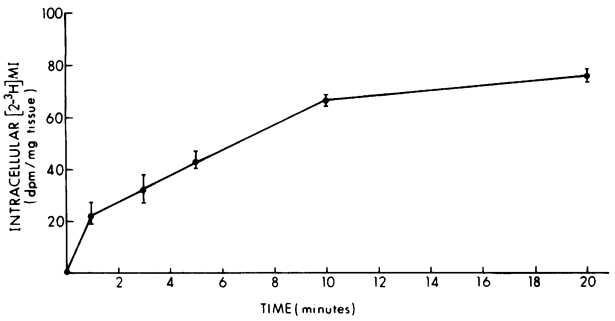

FIGURE 3 Intracellular uptake of [2-^{3}H]MI by epitrochlearis muscle as a function of time. [2-^{3}H]MI present in extracellular fluid has been subtracted from total uptake. Medium MI concentration was 25 μM. Mean±SE are shown, n = 3.

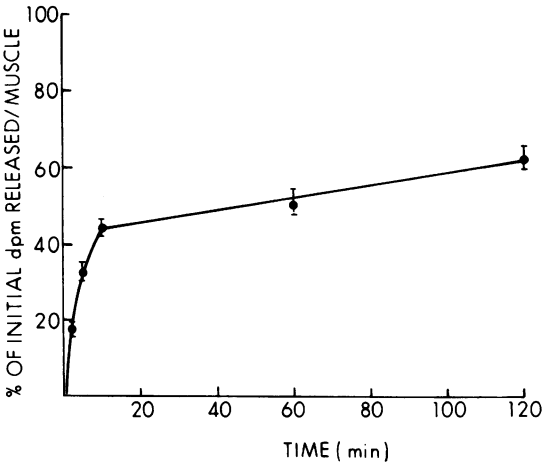

FIGURE 5 Epitrochlearis muscles were preincubated with [2-^{3}H]MI, 600,000 cpm and 25 μM MI for 1 h. Muscles were then transferred to medium that contained 25 μM MI without [2-^{3}H]MI. The percent loss of initial [2-^{3}H]MI as a function of time is shown. Mean±SE, n = 3.

The rate of release of intracellular MI was investigated by preincubating the epitrochlearis muscle with [2-^{3}H]MI and 25 μM MI. After correction for extracellular MI, the initial [2-^{3}H]MI radioactivity within the cell was 814±14 dpm/mg. When transferred to a medium without label and with the same 25 μM MI, there was a rapid loss of radioactivity from the muscle. The time-course of this disappearance is shown in Fig. 5. There was a rapid phase of [2-^{3}H]MI loss, which lasted 10 min, to be followed by a slower continuous loss, which remained linear during the study period of 120 min. At the end of the experiment, 217±40 dpm/mg remained within the muscle cells indicating that 63% of the initial radioactivity had been lost from the cells.

The exchange of MI between epitrochlearis muscle and the incubation medium was studied. Epitrochlearis muscle rapidly removed from the rat and immediately frozen had a MI concentration of 0.18±0.1 μmol/g wet tissue. This is similar to the MI concentration reported for diaphragm and heart (5). In comparison, pooled rat serum in our laboratory had a concentration of MI of 44 μM. Based on an intracellular water content of 60% wet weight, the intracellular water concentration of MI was calculated to be 0.3 μmol/g and the MI gradient between muscle and plasma water would be about 7:1. When the epitrochlearis muscle was placed in an incubation medium devoid of MI, there was a loss of 52% of the initial MI within 30 min (Fig. 6). After the muscles were transferred to a medium containing 55 μM MI, there was a rapid uptake of MI for 10 min to be followed by a slower linear increase in MI for the next 110 min. During this period, the loss of MI that had occurred during the period

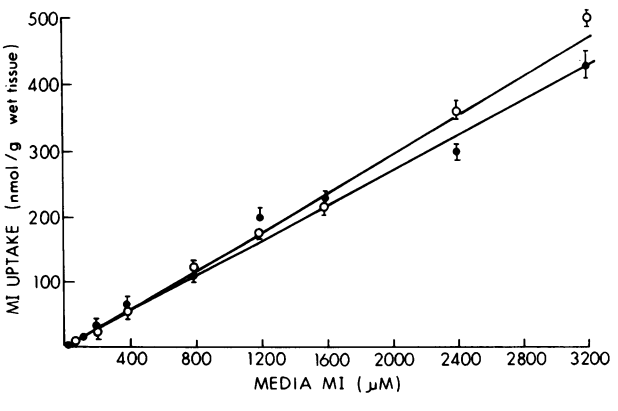

FIGURE 4 Intracellular uptake of MI at 10 min of incubation as a function of MI concentration. Uptake was measured as [2-^{3}H]MI uptake in disintegrations per minute divided by calculated medium specific activity. Uptake was measured at glucose concentrations of 5 mM (●) and 22 mM (○). Mean±SE, n = 3.

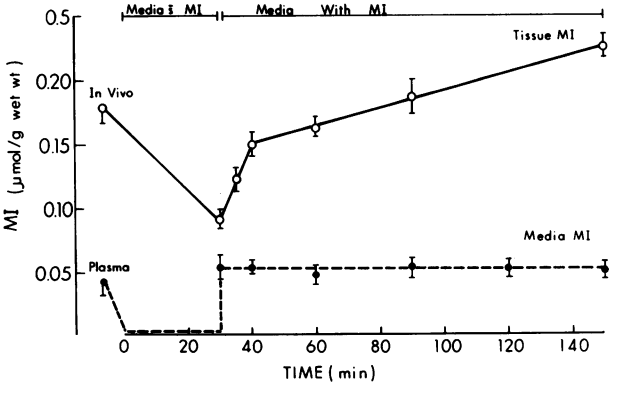

FIGURE 6 The concentration of MI in epitrochlearis muscle was measured immediately after removal from the rat, 30 min after incubation in a medium devoid of MI, and at intervals from 120 min of incubations in a medium that contained 55 μM MI. The upper curve shows muscle MI and the lower line shows for comparison plasma and medium MI concentration. Mean±SE, n = 3.

of incubation in the MI-free medium was completely restored.

We next investigated the effect of insulin on the incorporation of [2-^{3}H]MI into muscle phosphoinositides. In these experiments epitrochlearis muscle was incubated with and without the addition of 100 mU/ml insulin. Incorporation of [2-^{3}H]MI into phosphoinositides was measured with and without carrier MI, 50 μM. Incorporation of [2-^{3}H]MI was uninfluenced by insulin at either concentration of medium MI during 240 min of study.

DISCUSSION

MI is an essential constituent of cells, which may have important direct actions as well as serving as a precursor of phosphoinositides, which are critical constituents of cell membranes. Knowledge whereby intracellular MI concentration is maintained is essential for understanding its role in cellular biology. Many cells in culture cannot synthesize sufficient MI to maintain adequate intracellular concentrations to meet their growth requirements at low cell densities. Our finding that maintenance of intracellular MI is dependent on extracellular MI would explain this dependence on medium MI for growth.

We could find no evidence of an active transport mechanism that could explain the intracellular concentration of MI. MI uptake increased linearly over a concentration range that exceeded plasma concentrations by more than 60-fold. Therefore, no saturable carrier seemed to be involved. We also obtained evidence that MI uptake was not dependent on ATP or linked to Na^+ transport. The high concentration of high energy phosphate compounds in muscle makes it particularly difficult to deplete these reserves rapidly. We were able to reduce ATP levels by 75% and phosphocreatine levels by 92% with the conditions employed. If ATP were essential for MI uptake, some inhibition with 2,4-dinitrophenol should have been evident.

Ouabain is a potent inhibitor of Na^+ linked transport systems. It has been reported to inhibit MI transport in kidney (4), gut (10), and choroid plexus (11) but in our experiments was without effect on muscle MI uptake.

The loss of MI from muscle cells into the medium proved to be equally rapid either when measured by the loss of preloaded [2-^{3}H]MI or the loss of endogenous MI into a MI free medium. After 30 min, nearly half the original MI had left the muscle cells. Intracellular MI was rapidly restored when the muscles were returned to a medium containing 55 μM MI. These experiments show that intracellular MI in muscle is in rapid equilibrium with extracellular fluid MI.

If the concentration of MI in muscle cells cannot be attributed to active transport, what other possibilities need to be considered? It has been suggested that the gradient of MI in the nervous system might be maintained by *de novo* synthesis and limited MI escape from nerve cells (13). This seems unlikely in muscle because of the rapid exchange of intra- and extracellular MI and the low rate of MI synthesis from glucose.

We suggest that the high cellular content of MI could be related to MI binding within the cell. This would provide an explanation for the concentration gradient between muscle cells and extracellular fluid, the apparent lack of active transport of MI, and the rapid equilibrium established between extracellular fluid and muscle cells. The nature of the putative cellular component which binds MI remains to be identified. Kirazov and Lagnado (21) reported that brain tubulin could bind MI. Pickard and Hawthorne (22) confirmed the presence of bindng of MI by tubulin but considered the binding nonspecific because there was no evidence of a saturable binding site. The binding of MI by tubulin in these studies, however, was insufficient to account for the high intracellular concentration of MI in brain or other tissues. The possibility that other cellular components also bind MI needs to be examined.

We could find no evidence that elevated glucose concentrations or insulin affected entrance of MI into muscle. Insulin also did not increase incorporation of [2-^{3}H]MI into phosphoinositides. As muscle MI represents half of the body MI, these findings are consistent with the report of Daughaday et al. (19), that the disappearance of [2-^{3}H]MI was not significantly impaired in experimental canine diabetes, a finding which differs from the earlier observations of Clements and Reynertson (23) that total radioactivity disappeared more slowly from the serum of patients with diabetes mellitus than from normal subjects.

Although parathyroid hormone has a major effect on MI reabsorption in the kidney (19, 20), we did not detect an effect of parathyroid hormone or dibutyryl cyclic AMP on MI uptake in the epitrochlearis muscle.

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