1971

Hormone Induced Magnesium Transfer in Fat cell Plasma Membranes: A Mechanism for Effecting Changes in Cellular Metabolism

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HORMONE INDUCED MAGNESIUM TRANSFER IN FAT CELL PLASMA MEMBRANES; A MECHANISM FOR EFFECTING CHANGES IN CELLULAR METABOLISM

A thesis submitted to the Faculty of the Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
by
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June 15, 1971
The Rockefeller University
New York

APPROVED FOR PUBLICATION

Acknowledgements

These investigations were carried out during the tenure of a Rockefeller University Graduate Fellowship. I wish to thank Dr. Martin Rizack who gave enthusiastic support and valuable time to the work so as to encourage the development of confidence in pursuing research independently.

Abstract

A new method was developed to measure magnesium transfer across the plasma membrane. This technique made use of the ligand, 8-hydroxy-quinoline-5-sulfonic acid (8-OH-Q) which fluoresces in the presence of magnesium, and was used to demonstrate epinephrine stimulation of magnesium uptake.

The ligand was incorporated into plasma membrane vesicles prepared from rat epididymal fat cells. In the presence of calcium and magnesium but not in the presence of calcium alone, epinephrine and other lipolytic hormones stimulated an increase in fluorescence which was equivalent to an increase in intravesicular magnesium of 1.2 mM. The influx rate of magnesium entering the intravesicular space was calculated to be 10.2 micromoles of magnesium per gram of protein during the first thirty minutes. ACTH stimulation also caused an increase in fluorescence at a slower rate. Insulin and ouabain blocked the lipolytic hormone stimulated increase in fluorescence.

The uptake and retention of magnesium during epinephrine stimulation of plasma membrane vesicles was confirmed using radioactive magnesium (^{28}Mg). Epinephrine stimulation of magnesium uptake in whole fat cells was shown using ^{28}Mg and atomic absorption analysis of cold magnesium. Calcium potentiated the increase in magnesium retention following epinephrine stimulation.

Magnesium was shown to potentiate the decrease in fatty acid synthesis during epinephrine stimulation of fat cells. Uniformly labeled ^{14}C glucose was used for these studies.

These results are discussed in terms of a hypothetical model by which changes in intracellular magnesium concentration could account for changes in metabolism seen during lipolytic hormone stimulation of adipocytes.

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1. Introduction

Extracellular and intracellular concentrations of various salts affect the metabolism of the fat cells as well as other mammalian cells. Walter B. Cannon (1932) emphasized the extracellular fluid and the homeostatic mechanisms regulating its relatively constant composition. Recently Bygrave (1967) has emphasized the importance of intracellular ionic environment. Gevers and Krebs (1966) have drawn attention to the possibility that changes in calcium or magnesium concentration could have important consequences for the "direction" of the carbohydrate metabolism; the net metabolic effect could be mediated by the effect of the divalent cations on both enzymes and metabolic intermediates.

There have been many analyses over the years to determine the concentrations of the cations and anions in the intracellular fluid of the liver and muscle cell. Most of the isolated enzymes from these cells have been characterized in salt solutions and buffers which approximate the intracellular environment but usually do not reflect it exactly since it has been more important to find buffer conditions which maximize activity for kinetic studies of the enzyme itself than to try to approximate the true interior conditions of the cell. The activities of the cells enzymes have been analyzed in vivo or in semi in vivo states such as perfusion or isolated cell techniques. This has not allowed exact manipulation of the intracellular cation concentrations. The net result is a lack of precise information on how intracellular cation changes might alter metabolic patterns.

Gevers and Krebs (1966) have raised the possibility that changes in intracellular divalent cation concentration may alter metabolic patterns. Since peptide hormones and epinephrine are responsible for shifting the metabolic patterns at some of the same critical enzyme points affected by magnesium, the question is raised as to whether the peptide hormones and epinephrine alter the divalent cation concentrations inside the cell. This might explain part of the acute metabolic changes caused by such hormones.

Although the details of liver and muscle metabolism are known more

completely than that of the fat cell, the fat cell has two attributes which make it more useful than liver and muscle in our work. The first is its ease of isolation, free of connective tissue components, which allows a more exact analysis of the fat cell attributes. The second is that techniques exist for measuring end product metabolism which give information about the net activity of regulatory enzymes with more certainty than what one can conclude from analysis of the intermediate metabolites in liver and muscle.

Nagata and Rasmussen (1970) have emphasized how variations in the intracellular calcium concentration may be responsible for shifts in the renal cell metabolism under parathormone stimulation. Bygrave (1967) also emphasized the alteration in calcium metabolism. Gevers and Krebs (1966) and Wood et al (1968) pointed out how magnesium might alter the equilibrium of some of the critical enzymes which control the carbohydrate metabolism.

Since magnesium and not calcium is the predominant intracellular divalent cation and since calcium is known to block or inactivate enzymes it seems more likely that the physiological changes in enzyme activity may be induced by magnesium rather than calcium.

This thesis presents studies of hormone induced magnesium transfer in fat cells and plasma membranes derived from them. Before presenting our findings a review of the literature regarding the effect of cations on hormone regulation in the fat cell will be presented.

A. The Importance of Certain Ions in the Extracellular Fluid for Proper Fat Cell Hormone Response

The response to lipolytic hormone is significantly altered by deletion of K^{+} or Ca^{++} in so far as release of fatty acids is concerned.

It has been shown by Ho et al (1967) that either a potassium free medium or the presence of ouabain causes a marked reduction in ACTH or epinephrine induced c-AMP production with the result that free fatty acid release was impaired due to lack of lipase stimulation.

Mossinger and Vaughn (1967) and Yoshimura et al (1969) found that this potassium effect could be potentiated by removing calcium or magnesium from the medium.

Recently Efendic et al (1970) demonstrated that Ca^{++} had an enhancing effect in stimulation of lipase by c-AMP.

Apart from the studies of Mosinger and Vaughn there are no studies which have suggested that Mg^{++} in the extracellular fluid was important for the hormone stimulation of fat cells.

All of these studies relate to only one aspect of lipolytic hormone response, the release of fatty acids and glycerol. The effect of ion changes on the inhibition of fatty acid synthesis and the stimulation of glyceride glycerol synthesis by these hormones does not seem to have been studied at all.

In regard to insulin stimulation of the fat cell it was found that replacement of Ca^{++} or Mg^{++} with Na^{+} did not alter the basal metabolic activity nor did it alter the insulin responsiveness of cells (Jeanrenaud 1968). Omission of K^{+} increased basal activity but did not change the insulin response. If Na^{+} was omitted there was also an increase in basal activity and a marked decrease of the insulin effect on glucose metabolism.

B. Hormone Stimulated Ion Shifts in Mammalian Fat Cells

Although studies on the best extracellular ionic environment for the optimal hormonal stimulation of the fat cell have been in progress for some time very few studies have been carried out in the mammalian fat cell in regard to ion shifts until recently. There have been no studies of magnesium shifts into or out of the fat cell. The hormonal response in fat cells has been the subject of studies of electronegative potential, potassium flux, and calcium uptake. In addition the flux of the two anions, chloride and aminoisobutyric acid have been studied.

Biegelman and Hollander (1964) first showed that epinephrine and insulin both altered the electronegative potential of the fat cell.

Insulin caused a significant increase in electronegativity and although epinephrine tended to increase the resting potential as well, the amount of change was not as great as with insulin.

Touabi and Jeanrenaud (1970) found that stimulation of the fat cell by epiniphrine and ACTH caused a marked decrease in $^{42}K^{+}$ uptake by the fat cell. Perry and Hales (1969, 1970) also found that epinephrine affected the flux of potassium. They found that $^{42}K^{+}$ efflux from previously loaded fat cells was increased by epinephrine as well as ACTH, dibutyryl cyclic 3' 5' AMP and theophylline. The epinephrine stimulation was biphasic. The loss of potassium in the first five minutes was more rapid than in the last twenty minutes.

Calcium has been studied by Akgün and Rudman (1969) in relation to lipolytic hormone stimulation of fat cells. They found that in the intact rabbit, ACTH injection produced a dramatic decrease in serum calcium concentration at the same time that serum free fatty acid increased. They also studied the calcium shift under epinephrine stimulation in an <u>in vitro</u> system using rabbit adipose tissue slices bathed in Krebs-Ringer bicarbonate buffer with fatty-acid-free- albumin or with homologous serum. It was found that there was a decrease in calcium in the medium and an increase of tissue calcium if homologous serum was present. There was no similar shift if albumin was used even though there was still an epinephrine stimulated free fatty acid release.

Akgün et al (1969) also studied the serum magnesium concentration six hours after ACTH injection into rabbits. There was an increase of free fatty acids and serum magnesium from 3.3 to 5.5 mg/100ml while plasma calcium fell from 13.9 to 10.3 mg/100ml. βMSH produced a similar response. The magnesium was not studied in isolated tissues.

Chloride efflux was studied by Perry and Hales (1970). They found that epinephrine stimulated chloride efflux. Vassali and Jeanrenaud (1970) found that stimulation of lipolysis by epinephrine, ACTH or caffeine resulted in a marked inhibition of the net α-aminoisobutyric acid uptake by fat cells. The magnitude of the inhibition was proportional to lipolysis.

C. Magnesium and other Cation Shifts due to Lipolytic Hormone Stimulation in Organs other than Adipose Tissue

There have been several studies on the influence of hormones on cation shifts in various organs during more prolonged hormone stimulation. Plattner (1950) demonstrated that cold stress (a condition which includes sympathomimetic stimulation) for twenty-four hours in gold fish and turtles caused an increase of serum magnesium and a decrease of liver and muscle magnesium.

Lehr (1969) in a study of myocardial injury produced by sympathomimetic amines showed that as early as three hours, before there was any myocardial injury, there were significant shifts in total electrolyte concentration in heart and kidney. With epinephrine stimulation (β receptor) total magnesium in the heart decreased and kidney magnesium increased. This initial change was followed by an increase in calcium in both heart and kidney. During phenylephrine stimulation (α receptor) there was a decrease in magnesium and a later increase in calcium in both heart and kidney. The effects of calcium and magnesium could be blocked by propanolol in the case of epinephrine and by phentolamine in the case of phenylephrine.

Acute injection of epinephrine in pigs at levels sufficient to cause pulmonary hypertension and edema was correlated with a three fold rise in serum magnesium within one hour (Rowe and Elliott, 1965).

Lehr et al (1966) demonstrated a protective effect of parathyroid extract against cardiac necrosis produced by the sodium phosphate loading of parathyroidectomized rats. In the heart, parathyroidectomy produced a significant decline in heart magnesium levels. Parathormone restored it to normal. The calcium remained at the control levels. In the kidney, sodium phosphate loading caused a rise in the level of calcium but not magnesium. Parathyroidectomy caused a significant decrease in magnesium levels whereas total calcium decreased to the level of untreated controls. Administration of parathyroid extract caused a return toward normal levels of magnesium, while calcium again fell to the supernormal levels found in intact animals given sodium phosphate.

Nagata and Rasmussen (1970) suggested that much of the change in the metabolism of renal tubular cells induced by parathormone could be explained on the basis of parathormone stimulation of calcium uptake into renal tubular cells. Earlier Borle (1968) also showed that in renal tubular cells calcium uptake could be detected after parathormone exposure. There is not yet a clear understanding of how calcium increases in the cell can cause a stimulation of gluconeogenesis. The difficulty lies in finding a specific enzyme in the gluconeogenic sequence which is stimulated by calcium.

Friedman and Rasmussen (1970) studied isolated perfused rat liver and found that stimulation with either glucagon or c-AMP caused an efflux of Ca^{++} from liver previously loaded with $^{45}Ca^{++}$ and enhanced glucose formation from lactate or pyruvate. Changes in the Ca^{++} concentration of the perfusate did not affect gluconeogenesis but the addition of Mn^{++} stimulated gluconeogenesis without altering cyclic-AMP concentration. It was subsequently shown that this calcium efflux could be caused by perfusion with c-AMP (Friedman, 1971).

It is difficult to extrapolate these results from the liver and kidney to the fat cell since the metabolic response for any given hormone is distinctive for each tissue. Epinephrine or glucagon, for example causes release of glucose from liver and muscle glycogen but causes glucose uptake and synthesis of glyceride glycerol in the fat cell.

II. Experimental

Since changes in magnesium concentration in the cell may play a role in shifts of metabolism caused by hormones we designed a study to discover whether hormones regulate the transfer of magnesium across the plasma membrane of the fat cell.

A. Direct Measurement of Magnesium Flux Using a Fluorescent Ligand

In order to determine how much magnesium flux was occuring during hormone stimulation a new method was devised to measure the flux into plasma membrane vesicles directly. This was developed in order to measure magnesium transport into the cytoplasmic space under hormone stimulation. Other interfering ligands were kept to a minimum.

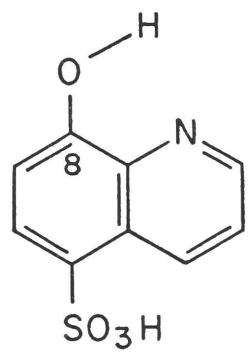
The method involves the use of a fluorescent ligand 8-hydroxyquinoline-5-sulfonic acid (8-OH-Q). With this ligand we can detect changes in the free magnesium. The concept of using ligands to measure free magnesium is new only in this application.

Walser (1960) and Berlin (1966) independently developed a method using eriochrome black T to determine free magnesium. Berlin found that a fairly reliable measurement of "free" magnesium could be made if the liganding indicator dye was low enough so that less than two percent of the magnesium was bound to it. In order to achieve this measurement of free magnesium, he used the ligand in the physiological pH range where the association constant of eriochrome black T for Mg^{++} was about three. This was less than for most other ligands present.

Burton (1959) and later Watanabe (1963) used 8-hydroxyquiniliol for the determination of the binding constants of ATP and ADP for magnesium. Bishop (1963) found it even more advantageous to use the water soluble form of the ligand, 8-hydroxyquinoline-5-sulfonic acid, (8-OH-Q) (Figure 1). Quinoline derivatives have an advantage because the magnesium salt fluoresces distinctively and the sensitivity of the assay is increased so that much less ligand has to be used.

Table I shows the chelation constants of 8-OH-Q for magnesium compared with the chelation constants of several other naturally occuring ligands for magnesium.

The 8-OH-Q chelation constant is logK=4.4. This is in the mid range of the other naturally occuring ligands shown and has a chelation constant which is approximately one-fourth that of ATP and one-eight



8 Hydroxyquinoline 5 sulfonic acid

Figure I. The magnesium ion, or other divalent cation, bonds to orbitals of the nitrogen in the ring and the oxygen radical of the dissociated hydroxyl group at the 8 position. The sulfonic acid group makes the ligand water soluble.

Ligand	log K
8 Hydroxyquinoline 5 Sulfonic Acid	4.40
ATP	4.78
ITP	4.90
ADP	3.78
Pyrophosphate	5.08
Pi	2.00
Citrate	3.63

Table I. Formation constants of divalent metal complexes of Mg^{++} and various ligands in 0.1 M TEA at $25^{o}C$. (Watanabe et al 1963)

that of pyrophosphate.

Methods Using 8-OH-Q

Attempts made to introduce the ligand directly into the fat cells were unsuccessful. It was decided to introduce the ligand directly into a plasma membrane vesicle obtained from the fat cell.

Plasma membranes were prepared using a modification of the method of McKeel and Jarett (1970) (Figure II). Cells were prepared as described in section B, page 20 and 21. After two washes with Krebs Ringer bicarbonate buffer with one percent albumin, the cell suspension was washed once with solution I containing 0.25 M Sucrose buffered with 0.1 Tris, pH 7.4 and 1 mM EDTA. This was used to reduce the calcium concentration. A second and third washing with solution II (0.25 M Sucrose, 0.1 M Tris, pH 7.4) and a final suspension in solution II removed most of the EDTA before homogenization.

The fat cell suspension was then homogenized with a loosely fitting teflon glass Thomas homogenizer at 800 revolutions per minute, with ten strokes.

The homogenized suspension was centrifuged in an International centrifuge in the cold room at 16,000 X g for 15 minutes. The centrifuge tube was thoroughly chilled in an ice bath after the centrifugation. The congealed fat on top was then easily removed with a spatula. The supernatant which contained the microsomal fraction was sedimented at 105,000 X g for 45 minutes in a Beckman 50 rotor in a model L centrifuge.

The pellet was washed and resuspended in solution II and centrifuged a second time to give a preparation of microsomes. They were found to contain no adenyl cyclase activity.

Pellet 3 containing plasma membranes, mitochondria and nuclei was suspended in solution II and the nuclei were sedimented by centrifugation at 1000 X g for 10 minutes. The supernatant containing plasma membranes and mitochondria was sedimented for twenty minutes at 20,000 X g and the sediment was resuspended in four ml of solution II and layered over a

PREPARATION OF FAT CELL PLASMA MEMBRANE VESICLES

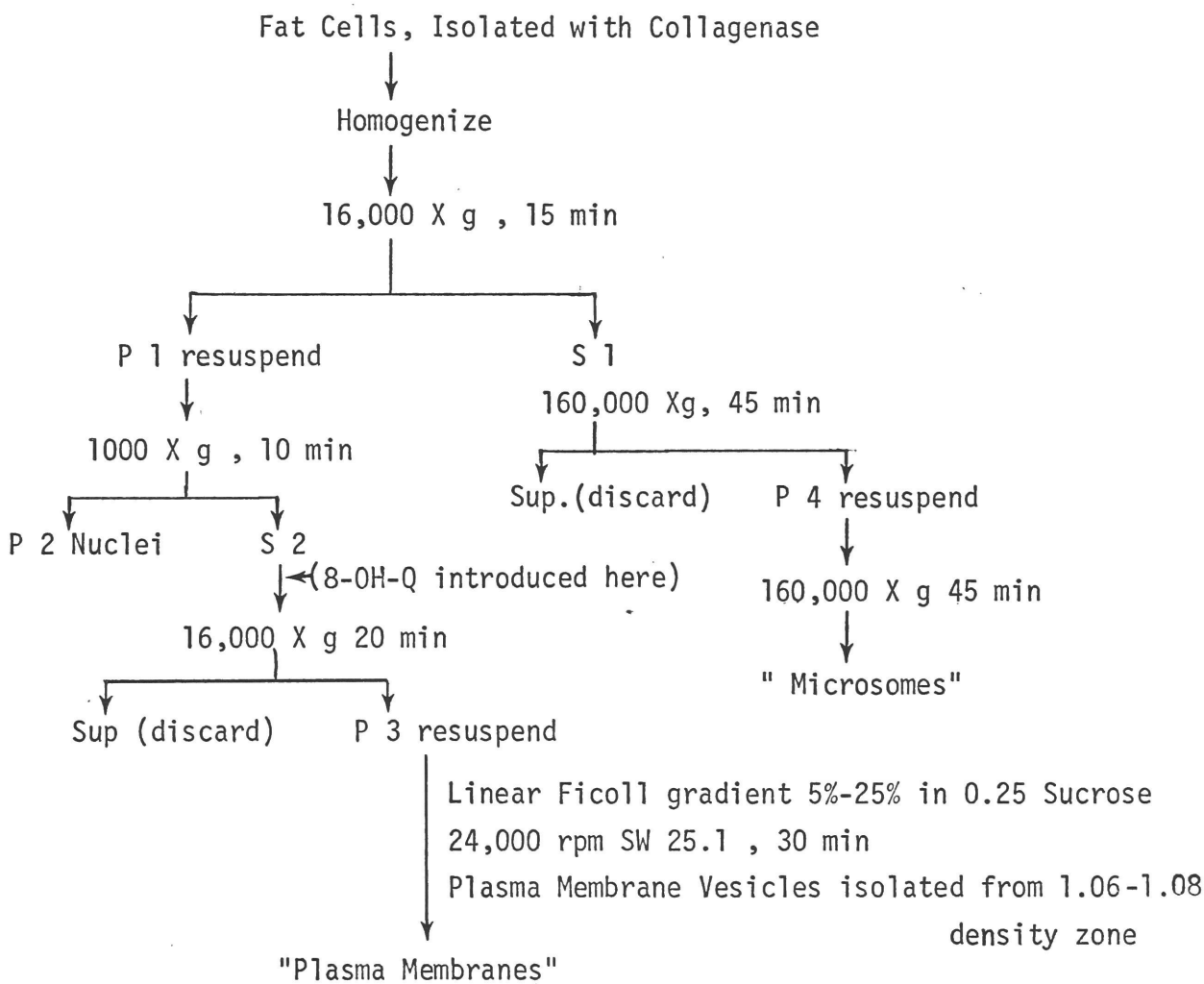


Figure II. The preparation of plasma membranes and microsomes from fat cells. Modification of the method of McKeel and Jarett (1970)

continuous Ficoll gradient, five to twenty five percent, in 0.25 M sucrose and centrifuged in a Beckman 25.1 swinging bucket rotor for 20 minutes at 63,000 X g.

After centrifugation there were two bands in the gradient. The upper band (density 1.06 to 1.08 g/ml and 1 to 1.5 cm from the top of the gradient) was found to contain the plasma membrane fraction as identified by adenyl cyclase activity. The second band near the bottom of the gradient contained mitochondria and was shown to have no adenyl cyclase activity. Adenyl cyclase was assayed in the plasma membranes and microsomal fractions by incubating with ^{14}C ATP and isolating cyclic 3'5' AMP by thin layer chromatography using PEI cellulose as previously described. (Rizack 1967)

The upper band was removed from the gradient, mixed with solution II to bring up to full volume and then centrifuged at 105,000 X g for 45 minutes to collect the plasma membrane vesicles. This was resuspended in solution II and centrifuged again under the same conditions.

In some experiments we introduced the ligand 8-hydroxyquinoline-5-sulfonic acid (8-OH-Q) into the plasma membrane vesicles, at the point indicated in Figure II, in the following manner. After the nuclei were removed by centrifugation, the suspension of particles in the remaining supernatant was added to a ligand solution containing 10 mM 8-OH-Q and 5 mM magnesium chloride; the final mixture with the particle suspension contained 2.5 mM of 8-OH-Q and 1.25 mM magnesium chloride in .25 M sucrose buffered with tris at pH 7.4. The mixture was homogenized vigorously with ten strokes at 6000 RPM while the eight ml teflon glass Thomas homogenizing tube remained immersed in an ice bath. The homogenized suspension was then layed over the Ficoll sucrose gradient and separation of the "plasma membrane" fraction was carried out as described above.

Subsequent sedimentation and resuspension resulted in a suspension of plasma membranes which in the Perkin-Elmer MPF 2A spectroflurometer were shown to contain a fluorescence peak exciting at 360 nM and emitting at 495 nM which was identical to the 8-OH-Q peak and was not present in

the blank plasma membrane suspension, where no 8-OH-Q had been added. Addition of more magnesium, 0.5 to 5.0 mM final concentration in the cuvette, resulted in no increase in fluorescence indicating that although the dye ligand was present, it was not available to magnesium added to the extravesicular space. Since saturating magnesium was not present, it is unlikely that all of the 8-OH-Q was saturated. This contention was supported by the evidence that when the plasma membrane vesicles were disrupted with sonication, there was an increase in 8-OH-Q fluorescence if additional magnesium was added or present in the suspending medium.

For these experiments, plasma membranes were suspended in modified Krebs Ringer triethanolamine buffer at pH 7.4 with varying concentrations of magnesium and calcium. The suspension was placed in a thermostated cuvette maintained at 37^{o}C in the Perkin-Elmer MPF 2A spectrofluorometer. Measurements were recorded as percent change in fluorescence.

In order to calibrate the changes seen in fluorescence during epinephrine stimulation of plasma membrane vesicles containing 8-OH-Q, the vesicles were prepared with three different concentration ratios of magnesium to 8-OH-Q. In the final homogenization mixture, 8-OH-Q was 2.5 mM with three magnesium concentrations of 0.125 mM, 0.75 mM and 1.25 mM. The difference in percent fluorescence of the 410 nM peak substracted from the 360 nM peak was plotted against the change in ratios and extrapolated for higher concentrations on the basis that the midpoint of the fluorescence titration curve is the point where magnesium is one half the concentration of the fluorescent ligand.

This calibration curve is shown in Figure III. Use of this curve allowed estimation of the change in magnesium concentration in the plasma membrane vesicles.

Results with 8-OH-Q

Nineteen experiments with 8-OH-Q plasma membrane vesicles were carried out in which a response to epinephrine and other hormones was measured. There were eight other preparations in which a response was not obtained due to various reasons.

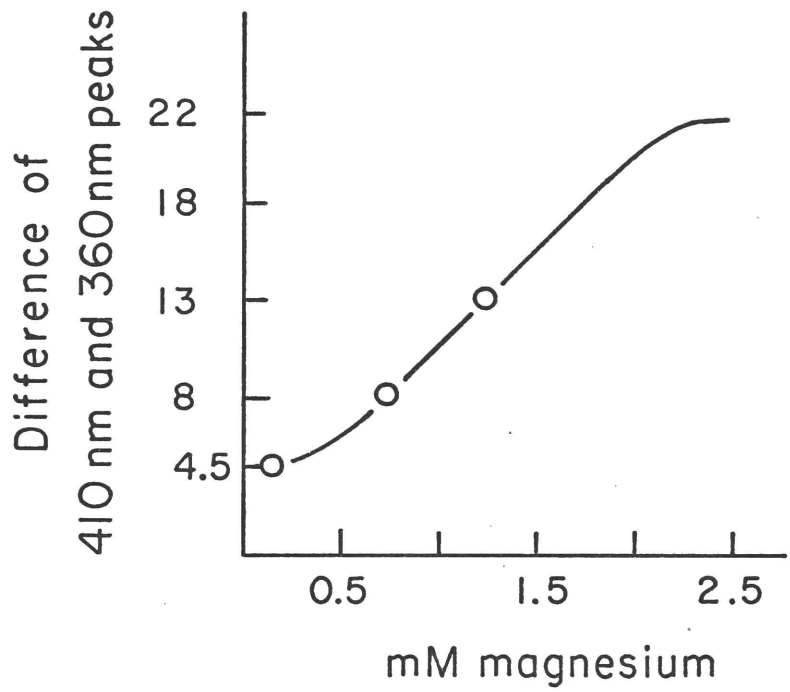


Figure III. Calibration curve for fluorescence of 2.5 mM 8-OH-Q with varying concentrations of magnesium incorporated into plasma membrane vesicles. Change in the fluorescence is the difference of the 410 nm and 360 nm peaks in percent fluorescence at sensitivity 4 on the MPF 2A where 20% of the scale is expanded to full scale.

Several requirements for a response to epinephrine were demonstrated. There is no response if ATP is absent from the medium. Both magnesium and calcium must be present for epinephrine to produce an increase in fluorescence. Since calcium causes little or no increase in fluorescence, though there is some binding to the ligand, an increase in fluorescence indicates that magnesium is being transported. In repeated experiments with epinephrine stimulation in the presence of calcium without magnesium, no increase in fluorescence resulted. Magnesium alone, without some calcium present did not lead to any epinephrine dependent fluorescence increase. It was demonstrated that there was no increase in nonspecific fluorescence in plasma membrane vesicles prepared without 8-OH-Q during epinephrine stimulation even if ATP, calcium and magnesium were present.

Insulin caused no response in the unstimulated vesicles. After epinephrine stimulation caused increasing fluorescence for twenty to thirty minutes, insulin or oubaine caused cessation of the increase in fluorescence and in some experiments, a slight decrease.

A typical experiment is shown in Figure IV. The tracings show the excitation spectrum with emission at 490 nM before and after 20 minutes of epinephrine stimulation with ATP, calcium and magnesium in the presence of 8-OH-Q prepared vesicles.

The tracing is at sensitivity 4 with other settings the same as in Figure III so that a direct estimate of the increase in intravesicular magnesium may be made. The difference between the 360 nM and the 410 nM peak was 7% of full scale at sensitivity 4 before epinephrine and 15% afterward. Based on the scale in Figure III, this represents an increase of a little less than 1mM [Mg^{++}]. After 15 more minutes, the difference between the two peaks had increased 5% more before the increase in the 360 nM peak slowed and then stopped.

The net increase in fluorescence is 13% over the 30 minute period. This increase is equivalent to an intravesicular increase of 1.2 mM [Mg^{++}]. Since the concentration of protein in the plasma membrane vesicles in the cuvette is known to be 180 micrograms per ml., the plasma membranes accumulated magnesium at the rate of approximately 10 micro-

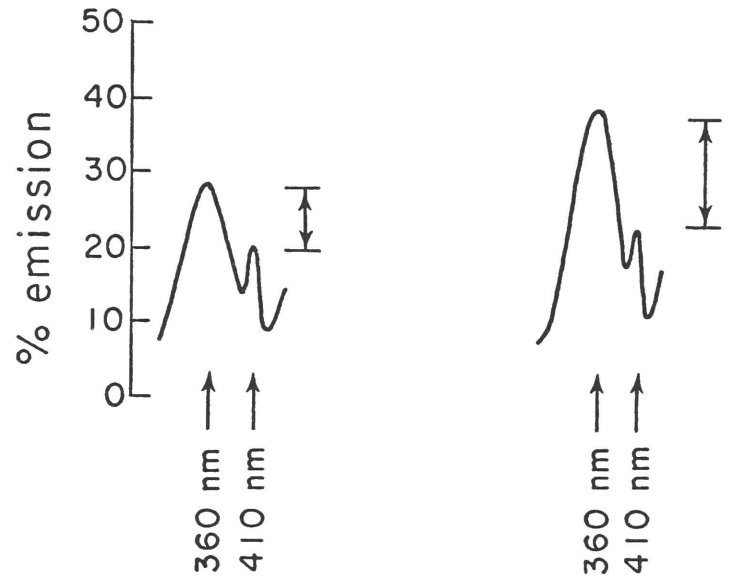


Figure IV. These two tracings show the excitation spectrum (300 to 410 nm with emission at 490 nm) in a preparation of plasma membranes containing 8-OH-Q, before and after exposure to 15 minutes of epinephrine at 2 μg/ml. The plasma membranes were suspended in a modified Krebs-Ringer triethanolamine hydrochloride buffer 0.05M, pH 7.4 with 1.0 mM [Mg^{++}] and 2.5 mM [Ca^{++}]. ATP was 1 X 10^{-4}M and protein concentration of the plasma membranes was 180 μg per ml. The abscissa is the excitation spectrum for each tracing in nanometers. The ordinate is the percent fluorescence intensity at sensitivity 4 with the excitation and emission slits each 10 nm. There is a net increase of 7% emission due to epinephrine after 15 minutes. This is equivalent to an intravesicular increase of 0.7 mM [Mg^{++}] (see Figure III).

moles magnesium/gram protein/30 minutes. In the calculation, the volume of the intravesicular space was assumed to be 1.5 microliters based on the pellet size of 2 X 2 X 0.4 mm and assuming all but 5% of the volume is intravesicular space.

Figure V shows the effect of ACTH stimulation on the fluorescence of plasma membranes containing 8-OH-Q. The response is slower initially than with epinephrine. Ouabaine (1 X 10^{-9}M) halted the increase in fluorescence in the plasma membrane preparation stimulated by epinephrine or ACTH.

Table II lists the average responses for the various hormones and ouabaine in terms of percent change at sensitivity four and its equivalent apparent intravesicular change in magnesium concentration. A calculation of the rate of uptake per gram of protein is made for two of the groups. The figures are averages from three typical experiments in each group.

Conclusions

A fluorescent ligand responsive to magnesium can be incorporated into the intravesicular fluid of plasma membrane vesicles. Under lipolytic hormone stimulation (epinephrine and ACTH) there is an increase in fluorescence indicating an increase in intravesicular magnesium. These hormones do not affect fluorescence in the absence of magnesium. This process requires ATP and is blocked by insulin and ouabaine. In a typical experiment with epinephrine stimulation, the apparent net increase in intravesicular magnesium concentration during the first 30 minutes stimulation was 1.2 mM over this time period. It was estimated that the net magnesium transport was 10.2 micromoles magnesium per gram of protein.

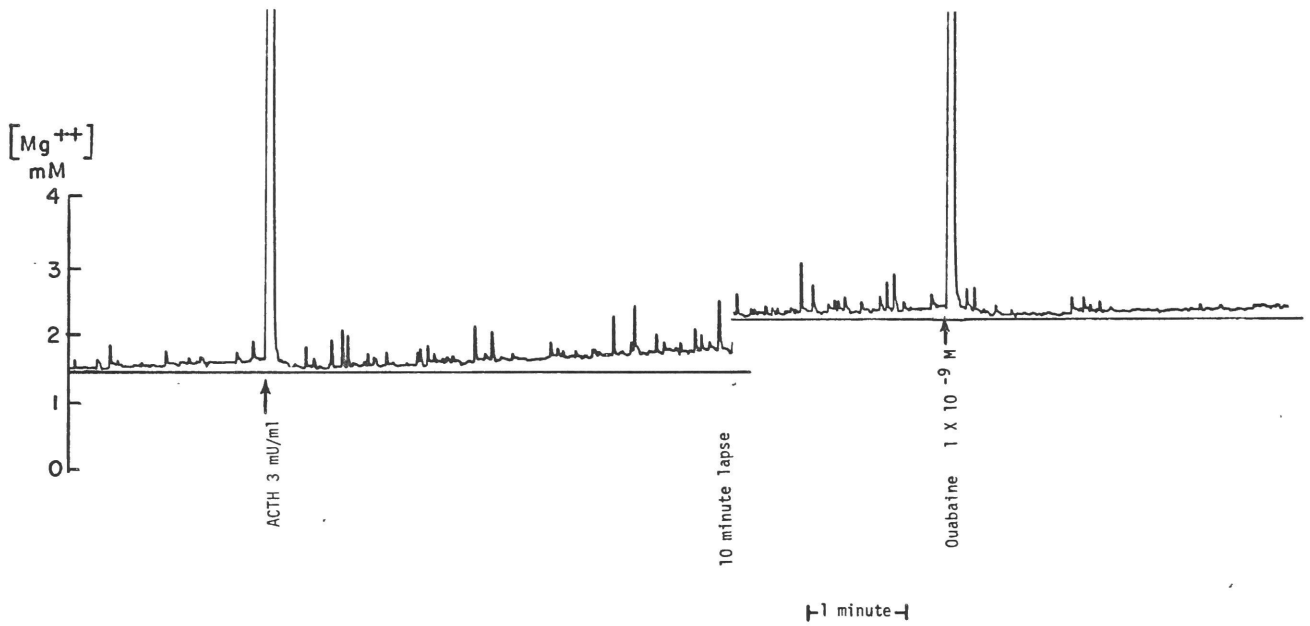


Figure V. The ACTH stimulated increase in fluorescence in 8-OH-Q loaded plasma membrane vesicles from fat cells. The increase is less rapid than with epinephrine. Ouabain blocks the fluorescence increase. Conditions are the same as in Figure IV except that the plasma membrane protein concentration is 120 μg/ml. Abscissa is time in minutes. The ordinate is the intravesicular [Mg^{++}] derived from the percent change in fluorescence (see Figure III). Excitation is at 360 nm and emissions at 490 nm. It was demonstrated that the excitation at 410 nm did not change after exposure to ACTH. The spike waves in the tracing are caused by the stirring bar entering the light path.

	% Change	$[Mg^{++}]$	Micromoles Magnesium per Gram Protein during First 30 Minutes
Epinephrine	13%	1.2 mM	10
ACTH	8%	.7 mM	10.6
Insulin	0		
Ouabaine	0		

Table II. The typical responses for various hormones and ouabaine in percent change and apparent intravesicular $[Mg^{++}]$. The typical experiments taken for epinephrine were at a concentration of 180 μg protein per ml and for ACTH at 120 μg protein per ml.

B. Direct Measurement of Magnesium

Using a complexometric titration method, Martin et al (1962) found they could not detect magnesium in adipose tissue. They concluded this was most likely due to a magnesium level below the limits of detection. We have found no other report of measurement of magnesium level or magnesium uptake in adipose tissue.

It is unreasonable to expect that there is no magnesium in fat tissue since the fat cell cytoplasm contains many of the enzymes found in other cells, some of which are known to be dependent on magnesium. Since the cytoplasm does comprise so little of the total cell mass (estimates range from 2.5 to 10% depending on the age of the rat and the size of the triglyceride droplet), it is understandable why it has been difficult to measure the magnesium. We assume the magnesium in the fat droplet is negligible.

Methods for Direct Measurement of Magnesium

Epididymal fat pads were obtained from Sprague Dawley strain of rats weighing 150-220 grams and five to six weeks old. The rats were anesthetized with Nembutal, (5mg/100 grams of body weight) with hexamethonium bromide (5mg/100 grams of body weight) added to block the release of epinephrine during agonal stress. After sedation, the chest cavity was opened and the heart incised so as to cause exsanguination. The epididymal fat pads were removed and placed in one milliliter of Krebs-Ringer bicarbonate buffer pH 7.4 maintained at 37^{o}C with one percent fatty-acid-free bovine serum albumin prepared by treatment with charcoal Chen (1967).

Isolated fat cells were prepared by a modification of the method of Rodbell (1965). One thousand units of purified collagenase (Worthington) were added to the fat pads in one ml of buffer in a polyethylene vial. Fat pads from eight to sixteen rats were pooled and prepared together. The plastic vial containing the fat pads and the collagenase was shaken for twenty minutes at two-hundred strokes per minute in an incubator maintained at 37^{o}C. The vial was then removed

and shaken vigorously by hand for about thirty seconds. This caused disaggregation of the fat pads and produced a smooth suspension of fat cells. Five milliliters of Krebs-Ringer buffer was then added and the shaking continued in the incubator for ten minutes more. The cell suspension was then filtered through course nylon stocking mesh to remove any remaining intact tissue elements.

The cell suspension was transferred to a water-jacketed plastic centrifuge tube equilibrated to a temperature of 37°C. This tube was centrifuged in an International Clinical Centrifuge at 3000 X g for one minute. The fat cells floated to the top of the heavier buffer. The debris from the interstitial matrix, fibrocytes, and red cells formed a sediment. The infranatant and the sediment were removed from under the fat cake with a plastic tipped syringe. The fat cells were washed twice more by suspension and centrifugation in the buffer to be used in the final suspension.

Both intact fat pads and isolated fat cells were analyzed after incubations with hormones. Fat pads were removed from the buffer, blotted dry and placed in tared weighing vessels for analysis. Samples of fat cells were placed in a narrow centrifuge tube and centrifuged at 3000 X g for 5 minutes. The infranatant was removed and stored for analysis while the cells were removed with a spatula and placed in tared weighing vessels for analysis.

It was possible in the case of whole fat pads to obtain a good measurement of the wet weight of the tissue. The magnesium in fat pads was assayed with greater reliability than isolated cells, but the presence of blood and other connective tissue elements raised the magnesium levels, so that the values were slightly higher than in the cells.

Fat was extracted from dried tissue with petroleum ether (30-80°C boiling range). The tissue was dry ashed in a muffle furnace for two hours at 500°C in platinum crucibles. Ashing for a longer period of time did not improve detection. The ash was dissolved in one drop of concentration HCl and then taken up with distilled deionized water into a volumetric flask and diluted so that the concentration was equivalent

to 5 mg of fat free dried tissue per milliliter. Aliquots of these solutions were used to measure calcium and magnesium by atomic absorption. Strontinum chloride 1.6% was used to further dilute specimens used for calcium determinations to decrease interference by anions. Magnesium and calcium were measured in a Jarrell Ash model 700 atomic absorption spectrophotometer.

Serum or extracellular fluid was diluted 12.5 fold with 1.6% strontinum chloride for magnesium or calcium determinations.

The amount of cells was determined by measuring the packed cell volume using samples taken in hematocrit tubes. This gave a measure of how much cell lysis and fat release was occurring, since the fat outside the cell sedimented differently than the fat cells themselves.

Protein was determined by the method of Lowry. This was correlated with a fluorescent peak emission at 310 nM with excitation at 285 nM. Measurement of the protein in this manner served very well when it was compared with appropriate standards. The fluorescence depends on the phenylalanine, tryptophan and tryosine amino acid content.

Results of Magnesium Determinations

Magnesium concentrations were determined in fat tissue and isolated fat cells using the above methods.

In addition fat cells were incubated with and without epinephrine and the magnesium was measured in the tissues and the incubation fluid.

Table III shows the results of the determination of magnesium in fat pads. By comparison, measurement of human muscle magnesium by these methods is 3.5 - .3 millimoles per 100 grams of fat free dry solids (FFDS) Elliott and Cheek (1968). Rat muscle tissue is slightly higher than human magnesium concentration and higher also than the magnesium concentration of fat pads.

In order to gain some idea of the magnitude and direction of change in cellular magnesium resulting from epinephrine stimulation of fat cells, experiments were done to determine the change in extracellular magnesium when fat cells were incubated with and without hormone.

mg per gram FFDS	.745 ± .080
mmoles per 100 grams FFDS	3.07 ± .37
mmoles per kg Fat Free Fresh	6.14 ± .73
micromoles per gram protein	41.0 ± 5.2

Table III. Magnesium concentration in rat epididymal fat pads. Eight fat pads from four different animals were used for the determination. Fat free dry weight was 18.5% of fat free fresh weight. This was calculated from data derived from weights of aliquots before and after drying and fat extraction. Protein was 15% of fat free fresh weight.

The incubation media was separated from sixteen paired aliquots of an isolated fat cell suspension incubated for thirty minutes with and without epinephrine. Changes in calcium and magnesium concentration in the extracellular fluid were measured. The results are shown in Table IV.

Epinephrine stimulates a significant decrease of 2.7% in the physiologic extracellular concentration of magnesium. There is a small decrease in the calcium level which is not significant.

At this concentration of magnesium, the method is near the limits of detection where the method for serum has a reliability of 2.5%, based on the determination of replicate samples.

With the large number of paired samples we have some confidence, however, that there is a significant decrease in extracellular magnesium uptake in the 20% cell concentration. If we assume that the cytoplasmic volume is 7% (estimates vary from 2.5% to 10%), then we find that there has been an apparent increase in intracellular magnesium concentration of 2.3 millimoles per liter of intracellular water.

With a concentration of protein in fresh cells of .018 grams per gram of fresh weight, we calculate a net influx rate of 8.9 micromoles per gram of protein during the 30 minute incubation.

Another approach was to use this indirect estimate with lower concentrations of magnesium in the media. A series of four experiments was done using 0.3 M magnesium, 20% of physiological concentration and measuring the change in concentration under hormone stimulation. One experiment is shown in Table V. Even with lower magnesium in the extracellular fluid, there is still an uptake of magnesium. The results are comparable to the indirect results estimated from the decrease in extracellular fluid magnesium. Determinations were done in duplicate and although the number of samples was not large enough for statistical comparison, there was no overlap in the two determinations from one group with any other group.

	Calcium	Magnesium
Control level	3.620 mM	1.46 mM
average decrease of epinephrine sample compared to control	- .014 mM	- .04 mM
T	-0.7	-2.74
15 df	$p<0.5$	$p< .02$
percent change due to epinephrine	0.48	2.78

Table IV. Comparison of the effect of epinephrine on extracellular fluid from 16 paired samples of a suspension of fat cells. The small decrease in calcium concentration is not significant but the decrease in magnesium concentration is significant. A statistical test of paired samples was used to determine significance from Mellits (1968) using the following formulae:

Y= Value of epinephrine treated extracellular fluid and X= control extracellular fluid D=Y-X

$$S_D = \sqrt{\frac{\sum_{i=i}^{n} D_1^2 - \frac{\left(\sum_{1=1}^{n} D_1\right)^2}{n}}{n-1}} \qquad T = \frac{\bar{D}}{S_D / \sqrt{n}}$$

	Change in $[Mg^{++}]$ in ECF in mM	Estimated $[Mg^{++}]$ in ICF in mM	Micromoles Mg^{++} Transported per Gram Protein
Epinephrine	-0.014	+ 2.65	8.2
Insulin	- .005	+ 0.95	2.8

Table V. The effect of epinephrine and insulin stimulation of fat cells with one fifth physiological concentration of magnesium (0.30mM) during a 30 minute incubation. The glucose concentration was 2 mg per ml, calcium 2.5 mM, and albumin 3%. The remaining electrolytes were the same as in Krebs-Ringer Bicarbonate buffer. The calculated change in the ICF (intracellular fluid) space was based on the assumption that the ICF is 7% of total cell volume. Micromoles magnesium transferred per gram of protein was based on a protein concentration of 0.018 grams per gram of fresh weight. Concentration of cell suspension was 7%, epinephrine 1 μg per ml, and insulin 50 μUnits per ml.

Table VI shows the magnesium concentration in fat cells measured directly following incubation for thirty minutes with and without ephinephrine in two different concentrations of magnesium. The cells were isolated according to the methods described and divided into several aliquots after being washed three times in the buffer to be used with the high or low magnesium concentration. One-half of the samples were treated with epinephrine and the remainder contained no hormone during the thirty minute incubation. After the incubation, the cells in each aliquot were isolated by centrifugation, weighed, dried and fat extracted. An aliquot of the fat free dried tissue was analyzed for protein. The remainder was ashed and analyzed for magnesium with atomic absorption spectroscopy.

This direct analysis of magnesium concentration in the isolated fat cell showed there to be a significant increase in magnesium concentration due to epinephrine in buffer containing normal magnesium. This was equivalent to 12.4 micromoles of magnesium per gram of protein. There were not enough samples in the low magnesium buffer for statistical comparison.

Conclusions

Indirect estimation of the changes in the intracellular magnesium by measurement of the extracellular fluid and the total fat cell magnesium indicates that epinephrine causes an increase in the fat cell magnesium concentration, presumably in the intracellular fluid, of from 2 to 3 mM during a 30 minute incubation. A net uptake in magnesium occurs which varies from 7 to 12 micromoles per gram of protein during the first thirty minutes of incubation.

Magnesium Concentration

Micrograms per mg FFDW	Millimoles per kg FFFW	μg per Gram Protein, Uptake
Extracellular fluid magnesium .30 mM		
Epinephrine 2μg/ml .430	3.55	
No Hormone .289	2.38	
+ .141	+ 1.17	Δ 7.6
Extracellular fluid magnesium 1.5 mM		
Epinephrine 2 μg/ml .853 ± .049	7.08 ± .44	
No Hormone .633 ± .073	5.17 ± .60	
.232 ± .043	1.91 ± .36	Δ 12.4 ± 2.3

Table VI. Direct measurement of fat cell magnesium after fat extraction. Fat free dry weight (FFDW) was 18.5% of fat free fresh weight (FFFW). An estimate of the net increase of intracellular fluid magnesium concentration was made. Intracellular fluid volume was assumed to be 60% of FFFW. With 0.30 mM magnesium, there was a net increase of 1.92 mM Mg per liter of cell water and with 1.5 mM magnesium 3.18 mM per liter of cell water estimated. Protein was 15% of FFFW.

C. Experiments with Magnesium 28

There have been no studies reported of magnesium 28 uptake in fat cells. The literature concerned with magnesium transport using $^{28}Mg^{++}$ uptake in other cell types is of some interest.

The most recent study is probably the most relevant. Wallach et al (1970) studied cellular transport of magnesium in rat liver using $^{28}Mg^{++}$. Liver slices were studied in a closed compartment system under steady state conditions. The influx and efflux coefficients governing transport between the extracellular phase and a rapidly exchanging cell fraction were determined and found to be 0.074 and 0.019 per minute, respectively. A ten fold increase in the extracellular concentration of Mg^{++} caused a 31% decrease in the influx transfer coefficient and a 30% increase in the efflux transfer coefficient. A five fold decrease in the magnesium concentration to 0.5 mM resulted in a 33% increase in the influx coefficient and a 16% decrease in the efflux coefficient. The alterations in the concentration of magnesium in the medium were of greater magnitude than the resultant changes in the transfer coefficients. The mean concentrations of the slices increased by 19.8 millimoles per kilogram in the 25 mM Mg^{++} and decreased by 3.8 millimoles per kilogram in the 0.5 mM Mg^{++}. At $0^{o}C$, both transfer coefficients were reduced by 65%. Increased pH and NaCN increased transport, whereas Ca^{++} reduced transport in both directions by 16% and decreased the concentration of the tissue slice by 28%. Reduced pH, altered Na^{+} to K^{+} ratio, Sr^{++}, glucose deletion, iodoacetate, ethanol and lactate had no significant influence on the transport coefficients.

The effects of the various hormones were not studied in this paper, but unpublished results cited were of interest. Thyroxine treatment of the rat has been found to stimulate both calcium and magnesium transport in liver slices. Parathyroid hormone was found to stimulate calcium but not magnesium transport in liver slices.

Another recent paper by Sparrow (1969) reported the interaction of $^{28}Mg^{++}$ with Ca^{++} and K^{+} in the smooth muscle of the Guinea Pig taenia coli. It was found that the Mg^{++} content rose to 15.4 millimoles per

kilogram fresh weight from an initial 6.56 mMoles/Kg. fresh weight during immersion in isotonic sucrose containing only $MgCl_2$. This effect was independent of the Mg^{++} concentration in this first solution but was depressed when K^{+} or Ca^{++} was added.

Magnesium uptake showed three separate phases, extracellular, intermediate and slow. The size of the extracellular phase was proportional to the Mg^{++} concentration in the solution but the size of the slow phase was constant. The size of the intermediate phase, exchanging with a half time of a few minutes, was depressed when K^{+} or Ca^{++} Ions were added.

Methods

Magnesium 28 was obtained from Brookhaven National Laboratory, immediately prior to use, on days when it was produced. Experiments could be run for up to three days after it was received before radioactivity decreased below acceptable levels. The half life is 21.3 hours.

It is possible to count magnesium 28 in either a gamma or beta scintillation counter. In each case, the windows must be carefully set to exclude radiation from aluminum 28, a daughter product, and ^{3}H a contaminant of the production procedure. With due care, counts from these contaminants can be eliminated by proper selection of the energy window.

One hundred microcuries of $^{28}Mg^{++}$ was received in three ml of solution containing 0.2 mg magnesium as the chloride. One tenth of a microcurie was used in replicate experiments with either whole cells or plasma membranes. The uptake of the isotope was between one to four percent of the total counts in the incubation media depending on the conditions used. This gave counts in the range of 50 to 2000 counts per minute in each sample depending upon the conditions used, if the gamma emission was counted. Twenty five percent of the counts were estimated to be due to exchange with magnesium in the cell or plasma membrane surface.

In experiments using whole cells, the incubation mixture was in most cases Krebs-Ringer bicarbonate buffer equilibrated with 5% CO_2 in air at

pH 7.4. Except as indicated, [Ca^{++}] was 2.5 mM and [Mg^{++}] 1.25, Glucose 2 mg per ml and albumin.

In experiments with plasma membranes, the incubation medium was 0.05 M triethanolamine - HCl buffer, pH 7.4. Unless otherwise specified, calcium was 1.25 and magnesium 9.6 mM. No albumin was added.

In experiments with whole cells, aliquots of cells were placed in plastic scintillation vials and incubated with shaking. At the end of the incubation period, the vials were immediately chilled in ice water and the cells filtered through a .45 micron millipore filter, which had been washed with cold buffer containing magnesium at a concentration slightly above the concentration in the incubation medium. The cells were washed three times with ice cold buffer containing magnesium at the same concentration as that used in the experiment. Adding one percent albumin did not lower the background. Washing is more efficient if completed while the cells are chilled, because at 37°C or room temperature the cells are quite plastic and tend to be sucked into or over the filter pores. This slows the washing and may cause cell disruption.

Plasma membranes were incubated with periodic agitation in cellulose nitrate tubes in a temperature block at 37°C. Plasma membranes were collected on 0.3 micron millipore filters. Washing was the same as for whole cells, but the plasma membranes were usually not chilled first. Plasma membranes were isolated as shown in Figure II.

Results of Experiments with $^{28}Mg^{++}$

Figure VI shows the time course for magnesium 28 accumulation in plasma membranes isolated and prepared without 8-OH-Q. There is an almost instantaneous binding or exchange of $^{28}Mg^{++}$ to the vesicle surface, which is equivalent to 17 micromoles of magnesium per gram of protein. After the first fifteen minutes, there is a total accumulation of 23 micromoles Mg^{++} per gram of protein. The net increase in the first fifteen minutes after instantaneous binding is 6 micromoles of magnesium per gram of protein.

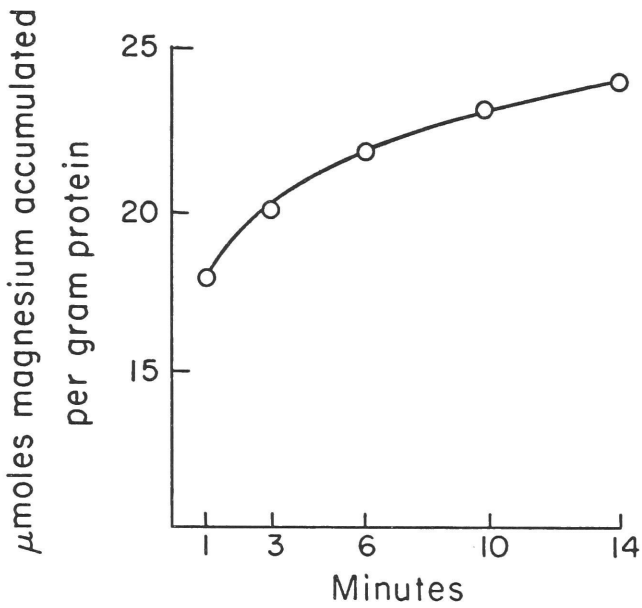


Figure VI. The time course for magnesium 28 accumulation by plasma membranes. Epinephrine concentration was 1 μg/ml, ATP 1 X 10^{-4}M and protein 40 μg/ml. Incubation was in TEA buffer with 2.5 mM calcium and 0.6 mM magnesium.

Figure VII shows the effects of variations in epinephrine or ATP concentration on the uptake of magnesium 28 by plasma membrane vesicles during a twenty minute incubation. Protein concentration was 80 micrograms per ml. The incubation medium was 0.05M TEA-HCl buffer, pH 7.4. Calcium concentration was 1.25 mM and magnesium 0.6 mM.

These studies demonstrate that even with no epinephrine, there is some binding of magnesium to the plasma membranes and even some slight accumulation. At 2.2×10^{-4} M ATP concentration, there is active accumulation of magnesium if epinephrine is present. At higher concentrations of ATP, uptake is inhibited. This may represent competition by the ATP for magnesium with the plasma membrane surface.

Table VII compares the uptake by plasma membranes of magnesium under varying conditions. It is shown that although cyclic 3'5' AMP and insulin cause some uptake above baseline binding, there is not the accumulation seen with ACTH and epinephrine. This suggests that cyclic 3'5' AMP is not the mediator of the magnesium uptake by the plasma membranes.

Figure VIII shows the influence of epinephrine (1 μg/ml) and insulin (500 μU/ml) on a time course for uptake of magnesium 28 by intact fat cells. Epinephrine causes a greater accumulation of magnesium 28 than the control while insulin causes a diminished accumulation.

Figure IX shows that magnesium 28 uptake is dependent on the presence of calcium. With epinephrine in the medium, magnesium 28 is again shown to accumulate at a greater rate than in controls.

Figure X demonstrates the increased retention of previously accumulated magnesium 28 when both epinephrine and calcium is present in the media. This may contribute to the net increase in fat cell magnesium during epinephrine stimulation.

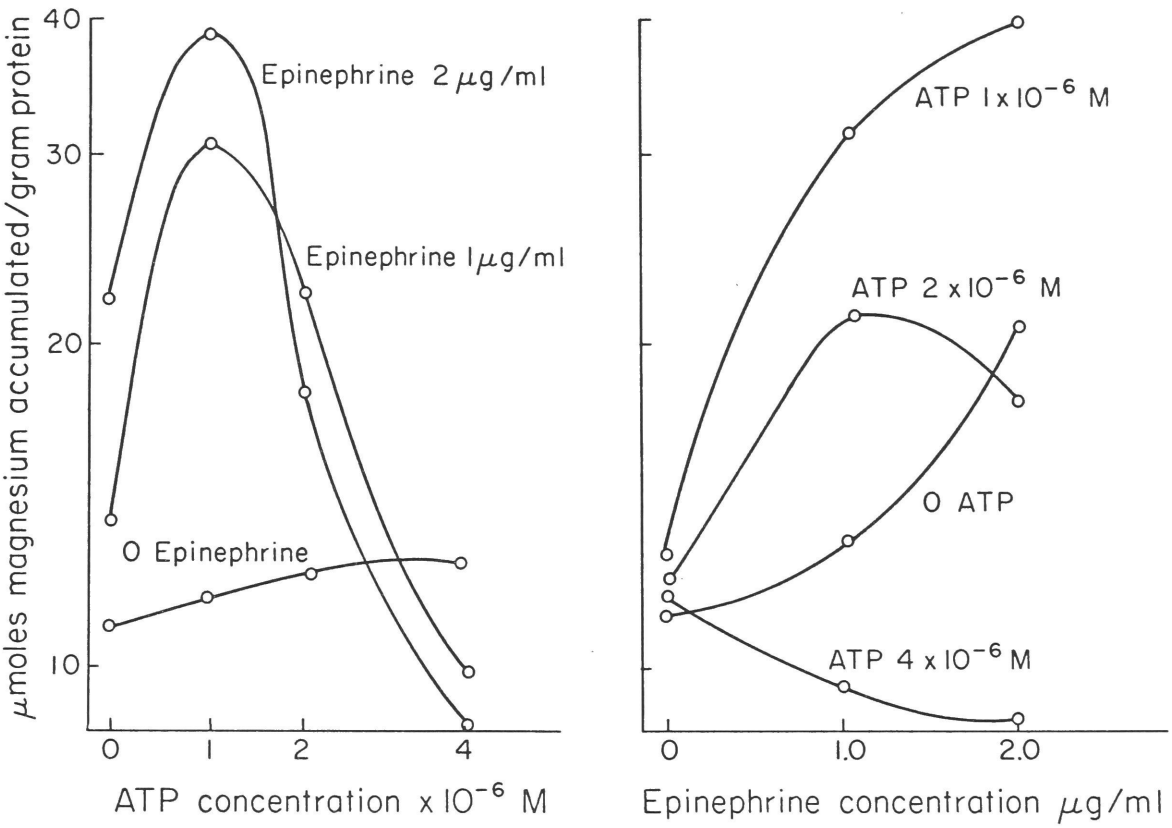


Figure VII. The effect of ATP on magnesium 28 uptake in plasma membranes at varying concentrations of epinephrine (left) and the effect of epinephrine on magnesium 28 uptake in plasma membranes at varying concentrations at ATP (right). Conditions are described in the text. Both are plotted from the same data.

Hormone	-	-	ACTH	EPI	Cyclic	Ins
ATP $2X10^{-4}M$	-	+	+	+	+	+
Plasma Membrane $[Mg^{++}]$ Micromoles/gm/protein	15.6	12.9	37.5	43	22	22

Table VII. Comparison of the uptake of magnesium 28 in plasma membranes. Incubations were for twenty minutes. ACTH concentration was 6 mU/ml, Epinephrine 2 μg/ml cyclic 3'5' AMP $1X\ 10^{-5}$ M and insulin 500 μU/ml, Incubations were in 0.05 M TEA-HCL buffer, pH 7.4 with 1.25 M Ca^{++}, 0.6 mM Mg^{++} and 80 μg/ml protein.

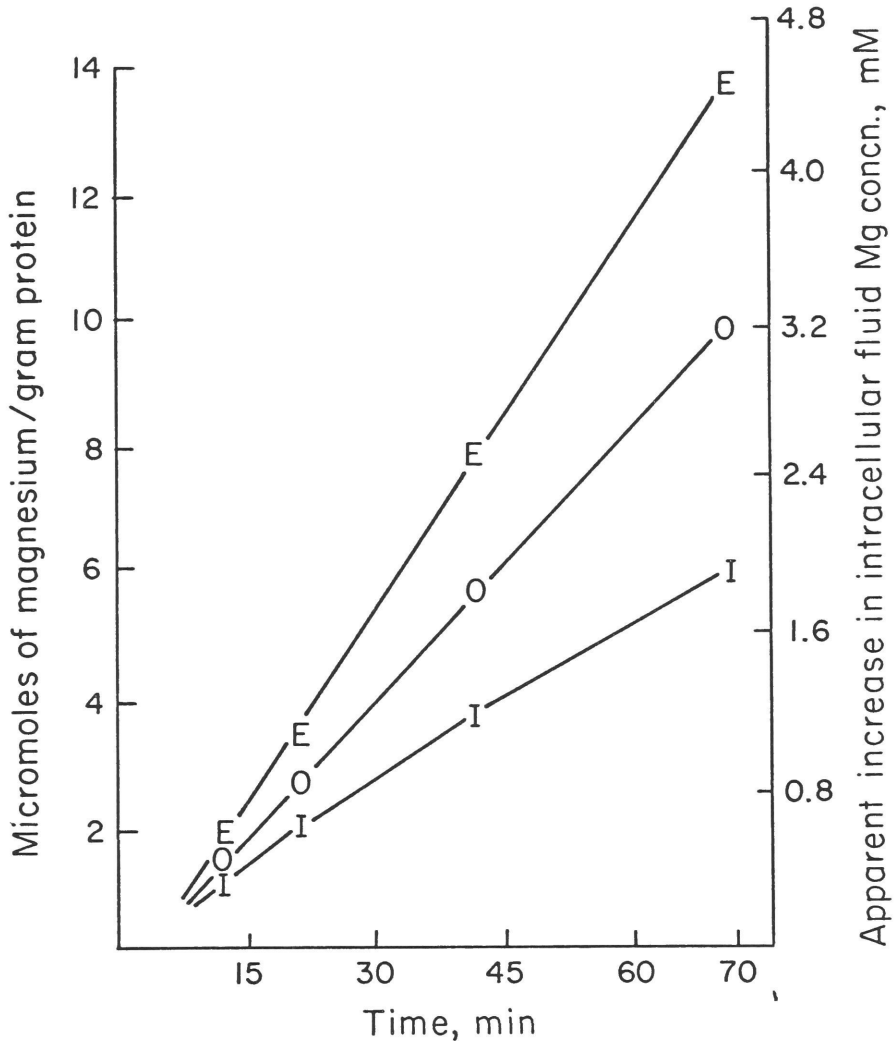


Figure VIII. Magnesium 28 uptake by isolated fat cells incubated with epinephrine (E), insulin (I) and control containing no hormone (O). Incubation was carried out in Krebs-Ringer bicarbonate buffer containing 1 mg/ml glucose, 3% bovine serum albumin, 2.5 mM calcium and 1.25 mM magnesium.

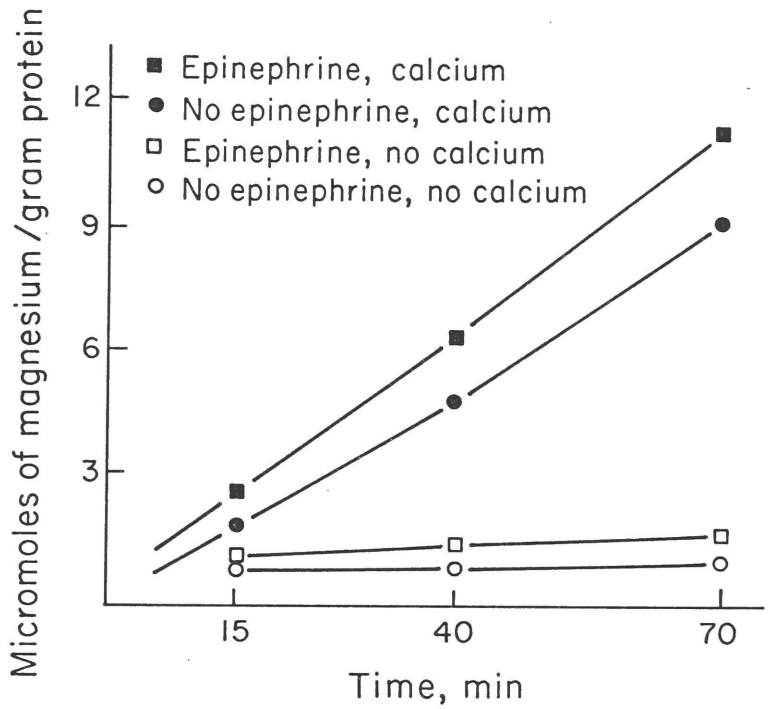


Figure IX. Magnesium 28 uptake by isolated fat cells incubated with and without epinephrine in the presence and absence of calcium. Calcium concentration was 2.5 mM and epinephrine 2 μg/ml. Remaining buffer conditions as in VIII.

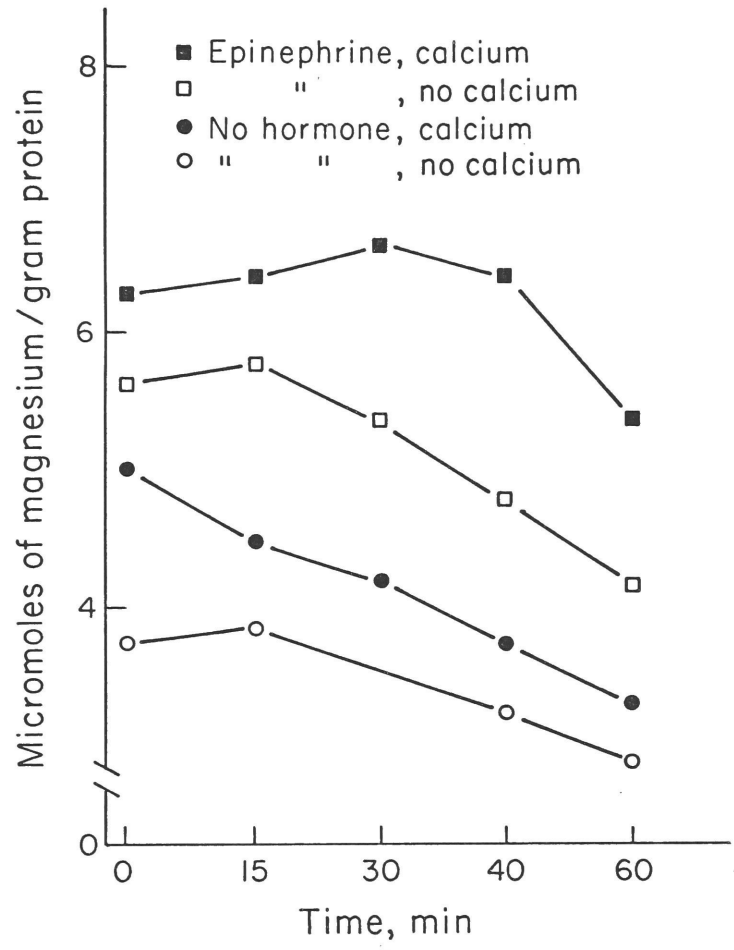


Figure X. The effect of epinephrine on magnesium 28 retention by fat cells previously loaded with the isotope. Each point is the average of two determinations. The cells were loaded with ^{28}Mg for thirty minutes with no hormone present, washed and divided into equal aliquots. Buffer contained .62 mM magnesium and 2 mg/ml glucose. When present, epinephrine concentration was 2 μg/ml and calcium 1.25 mM.

Conclusions

The magnesium 28 retention by plasma membrane vesicles and intact fat cells is stimulated by epinephrine and potentiated by the presence of calcium. Part of this retention may be due to the decreased efflux of magnesium seen in the presence of calcium and epinephrine.

D. The Potentiation of the Metabolic Effect of Epinephrine by Magnesium

Besides stimulating the release of fatty acids, epinephrine decreases fatty acid synthesis and increases the synthesis of glyceride glycerol.

Landau et al (1964) demonstrated this effect by measuring the distribution of the glucose taken up during epinephrine and insulin stimulation. These results are shown in Table VIII. With epinephrine stimulation, forty percent of the total glucose ^{14}C goes into glyceride glycerol and only seven percent into fatty acid. With insulin, on the other hand, fatty acid accounts for thirty six percent and glyceride glycerol for eleven percent. In controls with no hormones, the distribution is about equal between fatty acid and glyceride glycerol.

In view of our findings on the effect of epinephrine on magnesium accumulation in fat cells, we have done experiments to determine whether the presence of magnesium in the incubation medium has an effect on the metabolism of glucose carbon into triglyceride fatty acids or glyceride glycerol.

Methods

Lipid from fat cells incubated with $U^{14}C$ glucose was extracted by the method described by Dole (1965). Five ml of an extraction mixture consisting by volume of 40 parts of isopropyl alcohol, 10 parts heptane and 1 part of 1 N H_2SO_4 was added to a fat cell suspension after incubation. The mixture was agitated 4 times in 20 minutes with a Vortex Jr. Mixer. Four ml of heptane and three ml of distilled water were added to the mixture which was then agitated once again and centrifuged at high speed in an International Clinical centrifuge for five minutes,

	Control	Epinephrine	Insulin
Total	100	250	240
CO_2	29	30	34
Glyceride Glycerol	20	40	11
Fatty Acid	21	7	36
Lactate	30	23	19

Table VIII. Percent distribution of counts from uniformly labeled C_{14} glucose into various endproducts in fat cells under hormone stimulation, from Landau et al, (1965)

separating the mixture into an aqueous and a heptane phase. The upper, heptane phase was transferred to a glass centrifuge tube. Three ml of 0.16% NaCl was added and the tube centrifuged at high speed in the International Clinical Centrifuge to separate the phases. An aliquot of the washed heptane phase was transferred to a tared glass scintillation vial. The heptane was evaporated by a stream of warm air from a hair dryer. The vial was reweighed and the lipid weight calculated. Eighteen ml of the scintillation fluid was added to the vial and the total lipid ^{14}C counts were obtained.

^{14}C incorporated into adipose cell triglyceride fatty acids from U^{14}C glucose were measured as follows. An aliquot of washed heptane phase containing the total lipid extract was transferred to a test tube. The heptane was evaporated by simultaneously warming the tube and exposing its contents to a stream of nitrogen, leaving a total lipid residue. To this residue, two ml of a saponification mixture containing 0.5 ml saturated KOH in 50 ml 95% ethanol was added, the contents mixed and incubated at 60^{o}C overnight. Two ml of water and one drop of Brom Cresol Green (Water soluble, Amend Drug and Chemical Co.) in 100 ml of 20% ethanol were added to the mixture thus saponified and 1.0 HCl added to a blue yellow end point. The fatty acids liberated were extracted into 3 ml of heptane, and an aliquot of the heptane transferred to a glass scintillation vial. The heptane was evaporated, scintillation fluid added and triglyceride fatty acid ^{14}C counts obtained. A similar method for the analysis of ^{14}C triglyceride fatty acid production by isolated adipose cells has been described by Rodbell (1965).

^{14}C counts incorporated into adipose cell triglyceride glycerol from U^{14}C glucose were taken to be the difference between total lipid and triglyceride fatty acid ^{14}C counts. (Rodbell, 1965)

Results were calculated as follows. Fatty acids were expressed as micromoles of glucose carbon incorporated per mM glyceride glycerol or mM fatty acid. The results were also expressed as percent of glyceride glycerol counts in total triglyceride.

For the experiment described, each data point was the average of

duplicate determinations.

Results

Table IX shows the effect of magnesium in the extracellular fluid on glucose incorporation into fatty acids in the presence and absence of epinephrine. Magnesium decreases glucose incorporation into fatty acid whether epinephrine is present or not, but only the decrease in the presence of epinephrine is significant ($p < .01$). Viewed in another way, epinephrine causes a significant decrease in fatty acid synthesis whether or not magnesium is present. The decrease is greater with magnesium present, however.

Table X shows the effect of magnesium on uniformly labeled ^{14}C glucose incorporation into glyceride glycerol. Here, in the absence of hormone, magnesium caused a significant increase ($p < .01$) in glucose incorporation into glyceride glycerol. When epinephrine was added, magnesium caused no significant difference in the glyceride glycerol glucose incorporation. Of importance, however, is the observation that if glyceride glycerol incorporation is expressed as percent $U^{14}C$ glucose incorporation into triglyceride (Table XI), there is an increased percent incorporation when magnesium is present with ($p < .02$) and without ($p < .005$) epinephrine.

Conclusions

Though epinephrine causes a decrease in fatty acid synthesis in the absence of magnesium, the effect is markedly enhanced by the presence of magnesium in the incubation medium. Stimulation of glyceride glycerol production by epinephrine is not affected by extracellular magnesium. While magnesium stimulation of glyceride glycerol production in the absence of hormone is not significant when glyceride glycerol is viewed as a percent of ^{14}C glucose incorporated into triglyceride, magnesium causes a significant increase with or without hormone.

	Epinephrine 2 μg/ml	No Hormone	
No Magnesium present	2.97 ± .53	5.28 ± .58	t = 4.77 p < .01
Magnesium present	1.77 ± .34	4.24 ± .67	t = 6.46 p < .005
	t = 3.99 p < .02	t = 2.27 n.s.	

Table IX. Effect of magnesium on $U^{14}C$ glucose incorporation into fatty acid. Data in micromoles of glucose carbon per millimole fatty acid. Incubations were for thirty minutes with isolated fat cells in modified Krebs-Ringer Triethanolamine buffer, pH 7.4, albumin 3%, calcium 2.5 mM and magnesium, when present, 1.25 mM. There were five samples in each group. Data were calculated using paired t statistics. 5 pairs, 4 d.f.

	Epinephrine 2 μg/ml	No Hormone	
No magnesium present	14.75 ± 1.16	2.73 ± .23	t = 20.2 p < .001
Magnesium present	12.61 ± 1.75	3.84 ± .48	t = 13.01 p < .001
	t = 2.56 n.s.	t = 5.03 p < .01	

Table X. Effect of magnesium on $U^{14}C$ glucose incorporation into glyceride glycerol expressed in micromoles of glucose carbon per millimole of glyceride glycerol. Same conditions as in Table IX.

	Epinephrine	No Hormone	
Magnesium	87.74%	47.84%	t = 17.59
	± .80	± 4.82	p < .001
No Magnesium	83.30	34.10	t = 41.1
	± 1.94	± 1.60	p < .001
	t = 4.59	t = 6.29	
	p < .02	p < .005	

Table XI. $U^{14}C$ glucose incorporation into glyceride glycerol as a percent of total triglyceride. ^{14}C counts calculated from data from the same experiments as in Tables IX and X. Paired t statistics were used. 5 pairs 4 d.f.

III. Theoretical Considerations of the Effect of Magnesium on Cellular Metabolism

The metabolic patterns of the mammalian fat cell during hormone stimulation are well characterized. Insulin stimulates the uptake of glucose for use in the synthesis of fatty acid. Thus, in times of increased glucose supply, insulin stimulates storage in fat. Glycolysis via the hexose monophosphate shunt (HMP shunt) augments the pool of NADPH needed for fatty acid synthesis and yields CO_2 derived from the first carbon of glucose. The fatty acid synthesized is stored after conjugation with glycerophosphate as triglyceride. Glycerophosphate, the source of glyceride glycerol, is synthesized from glucose more slowly than fatty acid since almost all of the glucose is shunted directly into fatty acid synthesis. Thus, during insulin stimulation, the label from glucose may be found in the fatty acid moiety of the triglyceride predominantly and to a lesser extent in the glyceride glycerol.

In contrast to insulin, epinephrine and other lipolytic hormones such as ACTH and glucagon, stimulate the fat cell to release fatty acid and glycerol from its triglyceride fat stores. Epinephrine is released when there is a demand for increased energy substrates by the body and fat supplies most of these. The release of fatty acid is mediated by action of cyclic 3'5' AMP (C-AMP) on lipase. C-AMP production is stimulated by the action of the lipolytic hormone and adenyl cyclase, an enzyme present in fat cell plasma membranes. The lipolytic hormones also stimulate glucose uptake but instead of the glucose metabolites being directed into fatty acid synthesis, as in insulin stimulation, fatty acid synthesis is blocked almost entirely and the glucose metabolites are accumulated in the form of TCA cycle intermediates, lactate and glycerol phosphate. The glycerol phosphate is used to re-esterify fatty acids released from fat stores of triglyceride but not exported from the cell. The glycerol which is released during lipolysis is not used again since glycerol kinase is low or absent from the fat cell. Thus in epinephrine stimulation, glucose is converted

to the glyceride glycerol moiety of stored triglyceride or metabolised to CO_2 in the tricarboxylic acid (TCA) cycle. Since fatty acid synthesis is blocked during epinephrine stimulation, all fatty acid released is from triglycerides synthesized during insulin stimulation under conditions when glucose was plentiful.

These metabolic patterns are schematized in Figure XI from Jeanrenaud (1968). In summary, hormones influence the relative rates of synthesis of fatty acid and glyceride glycerol from glucose. Lipolytic hormones favor the production of glyceride glycerol. Insulin favors fatty acid synthesis. In addition to its role in lipase activation and fatty acid release, c-AMP may affect this balance between fatty acid synthesis and glyceride glycerol, but its known actions do not offer a complete explanation of the hormonal control of these processes.

To understand how hormones regulate the balance between fatty acid and glyceride glycerol production, we need to know which glycolytic and fatty acid pathway enzymes are stimulated or inhibited during hormonal stimulation. There is much evidence to suggest that the enzymes most important to this balance center around the metabolism of pyruvate as shown in Figure XII. Two enzymes, pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK) which together catalyze the synthesis of phosphoenol-pyruvate from pyruvate via oxaloacetate, may be involved in this control. (Reshef et al 1970, Ballard and Hanson 1967 and White et al 1968). In addition to their presence in mitochondria, these enzymes are found in fat cell cytoplasm as well. Jungas (1971) has suggested that pyruvate dehydrogenase may be a controlling enzyme influenced by cyclic AMP. This enzyme, however, is located only in the mitochondria.

Though c-AMP influences the balance between fatty acid and glyceride glycerol synthesis, there is no evidence that c-AMP directly activates or controls PC or PEPCK. C-AMP has been shown to have a direct effect only on the mammalian enzymes listed in Table XII. Three of these enzymes are concerned with the release of stored energy substrates such as triglycerides in the fat cell and glycogen in the liver, muscle and

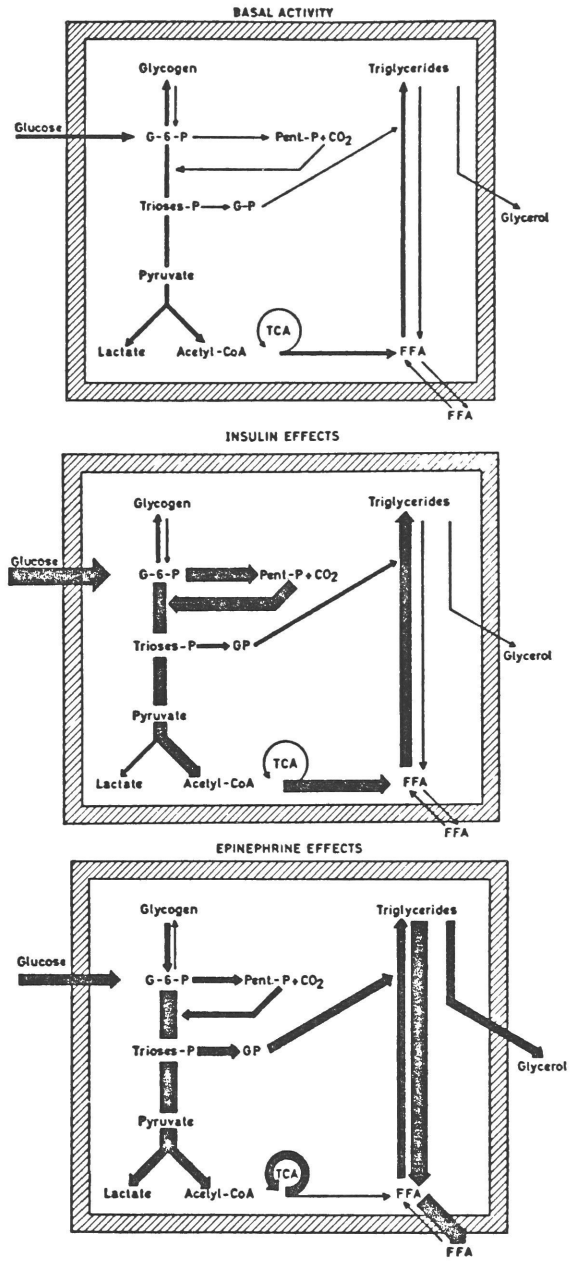


Figure XI. Diagrammatic representation of glucose metabolism in adipose tissue. Metabolic pattern induced by insulin or epinephrine. The thickness of the arrows schematizes the relative rates of metabolism. Abbreviations: FFA, free fatty acids; G-6-P, Glucose-6-phosphate; G-P, glycerophosphate; Pent.-P, pentosephosphate; TCA, Tricarboxylic acid cycle. From Jeanrenaud (1968)

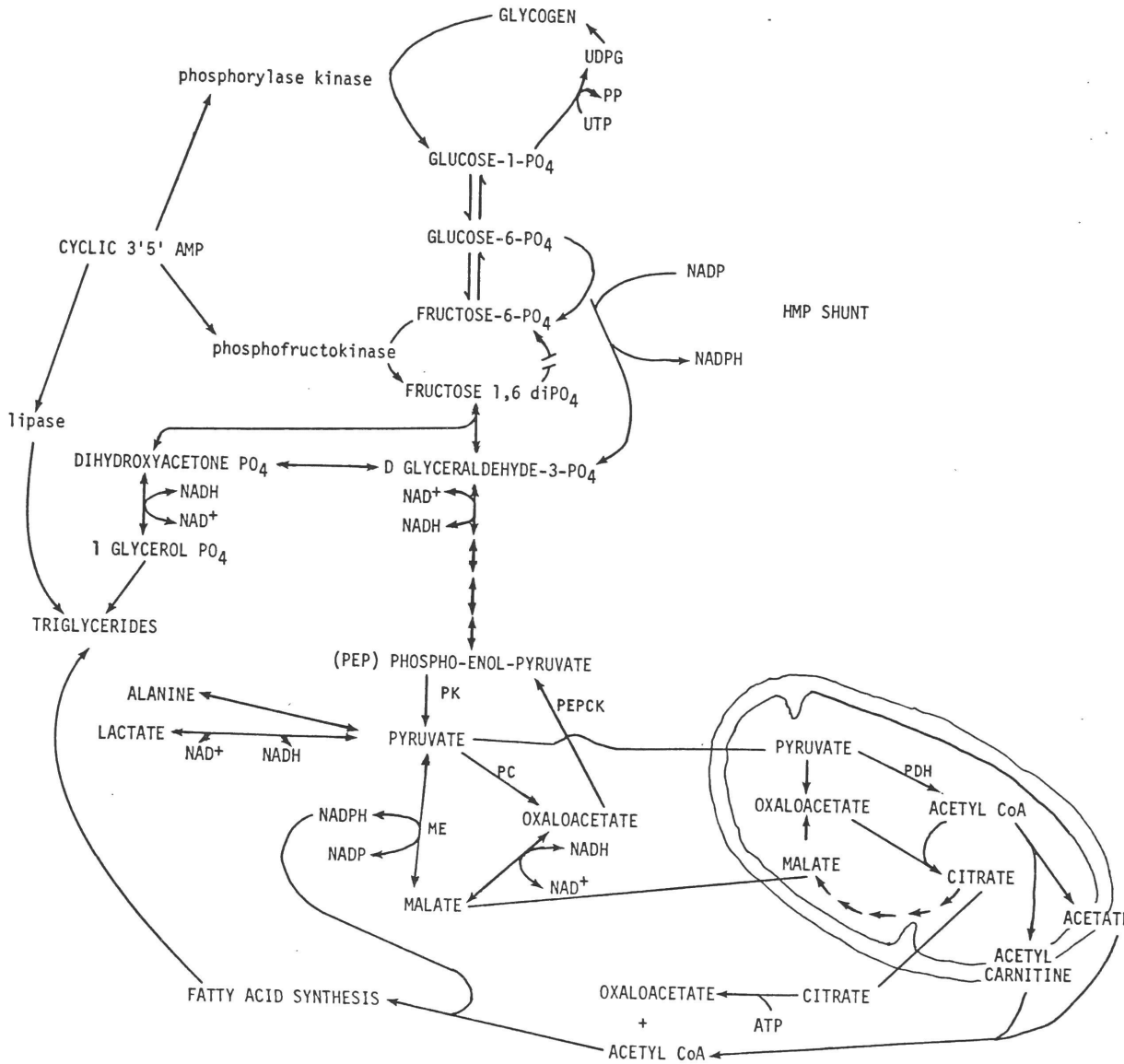


Figure XII. Metabolic pathways in the mammalian fat cell.

Enzyme	Action by c-AMP
Lipase, present in fat cell	stimulates
Phosphorylase kinase	stimulates
Glycogen synthetase	inhibits
Phosphofructokinase	stimulates
Fructose 1,6 di phosphatase not found in fat cell	inhibits

Table XII. Enzymes controlled by cyclic 3'5' AMP

fat cells. In addition, c-AMP has an effect on phosphofructokinase (PFK) and fructose 1,6 diphosphatase (FDP) which may exert control on the flow of carbohydrate into or out of the Embden-Mererhof-Parnas pathway. This may indirectly control the utilization of the hexose monophosphate shunt. (HMP shunt).

In E. coli, malic enzyme (ME) (Malate: NADP oxidoreductase, decarboxylating) has been shown to be inhibited by high (10^{-4}M) concentrations of c-AMP. There have been no studies on malic enzyme from mammalian sources with respect to c-AMP inhibition, but only 10^{-6}M c-AMP concentration is usually attained during lipolytic hormone stimulation of fat cells under physiological conditions. Even with caffeine, which blocks the degradation of c-AMP, c-AMP levels of 4×10^{-6}M per liter of cell water are obtained, Butcher et al, 1965. Thus, it seems that cyclic AMP does not offer any physiologically important control of malic enzyme in mammalian fat cells.

C-AMP may influence pyruvate metabolism in another way as demonstrated by Wicks (1971). He showed that c-AMP induces the synthesis of the enzyme PEPCK in tissue culture after one to two hours. The induction occurred only in soluble PEPCK and not in the mitochondrial enzyme. This induction may have caused the effect which Exton (1968) found during liver perfusion with c-AMP after one or two hours. He found a stimulation of some enzyme between pyruvate (Pyr) and phosphoenol pyruvate (PEP). While this induction of enzyme is probably physiologically important, in our studies we are looking for mechanisms which acutely shift the metabolism within 30 minutes, before synthesis of new enzyme has had time to occur.

All of these studies suggest that while cyclic 3'5' AMP is the predominant mechanism for controlling lipolysis in the fat cell, it does not seem to be the primary hormone activation mechanism controlling the balance between glyceride glycerol production and fatty acid synthesis.

The question remains: what other hormone induced mechanism contributes to the control of pyruvate metabolism as it affects synthesis of glyceride glycerol and fatty acid?

A thorough review of factors which may control pyruvate and other carbohydrate metabolism in liver and kidney was offered by Newsholme and Gevers (1967). This review as well as studies of other regulatory enzymes of pyruvate metabolism such as PC (Cazullo and Stopani, 1967), PDH (Jungas, 1971), PEPCK and PE (Wood et al, 1966), and pyruvate kinase (PK) (Boyer, 1969; T. Wood, 1968 and Holmsen and Storm 1969) all indicate one factor in common. This common denominator is that magnesium is a necessary co-factor and that it may play a role in enzyme regulation. In some, excess magnesium ion may inhibit the enzyme. In others, an increase in magnesium ion may decrease the inhibition caused by ATP and cause a stimulation of the enzyme.

We will discuss the relevance of magnesium to each of these reactions in the control of pyruvate metabolism during hormone stimulation later. What is important here is that magnesium seems to affect each of the regulatory enzymes around pyruvate (as well as some other regulatory enzymes along the pathways used during hormone stimulation). The "regulation" in some cases seems to be related to the total magnesium and in others, to the "free" magnesium concentration.

Wood et al (1966) raised the possibility that the free magnesium concentration might play a role in regulating the enzymes PC and PEPCK. They calculated the thermodynamic $\Delta F'$ values in calories under various pH and free magnesium ion concentrations using constants derived from studies with PC and PEPCK enzymes from propionic acid bacteria, (Table XIII). $\Delta F'$ analytical = $-RT \ln K'$ anal. Increase of free magnesium for either of these enzymes tends to decrease the movement of metabolites in the direction from pyruvate to oxaloacetate to phosphoenol pyruvate. The same concentration of magnesium has a greater effect on PEPCK than on PC. Since the calculations were done at or near physiological conditions for ionic strength, pyruvate, oxaloacetate and PEP concentrations as well as for ATP concentration, it would seem that the results reflect to some extent the physiologic conditions which exist in the bacterial cell and (to the extent that it can be applied) in liver

$\Delta F'$ anal. for Pyruvate Carboxylase

$$Pyr + CO_2 + ATP \rightarrow OxA + ADP$$

free Mg^{++} mM	pH 6	7	8
0	-0.6	-1.6	-2.9
0.5	-0.2	-0.8	-2.0
5.0	+0.3	-0.5	-1.8

$\Delta F'$ anal. for PEP Carboxykinase

$$OxA + ITP \rightarrow CO_2 + IDP + PEP$$

free Mg^{++} mM	pH 6	7	8
0	+0.8	+0.1	-1.2
0.5	+1.2	+0.8	-0.3
5.0	+1.7	+1.2	+0.1

Table XIII. Thermodynamic equilibria for PC and PEPCK under varying pH and free magnesium ion concentrations. A negative value indicates a more favorable equilibrium in the direction shown. Increase of either hydrogen ion or magnesium ion slows or, in some cases reverses the reactions. For similar sets of conditions, the PC is more favored than PEPCK in each instance.

$\Delta F'$ anal. = -RT ln K' anal. K' anal. is for 0.1 ionic strength of 25°C and total analytical concentration of reactants.

and fat cells as well. If they can be applied, then, the calculations indicate that increases in free magnesium would tend to block utilization of pyruvate and cause reversal of glycolysis.

We have demonstrated that the lipolytic hormones epinephrine and ACTH cause a net accumulation of Mg^{++} in the fat cell or plasma membrane vesicles prepared from the fat cell. This effect occurs promptly upon exposure to the lipolytic hormone and has been shown to continue for as long as seventy minutes.

There is some evidence that the net accumulation of magnesium leading to increased intracellular magnesium is enhanced by lipolytic hormone inhibition of magnesium efflux in addition to direct influx stimulation.

Insulin and ouabaine block this magnesium increase. There is no evidence that ligands such as ATP increase during lipolytic hormone stimulation. Indeed, Bihler and Jeanrenaud (1970) have shown a decrease in ATP levels in fat cells following epinephrine stimulation. Therefore, the increase in intracellular magnesium is probably reflected in an increased free magnesium as well.

We have shown that in intact cells, magnesium in the extracellular fluid depresses fatty acid synthesis, an additive effect to that of the hormone epinephrine. In terms of total distribution of $U^{14}C$ glucose into the components of triglyceride, magnesium enhances glyceride glycerol labeling.

Since many of the enzymes which are utilized during lipolytic hormone stimulation are magnesium dependent and show different thermodynamic properties depending on the magnesium level (Table XIII), it is of interest to examine what the net effect of change in free magnesium on metabolism would be. Is this change in the lipolytic hormone stimulated metabolism consistent with the metabolic effects of increased magnesium?

Figure XIII shows which of the pathways of fat cell metabolism may be influenced by an increase in magnesium concentration during epinephrine

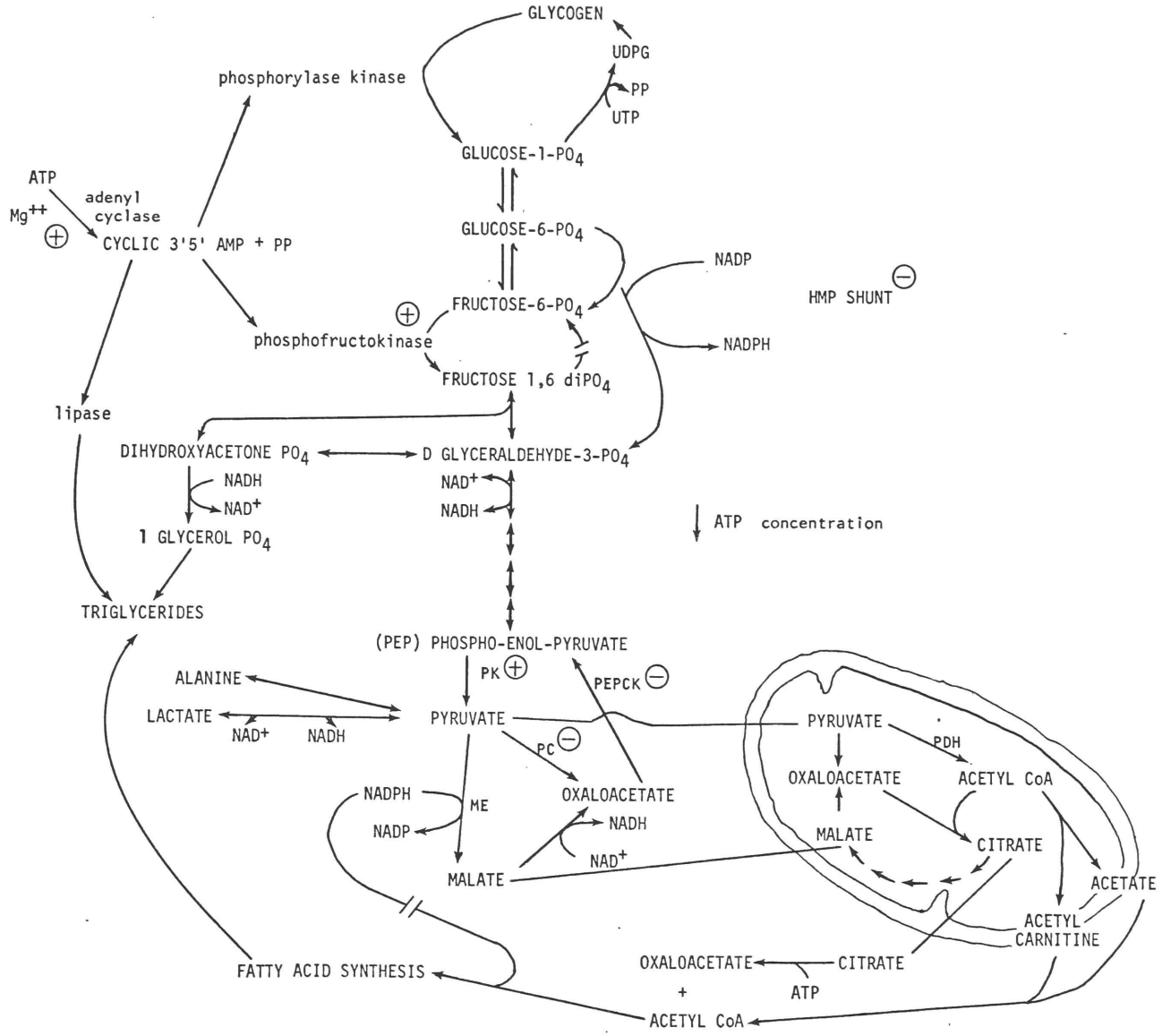


Figure XIII. Metabolic pathways in the mammalian fat cells during epinephrine stimulation. ⊕ Stimulatory effect of Mg^{++}. ⊖ Inhibitory effect of Mg^{++}.

stimulation. The net effect metabolism would be as follows.

First, magnesium is a requirement of adenyl cyclase. Drummond and Duncan (1970) have shown that in partially purified adenyl cyclase preparations from the heart, high magnesium levels promote this reaction. Thus, in hormone stimulation of adenyl cyclase, magnesium at higher concentrations allows a greater production of cyclic 3'5' AMP to occur.

In addition to the effect on adenyl cyclase, magnesium helps to relieve inhibition of phosphofructokinase by ATP, although the cyclic 3'5' AMP stimulation of phosphofructokinase is probably more important. ATP is low and there is probably little inhibition from it, though there could be significant ATP inhibition in the early moments of epinephrine stimulation when magnesium is increasing most rapidly.

Because phosphofructokinase is stimulated, there is a decrease in utilization of the hexose monophosphate shunt (HMP shunt) and so there is no increase in NADPH concentration from this source.

Pyruvate kinase (PK) is stimulated by increasing magnesium concentration. This shunts pyruvate into malate and oxaloacetate production which causes a decrease in NADPH and an increase in NADH concentration. This decrease in NADPH may be sufficient to stop fatty acid inhibition of fatty acid synthesis (Bihler and Jeanrenaud 1970) at the acetyl CoA carboxylase step. The increase in magnesium also inhibits phosphoenol pyruvate carboxykinase PEPCK even more than PC (Table XIII) and so there would be a tendency toward accumulation of oxaloacetate, malate and pyruvate. Thus product inhibition would soon block glycolysis back to glyceraldehyde-3-PO_4. Any new glyceraldehyde-3-PO_4 formed would simply be shunted to dihydroxyacetone phosphate leading to production of 1-glycerol-PO_4 and increased synthesis of glyceride glycerol as is seen in epinephrine stimulation (Table VIII). Glyceraldehyde-3-PO_4 conversion to glucose is impossible in the fat cell, because fructose 1,6 diphosphatase is not present. The increase in NADH from synthesis of oxaloacetate from malate will also contribute to increased production of glyceride glycerol.

In contrast to what we have just described, the effect of insulin is shown in Figure XIV. Since ATP is increased, free magnesium would decrease even if there is not a decrease in the total magnesium. A decrease in free magnesium would tend to inhibit production of cyclic 3'5' AMP. Increased ATP levels with decreased C-AMP and free magnesium would decrease phosphofructokinase activity. Since phosphofructokinase is inhibited, there would be increased utilization of the hexose-monophosphate shunt and increased production of NADPH.

Rising ATP levels would also inhibit PK. Pyruvate Carboxylase (PC) would be stimulated and although PEPCK would be slightly stimulated, the magnesium might not be low enough to relieve all of the inhibition. This would tend to increase oxaloacetate levels leading to malate and pyruvate production with an increase in NAD and NADPH. NADPH would stimulate fatty acid synthesis while increased levels of NAD would tend to inhibit production of l-glycerol phosphate and promote glycolysis as well as production of pyruvate from lactate. Increased pyruvate would cause an increase of pyrucate in the mitochondria by diffusion, which in turn would stimulate production of acetyl Co-A and ATP also stimulating fatty acid synthesis.

Thus changes in intracellular magnesium levels induced by hormones, could cause some of the alterations in metabolism which follow hormone stimulation. Our findings on the effect of Mg^{++} on fatty acid and glyceride glycerol synthesis during epinephrine stimulation, confirm this theoretical formulation. To prove this hypothesis, the effect of hormones on metabolites and enzymes in other portions of the affected pathways will have to be determined.

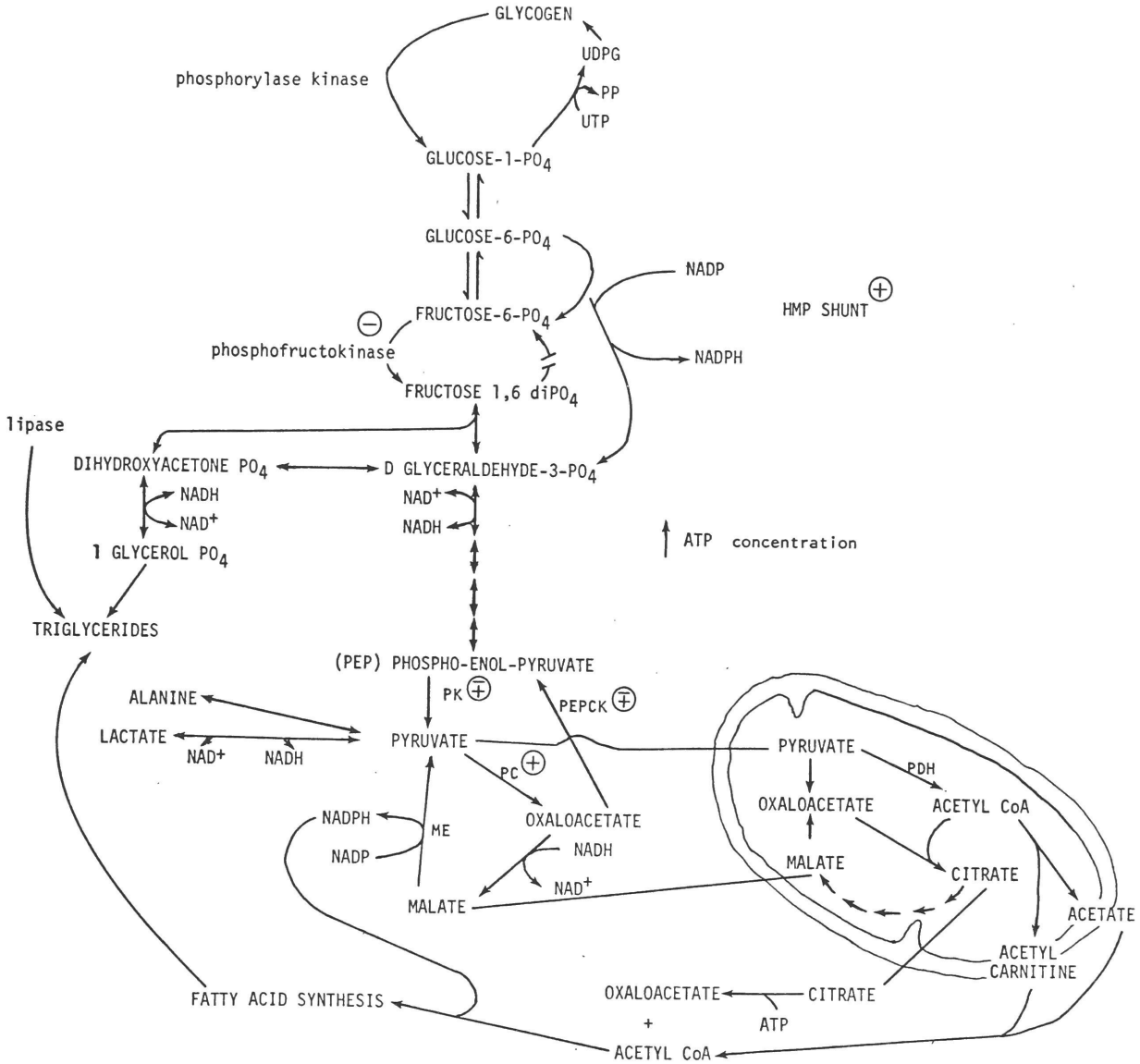


Figure XIV. Metabolic pathways in the mammalian fat cell during insulin stimulation. ⊕ Stimulatory effect of decreasing Mg^{++} concentration. ⊖ Inhibitory effect. ⊕⊖ An equivocal effect.

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