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THE EFFECT OF INSULIN ON GLUCOSE METABOLISM IN MUSCLE

ABSTRACT

Under two different experimental conditions it has been demonstrated that insulin stimulates directly glucose oxidation in the rat diaphragm independently of its effect on glucose transport. The site of this action was found to be mainly the phosphofructokinase reaction. Insulin has also a small stimulatory effect on the pyruvate dehydrogenase reaction. Data are presented which indicate the existence of more than one pool of glucose-6-phosphate and of phosphoglyceric acids in the rat diaphragm muscle. Insulin, *in vitro*, has no effect on gluconeogenesis in the rat diaphragm. It is suggested that in the rat diaphragm the pools of glucose-6-phosphate and of phosphoglyceric acids involved in glycolysis are separate from those involved in gluconeogenesis.

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by

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LIST OF ABBREVIATIONS

G-6-P	Glucose-6-phosphate
G-1-P	Glucose-1-phosphate
F-6-P	Fructose-6-phosphate
FDP	Fructose-1,6-diphosphate
3PGA	3-phosphoglyceric acid
2PGA	2-phosphoglyceric acid
PEP	Phosphoenolpyruvate
GA-3-P	Glyceraldehyde-3-phosphate

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CONTRIBUTION TO KNOWLEDGE BY THE AUTHOR

1. Insulin stimulates glucose oxidation in the rat diaphragm independently of its effect on glucose transport.

2. Changes in concentration and radioactivity of phosphorylated glycolytic intermediates after incubation with C^{14}-labeled glucose indicate that this effect is mainly due to stimulation of the phosphofructokinase reaction.

3. Insulin has also a small stimulatory effect on the pyruvate dehydrogenase reaction.

4. A method has been developed to measure the radioactivity and specific activities of phosphorylated glycolytic intermediates in the rat diaphragm.

5. The data suggest the existence of more than one pool of glucose-6-phosphate and of phosphoglyceric acids in the rat diaphragm.

6. Insulin, added <u>in vitro</u>, has no effect on gluconeogenesis (from pyruvate) in the rat diaphragm.

7. The data suggest that in the rat diaphragm, the pools of glucose-6-phosphate and phosphoglyceric acids of the glycolytic pathway are separate from those of the gluconeogenic pathway.

GENERAL INTRODUCTION

Although many years have passed since the discovery of insulin by Banting and Best in 1921, its mechanism of action is still not fully understood.

The multiplicity of insulin effects on responsive cells, makes it more and more impossible to explain them all on the basis of a single primary locus of action.

Following the demonstration of Levine in 1949 (1) of the action of insulin on cell membrane transport of sugar, and the subsequent confirmation and extension of this finding by others, it was most logical to adopt Levine's theory in 1955 (2) that this action of insulin was primary and that all other effects of the hormone are consequences of such an action. On this basis it seemed possible to account for increases in glucose oxidation, glycogen synthesis, formation of fat, esterification of fatty acids and protein synthesis which followed administration of insulin.

In recent years, however, data have been presented concerning insulin actions, which as Levine recently reconsiders (3): "make it difficult or well nigh impossible to deduce them from the effect on glucose transport." The glucose transport theory cannot account for the specific stimulation by insulin of muscle UDPG-glycogen transglucosylase activity (4), lipogenesis in chick embryo hearts (5), glucose utilization in rat (6, 7) and human (8) adipose tissue, and protein synthesis (9).

These recent reports indicating a variety of intracellular effects of insulin, have encouraged us to study whether in the mammalian muscle, insulin acts on glucose utilization independently of its action on glucose transport, and if so, to localize the site(s)of its action.

SURVEY OF THE LITERATURE

I. Insulin effects on carbohydrate metabolism.

A. Membrane transport of glucose.

1. Glucose transport mechanism.

There are many lines of evidence which point to the view that transport of glucose across the cell membrane, takes place by facilitated diffusion, i.e. carrier-mediated diffusion. Facilitated diffusion is the passage of glucose through the membrane by reversible association with a carrier molecule; it involves association with a carrier molecule in the membrane for transport across the cell, then dissociation on the inner side. The process is reversible.

Evidence for such a transport system has been found in numerous cell types, e.g. heart muscle (10), adipose tissue (11), erythrocytes (12, 13), sheep placenta (14), and recently, the liver (15, 16). There is no reported cell type in which sugars appear to enter only by simple diffusion. In mammals, except for intestinal mucosa and renal cortex, sugar transport has not been shown to be "active", i.e. against a concentration gradient. The lines of evidence for the mem-

brane carrier theory of glucose transport are:

a. Saturation kinetics:

When the uptake of sugar is plotted as a function of extracellular concentration, the curve seems to follow Michaelis-Menten kinetics for an enzyme-catalyzed reaction rather than the kinetics for simple diffusion (10). There is a rapid increase in transport rate at low concentrations and a plateau at higher concentrations. This can be explained by the saturation of a combining site, or carrier, necessary for transport (17).

Unidirectional transport into the cell can be expressed as follows:

$$Tinward = Tmax \frac{Ge}{Ge+Kt}$$

T = transport rate

Ge = extracellular glucose concentration

Kt = concentration at which the rate is half maximal.

Net transport is the algebraic sum of inward and outward transport (18) since the process is reversible.

$$T = Tmax \frac{Ge}{Ge + Kt} - Tmax \frac{Gi}{Gi + Ki}$$

where, T = rate of net transport, which is inward transport minus outward transport

Tmax = transport capacity

Ge = concentration of glucose on the outside of the cell membrane

Gi = concentration of glucose on the inside of the cell membrane.

Thus net transport is the difference between independent and symetrical

inward and outward transport processes, each of which conforms to Michaelis-Menten kinetics.

b. Specificity:

Preferential permeability is shown for substances of close structural relationship and size; for example, D-galactose enters the red cell and L-galactose is excluded (19), and in heart muscle, D-glucose enters and sorbitol is excluded (20). The process also shows stereospecificity with respect to insulin stimulation of glucose transport (21): the uptake of D-glucose is stimulated by insulin, but that of L-glucose is not. The stereospecificity has been studied particularly by LeFevre and Marshall (22) who concluded that the three-dimensional conformation of the sugar was the most important factor determining affinity for transport. D-glucose, the pyranose sugar which exists to the greatest extent in a chain conformation of the so-called C-1 type, has the highest affinity. Other hexoses and pentoses can be arranged in a decreasing order of affinity which correlates with the decline in the stability of the C-1 conformation in water. L-glucose does not exist at all in the C-1 conformation, but is largely in the mirror image conformation, 1-C. L-glucose has a very poor affinity indeed for the transport site and hardly penetrates most cells. Stereospecificity of the transport system is more readily explained on the basis of a membrane carrier than on the basis of another mechanism, such as membrane pores, since aqueous channels in the membrane would be unlikely to discriminate between such closely related substances.

c. Competition:

There is competitive inhibition of transport between various pairs of sugars both hexoses and pentoses. This provides another line of evidence for the existence of a membrane carrier. The sugar with the greater affinity for the carrier will be the one transported.

d. Counterflow:

This phenomenon is a special case of competitive inhibition. Counterflow is a facilitated movement of permeant down its electrochemical gradient, associated with movement in the opposite direction of a structurally analogous molecule up its electrochemical gradient (23).

Counterflow is demonstrable when the following conditions are satisfied:

1) two substrates must share a common facilitated diffusion system
2) there must be a concentration gradient of the driving substrate
3) the concentration of this substrate must be high in relation to its Michaelis constant.

In the isolated perfused rat heart, Morgan, Regen and Park (21) demonstrated clearly counter transport of 3-o-methyl-D-glucose and L-arabinose on addition of D-glucose to the perfusate. The isolated rat heart was perfused with a non-metabolized sugar, either 3-o-methyl-D-glucose of L-arabinose, until the internal concentration reached about half of the external concentration, at which time the rates of entry and exit from the cell would be equal. At this point, a high concentration of glucose was added. The 3-o-methyl-D-glucose (or the L-arabinose) then began leaving the cell. Since this sugar can-

not be metabolized, the addition of glucose had caused the 3-o-methyl-D-glucose to move out of the cell against a concentration gradient.

The fall in the intracellular 3-o-methyl-D-glucose can be explained as follows: On the external surface of the membrane glucose competes with the 3-o-methyl-D-glucose and reduces its entry; however, inside the cell glucose is phosphorylated by hexokinase and cannot leave the cell. Therefore it does not compete for exit; as a result the efflux of the nonmetabolized sugar exceeds the influx until a new equilibrium is established.

Counterflow argues for a carrier mechanism and against simple diffusion which can only proceed from a higher to a lower concentration.

e. Inhibition:

The rate of penetration may be specifically and markedly reduced by the presence of substances differing chemically from the permeant, these inhibitors being often chemical reagents also active as enzyme poisons; phloretin, phlorizin, and related compounds have long been known as specific inhibitors (12, 17). Inhibition has been demonstrated in red cells (12), muscle, striated and cardiac (21, 24), adipose tissue (25, 26), and liver (16). Transport in many cells is also very sensitive to low levels of sulfhydryl-blocking agents (27), and to dinitrofluorobenzene (28), suggesting participation of protein components in transport.

All the above mentioned lines of evidence point to a carrier syetem of some sort as being the most probable mechanism of glucose transport across the membrane.

The transport seems to involve combination of the sugar with a stereospecific membrane component or site. Efforts to identify and isolate chemical components of the transport system have not been successful. The reason for this failure lies mainly in the inability to assay for transport activity after a cell is broken.

All the recent efforts to search for transport components (29-31) have not yielded convincing results (32).

2. Evidence for insulin effect on glucose transport.

The fact that insulin accelerates glucose uptake in skeletal and cardial muscle, and in adipose tissue, has been amply proved by many workers (33-48). Indeed such an effect on glucose uptake by the rat diaphragm or adipose tissue, has served as the basis for estimation of plasma insulin (49-57)

a. The use of nonmetabolized sugars as models for glucose transport.

The use by Levine and co-workers (1, 2, 58-61), Park and co-workers (62, 63), and others of nonmetabolized sugars, afforded distinction between sugar transport and subsequent metabolism, including initial phosphorylation. Thus, infusion of certain nonmetabolized sugars into eviscerated nephrectomized dogs, resulted in their distribution in the extracellular water; insulin administration was followed by entry of the sugars into the intracellular water.

The conclusion was that insulin acted by facilitating the membrane transport of glucose into the cells. The nonmetabolized sugars compete with glucose for the same membrane transport system (19, 20, 64, 65); the stimulation by insulin, of membrane transport of these sugars therefore

made them useful models for glucose transport.

The studies using nonmetabolized sugars have been extended by *in vitro* experiments (66 - 68). Morgan et al. (68) using the perfused rat heart, have demonstrated that transport of L-arabinose, a nonmetabolized sugar, was stimulated strongly by insulin addition. Transport is a reversible process, sensitive to insulin influence in the outward as well as inward direction (19); when the perfused heart is preloaded with L-arabinose, then subsequent perfusion with a sugar-free medium causes outward transport of L-arabinose from the cell. The presence of insulin causes a three- to four-fold increase in the rate of outward transport. Insulin then can stimulate movement in either direction.

The lack of endogenous insulin, as in alloxan-diabetes, causes a decrease in transport. There is no accumulation of intracellular L-arabinose, outward transport of the sugar is decreased, and glucose uptake by the heart of alloxan-diabetic rats is reduced to about 40% of normal. Insulin *in vitro* can restore this to the normal level (69).

The increase in glucose transport produced by insulin is considered to result mainly from an increase in the maximal rate of transport. In the perfused heart of diabetic rats Tmax is increased by insulin about 13 times, and the transport constant Kt is increased about 5 times. In normal hearts Tmax is increased 5 times and Kt about 3 times (70).

b. Insulin affects glucose transport under conditions where hexokinase activity is decreased.

The use of nonmetabolized sugars led indirectly to the conclusion

that insulin affects glucose transport.

More direct evidence was brought by Park et al. (71-73): under conditions where transport of glucose exceeds the phosphorylating capacity of hexokinase system, i.e., low temperature or high concentration of glucose in medium, insulin caused a measurable rise in the free glucose content of muscle. Similar findings were obtained with the fat pad (74).

3. Possible mechanism of insulin effect of glucose transport.

a. Conformational change in the membrane.

(i) Binding of insulin to the cell.

In 1949, Stadie et al. (75) demonstrated that insulin binds to the isolated rat diaphragm; the diaphragm previously exposed to insulin showed an increased glycogen synthesis, this effect persisted after prolonged washing of the tissue with insulin-free solution. The question then arose whether this attachment is a specific binding to receptor sites in the cell or whether insulin attaches to the cell nonspecifically, similar to its attachment to many inert materials such as glass, paper etc. (76). Woltmann and Narahara (77) carried out similar studies to those of Stadie, using the frog sartorious muscle and the nonmetabolized sugar 3-o-methyl glucose. Muscles previously exposed to insulin were washed in insulin-free solution at 0°C. The insulin-enhanced permeability to the sugar was retained. But the insulin effect decreased when the washing was done at 19°C.

Schwartz et al. (78)proposed that insulin might react with a -SH group in the receptor by a disulfide interchange reaction,similar

to vasopressin. Park and coworkers (79) reported that in the perfused rat heart, a brief exposure to a -SH inhibitor N-ethyl meleimide (NEM), abolished subsequent stimulation of glucose or arabinose transport by insulin; such inhibition did not occur if the heart were first treated with insulin. Also, pretreatment of the isolated heart with NEM reduced the binding of I^{131} insulin. This strongly suggests that insulin attaches itself to the cell through a disulfide linkage formed by interchange between a disulfide bond in insulin and a thiol group in the cell membrane.

NEM, in concentrations which had no effect on basal levels, could inhibit the insulin-accelerated uptake of sugars by the rat diaphragm (64, 80) and the epididymal fat pad (81, 82). Much contradictory evidence, however, concerning the binding of insulin to these tissues was presented. Recently, Crofford (83), using the Rodbell isolated cell preparation of adipose tissue (84), has re-examined the question of how insulin binds to the cell. He employed native rather than labelled insulin, since the biological activity and binding qualities of labelled insulin are probably altered. The isolated cells were placed in a medium containing a physiological level of insulin and the fall in insulin concentration was followed. The following results were obtained: a) There was an immediate uptake of insulin, presumably reflecting binding to the cell surface, followed by a relatively very slow, progressive uptake, reflecting utilization of the hormone. b) The initial uptake was not reduced when incubation temperature was dropped from 37°C to 17°C, but the utilization was greatly slowed. c) The utilization of insulin required the presence of the cells. d) The initial binding could be prevented by

a short prior exposure of the cells to NEM, although this agent did not suppress the subsequent rate of insulin destruction.

The effects of maleimide in the fat cell (85) are similar to those observed earlier in the heart muscle (79): The stimulatory effect of insulin on glucose transport is suppressed by prior exposure to NEM but not by subsequent exposure.

Additional support for the disulfide interchange theory comes from the observations of Edelman et al. (86, 87). They concluded from their studies with skeletal muscle cell membrane preparation that insulin action involves a thiol-disulfide interchange reaction of the cyclic disulfide on the A chain of insulin and sulfhydryl groups on a receptor protein of the muscle cell membrane.

The combination of insulin with a specific receptor at the cell membrane, may lead to structural changes resulting in an enhanced transport. Rodbell et al. (88) have recently suggested that insulin may cause the transformation of plasma membrane lipoproteins from a laminated to a micellar form, which allows more solute to be transported. It might do so by interacting with specific receptor sites in the protein film of the plasma membrane, possibly by some interchange between the -SS- group on the A chain and the -SH group in the protein film.

(ii) Hydrolytic effects on the membrane.

Insulin-like effects were produced by using hydrolytic enzymes to change membrane structure. Rieser (89-95) has used proteolytic enzymes particularly chymotrypsin and trypsin. Both mimic insulin action by increasing sugar and amino acid transport and glycogen synthesis in rat diaphragm. Chymotrypsin acts more like insulin in increasing sub-

strate transport. It also produces hypoglycemia in the intact animal. The effects of chymotrypsin and trypsin on sugar transport were also confirmed by Dailey et al. (96). Pruit et al. (97) found chymotrypsin to be effective in the presence of insulin antiserum, showing that the results could not have been due to contamination of the enzyme preparation with insulin. The enzymes are thought to lower the cell membrane barrier to glucose and other products as a result of hydrolysis of peptide bonds in the cell membrane.

The suggestion was made that similar catalytically active residues might be involved in the insulin-like actions of the enzymes and in the hormonal activity of insulin. Insulin, in vitro, has been shown to catalyze the hydrolysis of quite a variety of proteins (90), although this has not been shown in vivo.

The hypoglycemic and enzymatic properties of both chymotrypsin and of insulin depend on the intactness of certain histidine residues. Furthermore, disruption of disulfide bridges leads to loss of both hormonal and enzymatic activity in insulin. The catalytic properties of chymotrypsin are known to depend on the association of three peptide chains joined by disulfide bridges (98).

Chymotrypsin not only acts like insulin, and insulin like a proteolytic enzyme, but there also exists a common structural basis for the hormonal and enzymatic properties of both molecules; they both contain a dihistidine cystine structure. This part of the molecule is constant among insulins of ungulate species. Intactness of the dihistidine cystine configuration in chymotrypsin and insulin of ungulate species is required for the expression of enzymatic and hypoglycemic actions of both

proteins.

In an attempt to arrive at a common mechanism of action of insulin and the variety of peptides and enzymes exhibiting similar biological activity, Rieser suggested the application of Hofmann's theory (99, 100) of peptide hormone action. Hofmann proposed that the peptide hormones exert their effects by interacting with cellular receptors to form active enzymes. Thus insulin and the insulin-like enzymes could complement the membrane-sited receptor to form an active enzyme.

Rodbell (101) has demonstrated on isolated dispersed fat cells that phospholipase C mimics insulin stimulatory action on glucose transport. He proposes that the primary action of insulin and phospholipase C on this cell is to alter the configuration of the plasma membrane lipoproteins.

b. Insulin and energy metabolism.

Randle and Smith (102, 103) found that a number of metabolic inhibitors, including anoxia, 2,4-dinitrophenol, arsenite, arsenate, cyanide and salicylate, all of which inhibit oxidative phosphorylation and therefore interfere with ATP generation, act like insulin in stimulating monosaccharide transport in muscle. Randle (104) suggested that the glucose carrier in the muscle membrane may exist in a phosphorylated form in which it is inactive, and in a dephosphorylated form in which it is active. According to this view, insulin increases glucose entry by preventing the transfer of ATP to the carrier and thus maintaining the carrier in a maximally activated form.

Cyclic 3',5'-AMP has been reported to increase glucose uptake by diaphragm (105). It was postulated that it might cause swelling of the transverse tubular extensions of the sarcolemmal membrane and of cells, thereby decreasing the interstitial space and expanding the pathways by which water, glucose, and amino acids could enter the cell. Whether this "insulin-like" effect can help in any way to clarify insulin action on transport remains unknown.

c. Question of relation to protein biosynthesis.

Enhancement of glucose uptake by insulin has been shown to be independent of protein biosynthesis since puromycin, which inhibits protein biosynthesis in mammalian cells, was found to have no influence on enhancement of glucose transport by insulin in the isolated rat diaphragm (106). This finding excludes the possibility that insulin effect on glucose transport could be due to de novo synthesis of a protein carrier.

B. Glucose phosphorylation.

1. Pathways of glucose phosphorylation.

a. Hexokinase and glucokinase:

The glucose after being transported across the cell membrane, is phosphorylated to glucose-6-phosphate via the hexokinase reaction. Two main types of hexokinase are recognized in animal tissues: a) The "low Km" hexokinase which has an apparent Km for glucose in the range 0.01 - 0.1 mM and is allosterically inhibited by its product glucose-6-phosphate (107). b) Glucokinase, which has an apparent Km for glucose of approximately 10 mM and which is not inhibited by glucose-6-phosphate (108-111).

The low Km hexokinase is of wide tissue distribution. The high Km glucokinase is virtually restricted to the liver. The hexokinase is present both in the soluble fraction of tissue extracts and also in a bound or particulate form, the per cent sedimentable depending on the tissue, e.g. brain hexokinase has been reported to be almost completely present in the particulate form (112). Recent studies with pig heart muscle (113), have indicated that an equilibrium exists between the soluble and particle-bound forms of hexokinase, the binding being affected by ionic strength, pH, and glucose-6-phosphate. The particulate hexokinase of ascites tumor cells (114) was shown to be mitochondrial; the activity was readily solubilized by glucose-6-phosphate, ribonucleoside triphosphates, high salt concentration and differing pH. Spydevold and Borrebaek (115) have recently demonstrated that rat epididymal adipose tissue hexokinase activity was associated with both mitochondria and microsomes; the enzyme activity could be released from the particles by addition of glucose-6-phosphate while subsequent addition of inorganic phosphate brought back a considerable part of the activity to the particle-bound state.

Chromatographic and electrophoretic fractionation led to identification of three isoenzymes of the low Km hexokinase, designed as types I, II and III (116, 117), which differ in certain kinetic and physical properties (118). Type IV, the most rapidly migrating form on starch gel electrophoresis, is identical with the high Km glucokinase found only in liver. This means that mammalian tissues contain altogether four hexokinases, including both the liver specific high Km glucokinase and the three low Km hexokinase isoenzymes; these

are present in differing proportions in various tissues.

The three purified isoenzymes of the low Km hexokinase differ with respect to apparent Km for glucose and probably for ATP, stability to heat, proteolytic inactivation, and degree of inhibition by glucose-6-phosphate and ATP. They are similar with respect to pH optimum, molecular weight, hexose and nucleotide specificity and Km for fructose (118).

Liver contains all four enzyme types. Diaphragm muscle enzyme consists primarily of type II with very slight amounts of type I. Adipose tissue contains types I and II (119, 120). In starvation type II decreases more rapidly than type I (119). Type II also shows a fall in activity in alloxan diabetic rats (121). Katzen (122) found that the type II enzyme could be separated into two subgroups: type IIa and IIb; only the IIa type was decreased in diabetes. These results were obtained with rat heart, adipose tissue, diaphragm and gastrocnemius muscle extracts assayed in the absence of mercaptoethanol (in most previous studies a thiol has been included for enzyme stabilization purposes).

The glucokinase is the main glucose-phosphorylating enzyme in the liver. It develops in the liver of the rat only after birth (123). The glucokinase content of liver decreases in starved animals and disappears in diabetic animals (124,109).

b. Other pathways.

Though hexokinase and glucokinase are the main phosphorylating enzymes of glucose, other phosphorylating pathways have been reported;

in the muscle a pathway that leads to the formation of glucose-1-phosphate from glucose, and does not have glucose-6-phosphate formation as an intermediate step, has been suggested (125). The two enzymes involved in this pathway are glucose-1-phosphate kinase and glucose-1-phosphate dismutase, previously known to exist in muscle (126, 127):

$$\text{Glucose-1-phosphate} + \text{ATP} \xrightarrow{\text{kinase}} \text{glucose-1,6-diphosphate} + \text{ADP} \quad (1)$$

$$\text{Glucose} + \text{glucose-1,6-diphosphate} \xleftrightarrow{\text{dismutase}} 2\ \text{glucose-1-phosphate} \quad (2)$$

$$\text{Glucose} + \text{ATP} \longrightarrow \text{glucose-1-phosphate} + \text{ADP} \quad (3)$$

No evidence has yet been provided for the operation of this alternative mechanism <u>in vivo</u>.

There are also several enzymes in the liver, able to phosphorylate glucose <u>in vitro</u>, whose physiological significance is unknown; One of them is the inorganic pyrophosphate-glucose phosphotransferase, catalyzing the reaction:

$$PP_i + \text{glucose} \longrightarrow \text{glucose-6-P} + P_i$$

The enzyme was discovered by Rafter (128). Nordlie and Arion (129) have shown that the enzyme resides in microsomes of rat liver and appears to be identical with glucose-6-phosphatase.

In addition, a group of nucleoside diphosphate- and triphosphate-glucose phosphotransferase activities were reported to be present in the rat liver microsomes and kidney homogenates (130), all catalyzing the transfer of phosphoryl group to the 6-position of D-glucose. Nordlie and Arion (130) postulated that the phosphotrans-

ferase reactions in the liver may constitute a physiologically significant compensatory mechanism for glucose phosphorylation in the diabetic animal; while the glucokinase is markedly depressed in the untreated alloxan diabetes, the phosphotransferase activities are significantly stimulated.

2. Insulin effect on glucose phosphorylation.

a. Liver.

(i) Induction of glucokinase.

Glucokinase activity disappears in diabetes, and returns after insulin administration to diabetic animals (131-133). The time required for reappearance of glucokinase activity in diabetic animals after insulin administration seemed too long to be due to enzyme activation. Insulin, in short in vitro experiments, did not cause the reappearance of the enzyme activity in liver of the diabetic animals. Puromycin and actinomycin D prevented an increase in glucokinase activity by insulin administration in the diabetic rat (131). This suggested that de novo synthesis of the enzyme is involved, i.e. that liver glucokinase is an inducible enzyme whose synthesis is induced directly or indirectly by insulin.

(ii) Direct activation of glucokinase.

In addition to the inductive effect of insulin on glucokinase, Vester and Reino (134) have demonstrated a direct effect of insulin, in vitro, on the hepatic glucokinase; particle free-extracts of rat liver, incubated with insulin showed an increased activity of glucokinase. The insulin effect was dose dependent. The effect was spec-

ific, as insulin inactivated with heat or alkali did not stimulate glucokinase activity. The concept of a specific insulin action on glucokinase was supported by the finding that liver supernatants prepared from diabetic animals which appear to possess only hexokinase activity, failed to respond to insulin.

(iii) Insulin effect on other phosphorylating enzymes.

The hexokinase activity of the liver is not changed in alloxan diabetic rats with or without insulin (132, 135). The inorganic pyrophosphate-glucose phosphotransferase, and the nucleoside diphosphate- and triphosphate-glucose phosphotransferase activities of the liver are all significantly stimulated in untreated alloxan diabetes, and are depressed by administration of insulin to diabetic animals (130). It is possible that this is an indirect action of insulin, the enzyme activities being depressed by the reduction in plasma glucose concentration.

b. Adipose tissue and muscle.

In the rat epididymal fat pad, hexokinase activity is reduced in alloxan diabetes, and restored upon administration of insulin (121, 136). Katzen (122), who found that the decrease in diabetes was in the type IIa hexokinase, demonstrated a restoration of activity upon insulin administration, both *in vivo* and *in vitro*, in adipose tissue, heart, diaphragm and gastrocnemius muscles.

Increased hexokinase activity was observed during incubation of epididymal fat pad, *in vitro*, by the addition of either glucose or insulin to the incubation media (136, 137). Additional increase in enzyme activity was observed when

glucose and insulin were added together. The action of insulin on adipose tissue hexokinase activity was clearly a separate effect, not dependent on the presence of glucose.

An interesting finding has been recently reported by Borrebaek and Spydevold (138), who found that insulin, in the absence of glucose, increases the per cent of mitochondria-bound hexokinase activity in rat epididymal fat pads. The authors suggest that the influence on localization of hexokinase may be of significance with regard to the role of insulin as a regulator of carbohydrate metabolism.

C. Glycogenesis and glycogenolysis.

1. Enzymes of glycogen synthesis.

The enzymatic reactions leading from glucose-6-phosphate to glycogen are:

$$\text{glucose-6-phosphate} \xrightleftharpoons{\text{phosphoglucomutase, } Mg^{++}} \text{glucose-1-phosphate} \quad (1)$$

$$\text{glucose-1-phosphate} + \text{UTP} \xrightleftharpoons{\text{UDPG pyrophosphorylase}} \text{UDPG} + PP_i \quad (2)$$

$$\text{UDPG} + \text{glycogen} \longrightarrow \text{UDP} + \text{glucosyl}(\alpha\text{-}1,4)\text{-glycogen} + H^+ \quad (3)$$

Reaction (3) is catalyzed by glycogen transferase (also named glycogen synthetase or transglucosylase) which is the rate-limiting step in glycogen synthesis. This enzyme incorporates the glucose moiety of UDPG into α-1,4 linkages of the outer chains of glycogen primer. This reaction is followed by the action of Amylo-(1,4 → 1,6)-transglucosylase (branching enzyme), which transfers glycosyl residues from α-1,4 to

α-1,6 linkages when the outer chain length has 7 residues (139, 140).

The glycogen transferase is present in 2 forms (141): The dependent form (transferase D) which requires glucose-6-phosphate for activity, and the independent form (transferase I) which is active in the absence of glucose-6-phosphate. The two forms are interconvertible (142). The transferase I is apparently the only form able to synthesize glycogen under physiological conditions (143). The Vmax for UDPG of the D form is increased by glucose-6-phosphate, but that of the I form is not changed. The effect of glucose-6-phosphate on the Km for UDPG of the D form shows a marked species variation. The Km for UDPG of the I form was found to be decreased by glucose-6-phosphate. The decrease in Km of the I form, rather than the increase in Vmax of the D form, appears to be the major physiological action of glucose-6-phosphate (143).

The conversion of D to I form is catalyzed by transferase phosphatase; glycogen inhibits this reaction (feedback inhibition) (144). The conversion of I to D form is catalyzed by transferase I kinase (145, 146). This inactivation reaction is a phosphorylation of a serine hydroxyl group in the transferase molecule and is reversible (147). The reaction requires the presence of ATP and Mg^{++}. The transferase I kinase was found in rabbit skeletal muscle in two distinct forms: one dependent on 3',5' cyclic adenylate for activity, the other active in the absence of this activator (143).

An irreversible conversion of transferase I into a transferase

D-like form occurs upon addition of Ca^{++}(10^{-4}M) together with a protein factor to a partially purified preparation of transferase from rat skeletal muscle (148, 149). This reaction does not require ATP, and is not stimulated by 3',5' cyclic adenylate. It has been proposed that this conversion by calcium may explain the rapid disappearance of transferase I during muscle contraction; however, the concentration of calcium required for the conversion of transferase I into the D-like form is much greater than that required for muscle contraction.

2. Enzymes of glycogen degradation.

The enzymes directly concerned with the breakdown of glycogen are:

(1) phosphorylase, which is specific for the phosphorylytic breaking of the -1,4- linkages in glycogen to yield glucose-1-phosphate:

$$P_i + \text{glucosyl}(\alpha\text{-}1,4)\text{-glycogen} \rightleftharpoons \text{glucose-1-phosphate} + \text{glycogen}$$

(2) Amylo-1,6-glucosidase (debranching enzyme), catalyzes the hydrolytic splitting of the -1,6- linkages.

Glycogen phosphorylase.

Phosphorylase is present in muscle in two forms: phosphorylase b - which is active only in the presence of 5'AMP, and phosphorylase a - which is active in the absence of 5'AMP.

ATP, ADP and glucose-6-phosphate were found to inhibit the activation of phosphorylase b by AMP (150, 151); Morgan and Parmeggiani (152) measured the activity of the two forms of phosphorylase using

concentrations of AMP, ATP, glucose-6-phosphate and inorganic phosphate similar to those present in resting muscle, and found that the activity of phosphorylase b under these conditions was insignificant, while phosphorylase a was fully active. Under different experimental conditions, phosphorylase b could be shown to be active (152). It appears, however, that phosphorylase a is the physiologically active form.

The regulation of glycogenolytic activity is mediated by the conversion of phosphorylase b to phosphorylase a. This reaction is catalyzed in skeletal muscle and heart by phosphorylase b kinase:

$$2 \text{ phosphorylase b} + 4 \text{ ATP} \xrightarrow[Mg^{++}]{\text{phosphorylase b kinase}} \text{phosphorylase a} + 4 \text{ ADP} + 4H^{+}$$

The inactivation reaction, namely the conversion of phosphorylase a to b is catalyzed by phosphorylase phosphatase (153, 154):

$$\text{phosphorylase a} \xrightarrow{\text{phosphorylase phosphatase}} 2 \text{ phosphorylase b} + 4 P_i$$

The phosphorylase phosphatase acts by removing inorganic phosphate groups from serine phosphates in the molecule of phosphorylase a (153, 154). Phosphorylase phosphatase from rabbit skeletal muscle is strongly inhibited by AMP and fluoride (155, 156). It is also inhibited by ATP, but activated by glucose-6-phosphate.

The phosphorylase b kinase is regarded as the key enzyme for regulation. This enzyme, like phosphorylase itself, exists in nonactivated and activated forms (157, 158). The activation of phosphorylase b kinase requires phosphorylation of the enzyme by ATP and Mg^{++} (159, 160).

Glycogen and 3',5' cyclic adenylate stimulate the rate of activation and phosphorylation of the enzyme. They act probably by different mechanisms since their effects are additive (160). 3',5' cyclic adenylate and glycogen stimulate activation of the kinase more than they affect its phosphorylation (160).

The nonactivated phosphorylase kinase can also be converted to the activated form in vitro, by preliminary incubation with Ca^{++} ions plus a protein factor from muscle (157, 158, 161, 162), or by treatment with trypsin (158).

The active phosphorylase b kinase can be dephosphorylated and inactivated by phosphorylase b kinase phosphatase. This enzyme is inhibited by glycogen (163).

3. Control of glycogen metabolism by insulin.

The fact that insulin increases glycogen formation was one of the earliest known effect of the hormone. This effect was demonstrated in muscle (33, 36, 164-167), in adipose tissue (168-170) and in liver (171, 172). Norman et al. (173) have demonstrated that in the rat diaphragm insulin exerts a direct effect on glycogen synthesis independent of its enhancement of membrane transport of glucose. They studied the insulin effect on glucose uptake and glycogen synthesis in hemidiaphragms incubated with varying concentrations of glucose; the effect of insulin in increasing glucose uptake reached a maximal at a certain concentration of external glucose, whereas the insulin effect on glucose incorporation into glycogen did not level off at this concentration of glucose.

Larner et al. (174) have shown that in the rat diaphragm glycogen synthesis was not increased by raising the glucose concentration from 140 to 280 mg per 100 ml, although glucose uptake was elevated. The dissociation in the effects of insulin in controlling glucose transport on the one hand and glycogen synthesis on the other, was recently demonstrated by Eboue-Bonis et al. (175); when hemidiaphragms were incubated with N-ethyl maleimide, the effect of insulin on glucose uptake was severely inhibited, whereas its effect to increase glycogen synthesis was maintained.

The direct effect of insulin on glycogen synthesis was localized to an action on glycogen transferase activity (4); the glycogen-transferase activity was found to be increased in extracts prepared from rat hemidiaphragm which had been incubated with insulin, when the activity was measured without the addition of glucose-6-phosphate. When glucose-6-phosphate was added in the assay, the activities of extracts prepared from control and insulin-treated diaphragm were the same. The increase in enzyme activity by insulin was not duplicated by incubation of diaphragms in an increased concentration of glucose.

These experiments demonstrated that insulin produces an increase in the percentage of transferase I without a change in total activity. These experiments were repeated with intact rat diaphragm yielding similar results (176). The increase in transferase I by insulin was also demonstrated in heart perfused in situ (177); intravenous injection of insulin caused an increase in the transferase I without significant change in total activity. No effect of insulin on phosphorylase activity has been observed (4, 177).

4. Possible mechanism of insulin action.

The above mentioned works indicate that insulin increases the percentage of transferase I activity. No in vitro effect of insulin on transferase has been found in broken-cell preparations (143). Therefore, this action of insulin must be an indirect effect.

Exton et al. (178) and Butcher et al. (179) have demonstrated that insulin reduces the level of 3',5' cyclic adenylate in liver and in adipose tissue when the concentration of 3',5' cyclic adenylate had been raised by previous treatment with epinephrine. Insulin by itself did not affect the 3',5' cyclic adenylate levels.

Schulz et al. (180) have reported that intravenous injection of insulin into alloxan diabetic rats causes an increase of phosphodiesterase activity of skeletal muscle and liver. Intraperitoneal injection of actinomycin D, prevents this increase, suggesting that insulin stimulates phosphodiesterase synthesis. The increase in phosphodiesterase activity would accelerate the hydrolysis of 3',5' cyclic adenylate. No measurements of 3',5' cyclic adenylate content were reported by these authors.

If insulin acts by reducing the concentration of 3',5' cyclic adenylate, this would lead to a decrease in transferase I kinase activity (which is known to be greatly enhanced by very low concentration of 3',5' cyclic adenylate), and the net result would be the conversion of transferase D to I. However, there was no lowering of 3',5' cyclic adenylate levels in rat diaphragm incubated with insulin either in the

absence or presence of epinephrine in the incubation medium (181). Insulin administration, *in vivo*, stimulated a rapid increase of rat skeletal muscle transferase I activity; this also was not accompanied by a decrease in 3',5' cyclic adenylate content of the tissue.

Villar-Palasi and Wenger (182) and Larner *et al.* (183) have recently reported results from their studies of the activities of the transferase interconverting enzymes in extracts of rat skeletal muscle after insulin injection. No difference in transferase phosphatase activity was detected. Transferase I kinase was shown to be decreased in muscle extracts of insulin injected rats, when assayed without 3',5' cyclic adenylate. There was no change in total transferase I kinase activity assayed in the presence of excess 3',5' cyclic adenylate.

If the physiologically active form of transferase I kinase is the 3',5' cyclic adenylate independent form, the reported effect of insulin could be explained by the conversion of this "active" (3',5' cyclic adenylate independent) form to "inactive" (dependent) form of transferase I kinase. This means that insulin acts at the kinase site to bring about a greater dependence on cyclic AMP and thus inactivate the kinase with no decrease in cyclic AMP tissue concentration. This in turn would result in the D form of transferase being converted to I form at an increased rate, leading to enhanced synthesis of glycogen.

There is no evidence, however, that the only physiologically active form of transferase I kinase is the 3',5' cyclic adenylate independent form.

D. Glycolysis and gluconeogenesis.

1. The enzymes unique to glycolysis.

The enzymes unique to glycolysis (from glucose-6-phosphate to pyruvate) are phosphofructokinase and pyruvate kinase.

a. Phosphofructokinase.

Phosphofructokinase catalyzes the phosphorylation of fructose-6-phosphate by ATP to produce fructose-1,6-diphosphate and ADP. Recently Lorenson and Mansour (184) have shown that crystalline sheep heart phosphofructokinase can also catalyze the reverse reaction, at a rate approximately 5% of that of the forward reaction. The same molecular state of phosphofructokinase catalyzes both reactions. The physiological significance of this finding is unknown.

Margreth (185) and Margreth et al. (186, 187) have reported data indicating that 25-30% of frog muscle phosphofructokinase is associated with the microsomal fraction of the muscle, the remaining portion is in the soluble fraction.

Phosphofructokinase is a complex enzyme, its enzymatic activity being regulated by a variety of agents. ATP (188-193) and citrate (194-197) are potent inhibitors of the enzyme. ATP inhibition can be overcome by small increases in ADP, AMP, inorganic orthophosphate (198), fructose-6-phosphate, 3',5'-cyclic AMP (189, 198), and fructose diphosphate (198). Likewise, NH_4^+, which increases during anoxia in certain tissues, is a potent stimulator of phosphofructokinase (192, 199). There exists in the enzyme a complex system of interacting binding sites for substrates, inhibitors and deinhibitors (200).

Lardy and co-workers (201, 202) have reported that phosphofructokinase from rabbit skeletal muscle consists of 4 identidal subunits (protomers) each of which contains 16-18 thiol groups. The sulfhydryl groups are essential for phosphofructokinase activity (203). Approximately 70% of the activity was reversibly lost when the enzyme was incubated with excess oxidized glutathione.

Phosphofructokinase undergoes a reversible conversion from active to inactive form (204); heart phosphofructokinase when incubated at pH 5.8 at 37° loses its activity. When the inactivated enzyme is incubated at pH 8.2 and 37° for 10 minutes, only a small part of the activity (lost during incubation at acidic pH) is recovered. When 10^{-3}M ADP or 10^{-3}M cyclic 3',5'-AMP is present during the incubation at pH 8.2, the amount of enzyme activity recovered is markedly increased. Less potent "reactivators" are AMP, ATP and GTP. Reactivation of the enzyme in an alkaline pH is likewise increased by fructose-6-phosphate and fructose 1,6-diphosphate. A combination of a nucleotide and a hexose phosphate is much more effective in reactivation of phosphofructokinase than either nucleotide or hexose phosphate alone. Both Mansour (204) and Paetkau and Lardy (203) concluded that at low pH the enzyme became dissociated into inactive subunits which reaggregated to an active form when the pH was raised in the presence of adenine nucleotides.

b. Pyruvate kinase.

Pyruvate kinase catalyzes the reaction:

$$\text{Phosphoenolpyruvate} + \text{MgADP} \xrightarrow{K^+} \text{Pyruvate} + \text{MgATP}$$

Pyruvate kinase of yeast (205), rat liver (206), bovine heart (205) and human erythrocytes (207), was found to be activated by fructose-1,6-diphosphate. The enzyme of muscle and rat liver is inhibited by ATP (208, 209). The enzyme is strongly dependent on K^+ ions. Pyruvate kinase from rabbit skeletal muscle has a mol.wt. of 237,000. It is a tetramer consisting of two identical particles or "protomers" each of which consists of two polypeptide chains similar in mass but probably not identical; each polypeptide chain is of mol. wt. 57,200. Each "promoter" has one site for phosphoenolpyruvate and for divalent metal (210).

There are two isoenzymes of pyruvate kinase: the (M) type, present in muscle, brain, heart, liver and kidney, and the (L) type, found only in liver and kidney (211). Type L appears to be under hormonal and dietary control.

Human pyruvate kinase exists in 3 isozymic forms (207), which have been designated in order of decreasing anodal mobility as pyruvate kinase I, present in erythrocytes and liver, pyruvate kinase II, present in kidney, and pyruvate kinase III, present in leucocytes, muscle, brain and thrombocytes.

2. The enzymes unique to gluconeogenesis.

a. Pyruvate carboxylase.

Pyruvate carboxylase catalyzes the carboxylation of pyruvate to oxaloacetate:

$$\text{Pyruvate} + HCO_3^- + \text{ATP} \xrightleftharpoons{\text{Acetyl-CoA, } Mg^{2+}} \text{Oxaloacetate} + \text{ADP} + P_i$$

The enzyme has been found in mitochondria of liver and kidney (212, 213) in various species. Some activity may be present also in brain and heart (213).

Pyruvate carboxylase requires acetyl CoA for activation (214). It exhibits also an absolute requirement for divalent cations such as Mg^{++} or Mn^{++}. The enzyme contains 4 moles of biotin per mole of protein (212).

The mechanism of the reaction, as proposed by Scrutton et al. (215) is as follows:

$$\text{Enzyme-biotin} + \text{ATP} + HCO_3^- \xrightleftharpoons{Mg^{2+},\ \text{Acetyl-CoA}} \text{Enzyme-biotin-}CO_2 + \text{ADP} + P_i \quad (1)$$

$$\text{Enzyme-biotin-}CO_2 + \text{Pyruvate} \rightleftharpoons \text{Enzyme-biotin} + \text{Oxaloacetate} \quad (2)$$

In purified preparations of pyruvate carboxylase, the presence of bound manganese has been demonstrated (216). The manganese is present in a stochiometry similar to that of biotin, i.e. 4 moles per mole of enzyme. The enzyme appears both to require a dissociable divalent metal ion, and to contain a bound metal ion. The bound manganese functions in step 2, while the added Mg^{+2} or Mn^{+2} functions in step 1 (217). Pyruvate carboxylase is inhibited by ADP (213) and acetoacetyl-CoA.

b. Phosphoenolpyruvate carboxykinase.

Phosphoenolpyruvate carboxykinase catalyzes the reaction:

$$\text{Oxaloacetate} + \text{GTP} \rightleftharpoons \text{Phosphoenolpyruvate} + \text{GDP} + CO_2$$

The enzyme utilizes GTP or ITP (218). It requires Mg^{2+} or Mn^{2+} for activity.

The intracellular location of the enzyme varies from species to species: in liver of rabbit and chicken the phosphoenolpyruvate carboxykinase is located in mitochondria, in rat and mouse liver it is in the cytosol, and in guinea pig liver it is in both compartments (219, 220).

c. Fructose-1,6-diphosphatase.

Fructose-1,6-diphosphatase is present in kidney and liver (221, 222). Newsholme and Randle (223) found that it is virtually absent from rat heart and diaphragm. Krebs and Woodford (224),however, found that the voluntary striated muscle of many vertebrate species possesses a fructose diphosphatase.

The enzyme catalyzes the hydrolysis of fructose-1,6-diphosphate and of sedoheptulose-1,7-diphosphate. It requires Mg^{++} or Mn^{++} for activity. The enzyme is active at neutral or alkaline pH (225). It is strongly inhibited by AMP (226-229) and by high concentrations of fructose-1,6-diphosphate (229, 230).

d. Glucose-6-phosphatase.

The enzyme is present in microsomes of liver, kidney and in small intestine (231, 232).

The hydrolysis of glucose-6-phosphate, of pyrophosphate, and the transfer of phosphoryl group from mannose-6-phosphate to glucose, or from pyrophosphate and from nucleosides di- and tri-phosphate to glucose, are likely to be catalyzed by the same protein (129, 130). All these activities are increased after cortisone administration

in adrenolectomized rats (233). They are also increased in starvation and alloxan diabetes (129, 130, 234). Administration of insulin to alloxan diabetic rats depresses their activities. Nordlie (130) suggests that these activities may constitute a compensatory mechanism for glucose phosphorylation in the diabetic animal - as in this condition glucokinase is markedly depressed, and these activities are increased. Having a high Km for glucose, this system could be well operating in diabetes where blood glucose is high.

3. Proposed pathways for phosphoenolpyruvate synthesis in gluconeogenesis, in different species and different tissues.

a. The mitochondrial-cytosol pathway.

In the rat and mouse liver, the phosphoenolpyruvate carboxykinase is located in the cytosol. The oxaloacetate formed in mitochondria from pyruvate by the pyruvate carboxylase reaction cannot diffuse to the cytoplasm (235, 236). It has to be converted to aspartate by the mitochondrial glutamic-oxaloacetic transaminase or to malate by malic dehydrogenase. Aspartate and malate can readily diffuse into the cytosol, where the same two enzymes, which are present in the cytosol, regenerate oxaloacetate through the reverse action. The phosphoenolpyruvate carboxykinase in the cytosol acts then on the oxaloacetate to form phosphoenolpyruvate. The NADH formed in the cytosol from the conversion of malate into oxaloacetate is used for the reduction of 1,3-diphosphoglycerate to glyceraldehyde-3-phosphate.

The concept of a mitochondrial-cytosol pathway of phosphoenol-

pyruvate synthesis is based on the assumption that pyruvate carboxylase is located primarily within the mitochondria in all species, as has been demonstrated for avian liver (237). However, in rat liver and kidney, this enzyme was found both in the mitochondria and the cytosol (238). This indicates that in the rat liver and kidney, synthesis of glucose-6-phosphate from pyruvate could also occur in the cytosol.

b. The mitochondrial pathway.

In the liver of rabbit and chicken, phosphoenolpyruvate carboxykinase is located in the mitochondria (219, 220); in these species the phosphoenolpyruvate is most probably synthesized in the mitochondria. It then diffuses to the cytosol where it serves as a precursor for glucose-6-phosphate synthesis. In these species, malate may serve only as a carrier for reducing equivalents.

c. The pyruvate kinase pathway in muscle.

The main gluconeogenic pathway in liver and kidney involves the pyruvate carboxylase and phosphoenolpyruvate carboxykinase reactions. In muscle, there is no pyruvate carboxylase (213, 239), so there is probably no dicarboxylic acid shuttle. Gluconeogenesis must proceed through the reversal of the pyruvate kinase reaction (240-242). This enzyme in muscle is far more active than in liver and kidney (243).

4. Control of glycolysis and gluconeogenesis.

Present ideas on the general control mechanisms for the processes of glycolysis and gluconeogenesis are that the control is exercised through the regulation of the activities of the key enzymes. Measure-

ment of the levels of hexose phosphates and the rate of glycolysis in a large variety of tissues have indicated that the activity of phosphofructokinase controls the glycolytic rate. The activity of phosphofructokinase and the rate of glycolysis are increased in vitro by agents which impair ATP synthesis, e.g. anoxia; this was demonstrated in heart muscle (244), brain (245), yeast (197), ascites tumor cells (246), erythrocytes (247), diaphragm (248) and kidney cortex slices (249). An activation of phosphofructokinase also occurs when glycolysis is increased during muscular activity (250, 251), and in heart muscle stimulated by epinephrine (252). All these observations suggest that the control at the phosphofructokinase step is a uniform property of the glycolytic system. The enzymes from different sources are similar in being inhibited by ATP and citrate, and activated by AMP, ADP, 3',5' cyclic AMP, fructose-6-phosphate, fructose-1,6-diphosphate and inorganic phosphate (188-200).

Pasteur effect: In yeast, the transition from anaerobic to aerobic conditions, which evokes the Pasteur effect of inhibiting glycolysis, raises the citrate level markedly (194), whereas the ATP-ADP ratio changes little. Citrate inhibits phosphofructokinase and it was regarded as the immediate cause of the Pasteur effect in this organism. Similar results have been obtained for mammalian cells; in liver, citrate is more inhibitory as the ATP level increases (253). It appears that the role of citrate is to potentiate effects produced by changes in the ATP level.

No changes in citrate level were observed in the perfused rat heart during transition from aerobiosis to anoxia (244). The stimulation of phosphofructokinase under these conditions was found to be re-

lated mainly to the rise in AMP and inorganic phosphate levels. In yeast, unlike heart, a decrease of citrate level seems to be the more important mechanism for phosphofructokinase activation (197).

A marked increase in citrate coincident with a decrease of phosphofructokinase activity has been observed in normal heart after the addition of fatty acids or ketone bodies (254), pyruvate or acetate (254, 255), and in hearts from diabetic (254, 256) and starved (254) animals.

AMP stimulates and ATP inhibits phosphofructokinase (189, 192). On the other hand, ATP stimulates and AMP inhibits fructose-1,6-diphosphatase (226-229). On the basis of these opposite effects, Krebs (257) suggested that ATP plays a decisive role in determining the direction of metabolism: glycolysis is favoured when the ATP level is low, and gluconeogenesis when it is high.

The phosphofructokinase and fructose-1,6-diphosphatase are also oppositely affected by the concentration of fructose-1,6-diphosphate. Phosphofructokinase is stimulated (198), whereas fructose-1,6-diphosphatase is inhibited (229, 230). The autocatalytic activation of phosphofructokinase is due to the powerful antagonistic effect of fructose-1,6-diphosphate to ATP and citrate inhibition (196, 198). Fructose-1,6-diphosphate further activates the pyruvate kinase by "feed-forward" activation (205, 206). Pyruvate kinase also was shown to be inhibited by ATP in heart and liver (208, 209). ATP is also an inhibitor of DPN-linked isocitric dehydrogenase (258) and of citrate

synthetase (259), and AMP or ADP are activators for these enzymes. This provides a link between glycolysis and the Krebs cycle; it seems that an increase in AMP and ADP accompanied by a decrease in ATP, will stimulate the glycolytic pathway and Krebs cycle, whereas an increase in the ATP:ADP(AMP) ratio will favour gluconeogenesis.

5. Fatty acid oxidation and the control of glycolysis and gluconeogenesis (the glucose-free fatty acid cycle).

a. Muscle.

Randle and co-workers (260-265) have demonstrated that addition of fatty acids or ketone bodies to incubation medium of isolated rat heart or rat diaphragm, diminishes glucose uptake and glucose utilization via glycolysis and oxidation in Krebs cycle. The same abnormalities in glucose metabolism are observed in alloxan-diabetes and in starvation. It was shown by Garland and Randle (265) that there is increased provision of fatty acids for respiration in heart and diaphragm muscle of alloxan-diabetic and starved rats, due in part to an increased rate of lipolysis and possibly in part to a diminished rate of re-esterification of fatty acids. This increased availability of fatty acids in alloxan-diabetes and starvation most probably causes the abnormalities in glucose metabolism in muscle. Garland and Randle (265) showed that in perfused hearts from diabetic or starved rats, or in hearts from normal fed rats perfused with medium containing fatty acids or ketone bodies, the concentrations of acetyl-CoA and citrate are increased. The disturbances in glucose metabolism under these circumstances may all be explained by the elevation in concentration of acetyl-CoA. The rise in the concentration ratio acetyl-CoA/CoA in-

hibits (feedback inhibition) pyruvate dehydrogenase, and this is believed to account for the inhibition of pyruvate oxidation. A rise in the acetyl-CoA/CoA ratio may also lead through the citrate synthetase reaction to a rise in the concentration of citrate, seen in these conditions. Citrate is an inhibitor of phosphofructokinase and hence glycolysis. Finally, the inhibition of hexokinase is believed to be secondary to phosphofructokinase inhibition, which leads to accumulation of glucose-6-phosphate a known inhibitor of hexokinase.

More direct evidence that phosphofructokinase is inhibited in heart from alloxan-diabetic or starved rats, or in hearts from normal rats perfused with medium containing fatty acids or ketone bodies, was obtained by measurement of hexose phosphates (262); the concentrations of fructose-6-phosphate and glucose-6-phosphate were increased in these states, and that of fructose-1,6-diphosphate was decreased.

b. Adipose tissue.

There is a striking difference between the muscle and adipose tissue in their response to fatty acids and ketone bodies. Fatty acids added to incubation medium depress the glucose uptake and utilization by rat diaphragm and perfused heart, but stimulate glucose uptake and oxidation to CO_2 by rat fat pad (266). Acetoacetate depresses glucose uptake and oxidation by muscle, but stimulates glucose uptake and G-1-C^{14} oxidation by rat epididymal fat pad. It impairs the insulin responsiveness of striated muscle but does not alter the insulin responsiveness of adipose tissue (267).

c. Liver and kidney.

In recent years it has been demonstrated that fatty acids stimulate the gluconeogenic process in liver and kidney; a stimulation of net glucose production by fatty acids has been shown in rat liver slices (268), kidney cortex slices (269), perfused rat liver (270-277) and rat liver *in vivo* (275, 278, 279). Weber *et al.* (209, 280-282) have demonstrated that fatty acids selectively inhibit the key enzymes of glycolysis and lactate production by liver. Fatty acid oxidation in isolated rat liver mitochondria inhibits citrate synthetase, pyruvate dehydrogenase, and both MAD and NADP-specific isocitrate dehydrogenase; it activates pyruvate carboxylase, and stimulates acetoacetate synthesis (283).

The stimulation of gluconeogenesis by fatty acids is mainly attributed to the increase in the acetyl-CoA/CoA and NADH/NAD^+ ratios by fatty acid oxidation.

(a) Regulation by acetyl-CoA/CoA ratio:

Increased intramitochondrial content of acetyl-CoA activates pyruvate carboxylase (284); Williamson *et al.* (285) have reported from their study of the effects of oleic acid infusion on the levels of metabolic intermediates of the gluconeogenic pathway in perfused rat liver, that fatty acid oxidation increases gluconeogenesis by enhancing the action of pyruvate carboxylase. Acetyl-CoA was also shown to exert an inhibitory action on hepatic glucokinase and on hepatic and muscle pyruvate kinase (280). An increase in the ratio of acetyl-CoA/CoA also inhibits pyruvate dehydrogenase (286, 287). Thus an elevation of acetyl-CoA/CoA ratio will favour gluconeogenesis and decrease glycolysis and

pyruvate utilization through the citric acid cycle.

(b) Regulation by NADH/NAD^+ ratio:

Williamson et al. (275, 285) have reported that when alanine is used as a gluconeogenic precursor in perfused rat liver, control of gluconeogenesis by fatty acid oxidation is exerted at the glyceraldehyde-3-P dehydrogenase step through elevation of cytoplasmic NADH/NAD^+ ratio (220, 287, 288), as well as by activation of pyruvate carboxylase. Thus the increase in both acetyl-CoA/CoA and NADH/NAD^+ ratios by fatty acid oxidation favours gluconeogenesis.

6. Insulin effect on glycolysis and gluconeogenesis in liver.

Insulin, in vitro, suppresses glucose output by the liver (178, 289) and enhances glucose oxidation in the perfused rat liver (290, 291).

Insulin in the liver induces synthesis (slow action) of the key glycolytic enzymes. In states of hypoinsulinism (alloxan diabetes and starvation) the key enzymes of glycolysis decrease (133, 135, 292, 293); insulin treatment of diabetic rats, or refeeding of starved rats, returns the activities to normal. The insulin-induced rise in the key glycolytic enzyme activities was shown to be dose-dependent (292). This insulin effect is blocked by administration of inhibitors of protein biosynthesis suggesting that it is due to de novo enzyme synthesis (133, 135, 292, 293).

Weber et al. (294-296) have provided evidence that insulin also acts as a suppressor of the biosynthesis of the key enzymes of gluconeogenesis. In starvation and alloxan-diabetes, when the insulin level decreases, the gluconeogenic enzymes are increased (295, 297-299) ;

insulin administration returns the gluconeogenic enzymes to normal. Injection of adrenocorticoid hormones increases the hepatic gluconeogenic enzymes (297); concurrent treatment with insulin can prevent the corticosteroid-induced enzyme biosynthesis (295).

7. Insulin effect on glycolysis in muscle.

Muscle apparently lacks the oxidative enzymes involved in the hexomonophosphate shunt, and because of the consequent failure to form TPNH, there is essentially no fatty acid synthesis. There is also a probable absence of dicarboxylic acid shuttle. The main pathways of glucose metabolism in muscle are the glycolysis and oxidation through the Krebs cycle, and glycogenesis.

Insulin increases the overall glycolysis in muscle as demonstrated by increased lactate formation from glucose in the rat diaphragm (300). Insulin also increases glucose oxidation to CO_2 by rat diaphragm (37, 165).

In the isolated frog sartorious muscle incubated in the absence of glucose, insulin lowered the concentration of glucose-6-phosphate during incubation in the presence of epinephrine. Under anaerobic conditions insulin also increased the formation of lactate (301).

8. Insulin effect on glycolysis in adipose tissue.

Glucose oxidation in adipose tissue is stimulated by insulin via both the Embden-Meyrhof pathway and the pentose cycle (302). Oxidation of glucose to CO_2 is decreased in adipose tissue from alloxan-diabetic (303) or pancreatectomized rats (304). Insulin prevents this defect.

Addition of insulin also causes an increase in lactate production by the isolated rat epididymal adipose tissue (42).

Lynn et al. (6) have shown that when adipose tissue is preincubated with digitonin and hyaluronidase, in the presence of large amounts of glucose, a condition in which glucose rapidly and completely equilibrates with tissue water, the addition of insulin caused an increased production of either lactate (anaerobically) or CO_2 and triglyceride (aerobically) from glucose. These results indicate that insulin may stimulate glycolysis in adipose tissue independently of its effect on glucose transport.

II. Insulin effect on lipid metabolism.

A. Fatty acid synthesis and esterification.

In the rat adipose tissue insulin increases the incorporation of glucose carbon into glyceride-fatty acid and glyceride-glycerol (305-308), reflecting effects on both fatty acid synthesis and esterification.

In human omentum, from adult subjects, insulin was shown to increase mainly the incorporation of glucose into glyceride-glycerol (308). The response of human adipose tissue to insulin shows age dependency; the most pronounced increase by insulin of incorporation of glucose-1-carbon into fatty acid was demonstrated in adipose tissue of children (309).

In the rat adipose tissue insulin, in the presence but not in the absence of glucose, stimulates lipogenesis from acetate or pyruvate (310).

Bally et al. (311)have demonstrated that insulin, in the presence, but not in the absence of glucose in the medium, increases the incorporation of palmitate-1-C^{14} into adipose tissue lipid, and depresses fatty acid oxidation.

The effect of insulin on triglyceride synthesis in adipose tissue appears to be dependent on its effect on glucose utilization which generates acetyl-CoA, NADH, and NADPH (via the pentose cycle), all of which are required for the synthesis of long chain fatty acids, and which also provides α-glycerophosphate for fatty acid esterification (312, 313) (adipose tissue has no glycerokinase activity (314, 315).

Foa et al. (5) have shown that insulin stimulates incorporation of acetate-1-C^{14} into lipids of 5-day-old chick embryo hearts, that is several days before the age when an insulin-sensitive transport system for glucose may be demonstrated. The effect was obtained also in hearts incubated in glucose-free medium. This suggests that the effect of insulin on the synthesis of fat from acetate in the heart muscle is not dependent upon the acceleration of glucose uptake.

B. Lipolysis and release of fatty acids.

Insulin diminishes the release of fatty acids from adipose tissue (316). The rate of fatty acid accumulation in adipose tissue, in vitro, may be affected in two ways by the action of insulin: first, insulin increases the rate of reesterification of fatty acids by increasing the provision of α-glycerophosphate from glucose. Second, insulin also appears to have a specific inhibitory action on the process of lipolysis initiated by catabolic hormones such as epinephrine, norepinephrine, adrenocorticotropin, cortisol and glucagon (317). The anti-lipolytic effect of insulin is independent of its effect on glucose uptake (318) as it can be demonstrated in the absence of glucose in the incubation medium.

The mechanism of insulin's anti-lipolytic action is probably dependent on its ability to inhibit production of cyclic AMP which is necessary to activate lipase. Butcher et al. (319) have reported that insulin inhibits epinephrine-induced increases in cyclic AMP levels in rat epididymal fat pads. Pretreatment of adipose tissue

with insulin resulted in a 30% reduction of adenyl cyclase activity (320). The antilipolytic effect of insulin is attributed to inhibition of adenyl cyclase activity, decreasing cyclic AMP formation and thus decreasing lipolysis (321).

The action of insulin on adenyl cyclase is indirect as no effect on the enzyme activity could be shown in homogenates of adipose tissue (320).

III. Insulin effect on protein synthesis.

A. Insulin effect on protein synthesis unrelated to its effect on glucose transport.

Insulin added, in vitro, was shown to stimulate protein synthesis in muscle under conditions where there was no extracellular glucose (322, 323). Thus insulin enhances protein synthesis in muscle independent of its action in facilitating entry of glucose.

B. Insulin effect on protein synthesis unrelated to its effect on amino acid transport.

Kipnis and Noall (324) were the first to demonstrate that insulin increases the accumulation of α-aminoisobutyric acid, a non-utilizable amino acid, in muscle. Later it was demonstrated that insulin can increase the entry of most, if not all, amino acids into the cell (325-331).

The stimulation of protein synthesis by insulin is independent of the hormone effect on amino acid transport (9). Thus, insulin in vitro, in the absence of substrate in medium, increased amino acid incorporation into protein in diaphragms from rats that had been previously injected with the labeled amino acid (332). Subsequently, Manchester and Krahl (333) and Wool and Krahl (334) demonstrated that insulin increased the incorporation into muscle protein of radioactivity from a variety of labeled carboxylic acids, which are precursors for the intracellular synthesis of amino acids. From these works it can be concluded that the insulin effect on protein synthesis is not related to its effect on amino acid transport.

C. Insulin effect on protein synthesis unrelated to its effect on RNA synthesis.

Insulin increases RNA synthesis in muscle (335-337). This increase, however, was also proved not to be required for the hormone-mediated stimulation of protein synthesis (338, 339), as actinomycin which suppresses RNA synthesis, did not interfere with insulin's effect to increase protein synthesis. It is concluded that although insulin increases glucose transport, amino acid transport and RNA synthesis, none of these effects can account for its stimulatory effect on protein synthesis (340).

D. Effect of insulin on protein synthesis in isolated muscle ribosomes.

Wool and co-workers (340-343) studied the effect of diabetes and insulin on the behavior of muscle ribosomes. The results obtained supported their suggestion that "the locus of action of insulin in stimulating protein synthesis is the ribosome" (343).

Wool et al. (340) have recently demonstrated that skeletal muscle ribosomes from diabetic animals have a reduced ability to catalyze protein synthesis as compared to ribosomes from normal animals. Treatment of diabetic animals with insulin (0.1 unit I.P.) reversed the defect due to diabetes within 5 minutes. However, insulin added in vitro to muscle ribosomes was without effect on their ability to synthesize protein.

The fact that insulin acted only when administered in vivo raised the possibility that insulin might have acted initially on a tissue other than muscle to cause release of some substance that then

affected the muscle ribosome. Another possibility was that the effect on the ribosome was secondary to an increase in transport of the substrate, in vivo, prior to the isolation of the ribosomes. These two possibilities were excluded when it was demonstrated that ribosomes from hearts of diabetic animals perfused with insulin, but without added substrate, were more active in carrying out protein synthesis than were ribosomes from hearts perfused without insulin (344). Thus "the effect of insulin to increase ribosomal protein synthesis is independent of net transport of substrate and is exerted directly on muscle" (340).

As pointed out before, the effect of insulin to increase ribosomal protein synthesis does not require RNA synthesis; actinomycin did not affect the ability of insulin to increase protein synthesis by ribosomes from diabetic animals. Puromycin and cyclo - heximide, however, inhibited the stimulation by insulin of ribosomal protein synthesis.

On the basis of these experiments Wool and co-workers (340) came to the hypothesis that either protein synthesis per se may be required for the action of insulin, or that insulin acts to bring about the synthesis of a "specific protein" ("translation factor"). This "translation factor" is then responsible for the binding and translation of natural messenger RNA by the ribosomes.

The chemical identity of the "translation factor" is unknown; this may be a protein or a product of a protein enzyme.

Cyclic AMP has no effect on skeletal muscle ribosomes from normal or diabetic animals (340), so it is most unlikely that cyclic

AMP is the "translation factor". The "translation factor" also does not appear to be a proteolytic enzyme as trypsin treatment of skeletal muscle ribosomes, inhibits rather than increases protein synthesis, in contrast to its stimulatory effect on protein synthesis in isolated ribosomes from unfertilized sea urchin eggs (345).

In their further search to identify the "translation factor" Wool et al. (340) have reported that by incubating diaphragm muscle from diabetic animals with radioactive amino acid, insulin,added in vitro, increased the radioactivity of all the proteins from the soluble fraction separated by discontinuous electrophoresis on polyacrylamide gel. However, one of the protein fractions showed a disproportionate increase as a result of the hormone. The function of this ribosomal protein(s) whose synthesis is so markedly stimulated by insulin is still unknown.

Wool et al. (340) compared the chemical and physical properties of muscle ribosomes from normal and diabetic rats; they found no differences in the RNA or in the ribosomal proteins. However, normal and diabetic ribosomes differed in their sedimentation properties: the ribosomal preparations from alloxan-diabetic animals contained a far smaller proportion of polysomes and more of the smaller particles. Administration of insulin to diabetic animals caused within 5 minutes reaggregation of the smaller particles to form polysomes. Administration of acinomycin to diabetic animals did not alter the insulin-induced formation of polysomes, indicating that insulin action does

not require RNA synthesis. Insulin probably affects the binding and translation of preformed messenger RNA.

Wool et al. (340) propose that the specific effect of insulin on ribosomal protein synthesis "is to increase the number of active ribosomes by initiating the formation of a 'translation factor' in a process that itself requires protein synthesis but not transcription of DNA.

METHODS AND MATERIALS

A. Animals.

Male Sprague-Dawley rats weighing 110-130 gm were used; they were fed ad libitum with Purina laboratory chow. The rats were given tap water to drink. They were kept in stock cages, 4-6 rats per cage, at room temperature of 75°F, humidity 50%, with the lights on from 7 a.m. - 7 p.m. Care was taken not to excite the rats prior to the experiment.

B. Preparation and incubation of rat diaphragms.

The rats were decapitated with a guillotine; the abdomen was opened and the diaphragm carefully excised. It was placed immediately in ice-cold Krebs Ringer bicarbonate (KRB) buffer (346). The diaphragm, while so immersed, was divided into two approximately equal parts, the thick posterior portion being discarded. Each hemidiaphragm was then gently blotted on moist filter paper and rapidly weighed on a torsion balance.

The hemidiaphragms were incubated at 37°C in 2 ml KRB buffer previously gassed with 95% oxygen: 5% CO_2 to pH 7.4 (346). One vessel of each pair of hemidiaphragms contained insulin; to the other flask was added an equal volume of HCl diluted to pH 3.5.

A stock insulin solution was prepared by dissolving 1 mg

(approximately 25 units) of crystalline insulin in 1 ml of HCl, pH 3.5; one drop of 1N HCl was added to dissolve the insulin completely. From this stock solution dilutions were made with HCl, pH 3.5.

The medium was supplemented with glucose and glucose-u-C^{14} when indicated in the experiments.

C. Estimation of $C^{14}O_2$, C^{14}-lipid fractions and C^{14}-glycogen.

These were estimated by methods described by Kahlenberg, Rubinger and Kalant (308):

1. $C^{14}O_2$.

The $C^{14}O_2$ was absorbed by hyamine (0.2 ml, 1.0 M), which had been injected at the end of the incubation period, through rubber cap of the Warburg flask into a removable vessel previously placed in the centre well. The metabolic activity was stopped and the CO_2 released from the incubation medium by tipping in trichloroacetic acid from a side-arm, to provide a final concentration of 10%. After the flask had been shaken for one additional hour at 37°C to facilitate absorption of the $C^{14}O_2$ by hyamine, the removable vessel was transferred to a counting vial containing 10 ml of scintillation fluid (prepared from 650 mg PPO and 39 mg POPOP in 100 ml toluene). The radioactivity of the solution was measured in a three-channel liquid scintillation counter, using the channels-ratio method for quench correction.

2. C^{14}-lipid fractions.

The C^{14}-lipid fractions were extracted from the tissue by the method of Vaughan (347). The total tissue lipid was extracted overnight in Dole's extraction mixture (348). Heptane and water were added. The mixture was shaken vigorously, then allowed to separate into two phases. The radioactivity of the total lipid in the heptane phase was determined. Another part of this heptane phase was extracted with Borgström's alkaline ethanol mixture (349) to remove free fatty acids; one portion (referred to as the triglyceride fraction) was assayed for radioactivity, another was saponified with alcoholic KOH, acidified, and extracted with heptane to obtain the glyceride fatty acids for determination of radioactivity. The C^{14}-total lipid, triglyceride, and triglyceride-fatty acid fractions were counted, after evaporating the heptane phase, in 10 ml of toluene counting solution described above.

3. C^{14}-glycogen.

Glycogen was released by digestion of tissue in KOH, by the method of Good, Kramer and Somogyi (350), precipitated with ethanol in the presence of 5 mg of carrier glycogen and hydrolyzed in 1 ml of 2N H_2SO_4. Aliquots (0.5 ml) of the hydrolyzate were assayed in the liquid scintillation counter, with 15 ml of dioxane counting solution (351).

D. Determination of glucose.

Glucose in the incubation media was determined by the glucose oxidase method of Washko and Rice (352) with the micro adaptation of Meites and Bowman (353). The method was modified in the present study as follows:

(a) The deproteinization step was omitted.

(b) 25 µl of sample or standards were added to 1 ml of water, followed by the addition of 1 ml glucose oxidase reagent and incubation for 30 minutes at 37°C. 4.0 ml of 7.6N sulfuric acid were added, and the color was measured spectrophotometrically at 540 mµ.

The standard curve of glucose estimated by this method is shown in fig. 1. A straight line relationship between the sample glucose content and the optical density existed in the range of glucose concentration tested.

The uptake of glucose by the hemidiaphragms was calculated from the difference, after incubation, between glucose concentration in control vessels containing only the incubation medium but no tissue, and the glucose concentration in the medium of the test vessels with the hemidiaphragms. The control and the test vessels were incubated simultaneously under identical conditions.

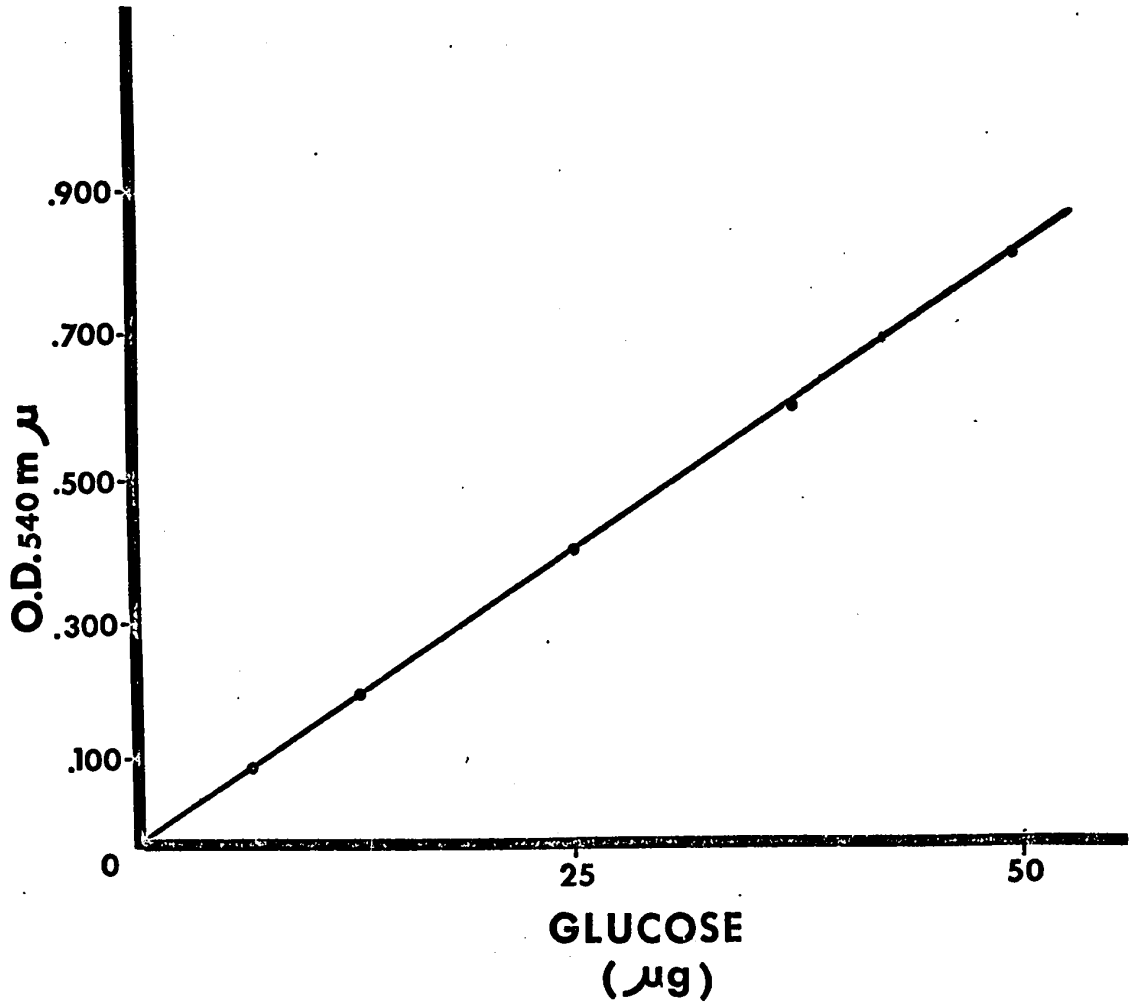

Figure 1

Glucose standard curve. Each value plotted is the mean of 10 determinations.

E. Method for the estimation of specific activity of the phosphorylated glycolytic intermediates.

A method was developed for the estimation of specific activity of the phosphorylated glycolytic intermediates of the rat hemidiaphragms incubated in medium containing glucose and glucose-u-C^{14}. The method consists of the following steps: extraction of the phosphorylated glycolytic intermediates from the tissue with perchloric acid, isolation by barium-alcohol precipitation, separation by thin layer chromatography, and the determination of amount and radioactivity.

1. Preparation of tissue extracts.

After incubation the vessels were placed in ice, the hemidiaphragms were removed, washed in cold KRB buffer, blotted , and rapidly frozen in a porcelain mortar immersed in liquid nitrogen. The frozen tissues were then pulverized in the mortar at liquid nitrogen temperature. A Potter & Elvehjem (354) homogenizer tube containing 2.5 ml of 3N perchloric acid (355) and a glass rod were preweighed. The tube was then placed in a container of solid CO_2 until the contents were frozen. The frozen powdered hemidiaphragms were placed on top of this frozen acid. The tube with the powder was then transferred to an alcohol bath kept at -10°C. The sample was stirred with the glass rod in the bath until the acid had com-

pletely penetrated the powder. This method of extraction prevents thawing of the tissue before acidification (356). The mixture in the test tube with the glass rod was reweighed (the difference giving the wet weight of tissue). 7.5 ml cold twice-distilled water were added to the mixture (355). The mixture was then homogenized in the cold for 10 minutes using a pestle attached to an electric motor at 1800 r.p.m. The test tube with the mixture was centrifuged at 4°C for 10 minutes at 3000 g. The supernatant was taken out and stored on ice. The precipitate was washed with 1 ml of 0.3N perchloric acid, centrifuged, and the supernatant was added to the first. The extract was then neutralized with saturated KOH to pH 6-7 and chilled on ice for 20 minutes. The potassium perchlorate was removed by centrifugation. The supernatant was stored overnight at -20°C.

2. Isolation of phosphorylated glycolytic intermediates.

The phosphorylated intermediates were isolated from the tissue extract by precipitation with barium and alcohol at pH 8.2, as follows: The extract was adjusted to pH 8.2 by the addition of 30 µl of 1% phenolphthalein and addition of KOH to a just discernible pink (357). The barium insoluble and alcohol insoluble phorphorylated compounds were precipitated together (358) by adding 0.4 ml of 25% barium acetate and 4 volumes of ethanol 95 per cent, and chilling at 4°C for 30 to 45 minutes. The pH was kept at 8.2 throughout the precipitation

by adding more of 1% phenolphthalein (100 µl) and adjusting the pH with 5% KOH. The precipitated material was isolated by centrifugation, washed with 80 per cent ethanol, and dried in air. The dried precipitate was then dissolved in 1 ml of 0.1N HCl. In order to remove barium ions from the isolated phosphorylated intermediates, the solution was shaken with an excess (0.76 g) of washed Dowex 50W-X_2 resin in the H^+ form (50-100 mesh). The Dowex was then removed by centrifugation and the solution was freeze dried, redissolved in 0.175 ml water and applied to a thin layer chromatograph for separation of the phorphorylated intermediates.

3. Separation of the phosphorylated glycolytic intermediates.

The phosphorylated glycolytic intermediates were separated by a thin layer chromatographic procedure based on the method of Grassetti et al. (359). Single plates instead of the "sandwich" method were used as no advantage was found by us by the use of the latter technique. The cellulose employed was Macherey - Nagel Type MN 300. The plates were coated with the slurry (15 g cellulose and 90 ml distilled water) which had been allowed to stand overnight. The plates were coated to a thickness of 0.3 mm and dried at 105°C for 1 hr. The plates were washed with the solvent to be used for development (acetone-acetonitrile-1N hydrochloric acid 64:26:10, v/v) until all the yellow-brownish impurity in the cellulose was accumulated on a filter paper in contact with the

cellulose layer at the top of the plate.

2 µl of standard solutions of the glycolytic phosphate ester salts containing 4 mg/ml of each compound were applied to the plate. After the development (at room temperature) the plates were dried. Visualization was achieved with the spray reagent of Hanes and Isherwood (360), followed by 10 minutes exposure to an ultraviolet lamp (Mineralight, Mod. No.B 50) at a distance of 10 cm.

The separation obtained by allowing the solvent to rise 18 cm from the bottom,is shown in fig. 2. It agrees well with the reported results of Grassetti et al. (359).

In order to improve the separation this method was modified. As Grassetti et al. (359) pointed out, 3-phosphoglyceraldehyde and dihydroxyacetone phosphate were found to give a streaky spot which contaminated other phosphorylated intermediates. These triose-phosphates were therefore removed by incubating the tissue extract with an equal volume of 2N KOH, for 20 minutes at room temperature and subsequently neutralizing with HCl (357). Under these conditions the triose phosphates are hydrolyzed without affecting the other phosphorylated intermediates (357). Fig. 3 shows the separation of the compounds obtained before and after KOH treatment.

As seen in figures 2 and 3, the method of Grassetti et al. (359) does not enable separation of fructose-6-phosphate from fructose-1,6-diphosphate. We succeeded in separating these two compounds

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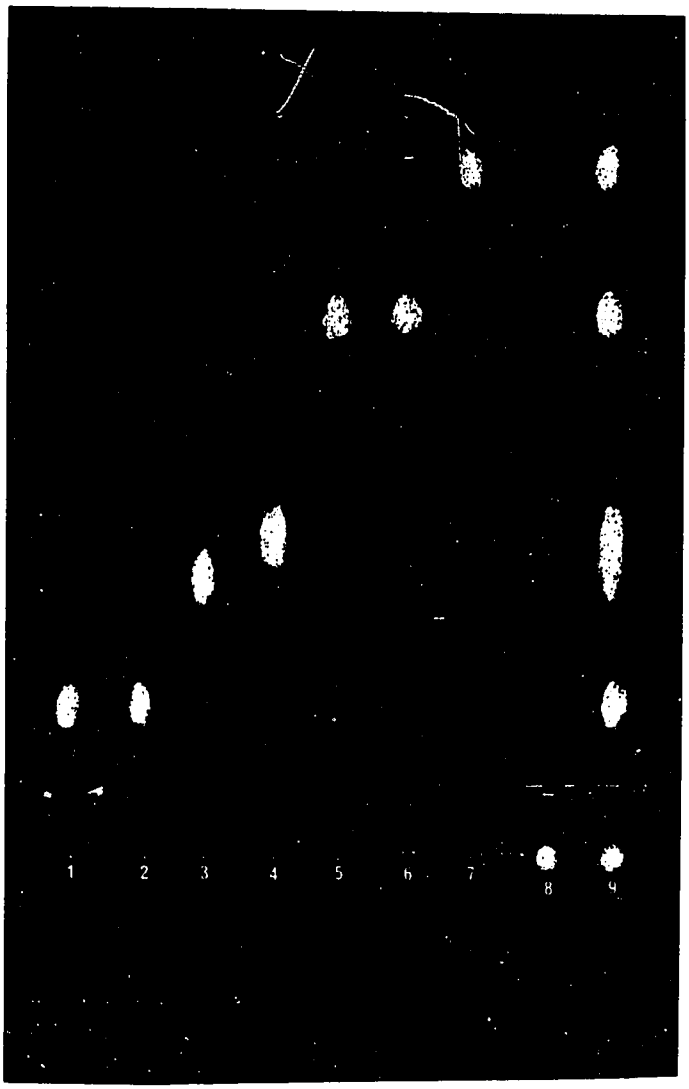


Figure 2

Drawing of thin-layer chromatogram of phosphorylated glycolytic intermediates. (1) = G-1-P; (2) = G-6-P; (3) = F-6-P; (4)=FDP; (5) = 3PGA; (6) = 2PGA; (7)=PEP; (8)=ATP; (9) = Mixture.

20 cm plate, coated with cellulose Macherey-Nagel Type MN 300.

Solvent: acetone-acetonitrile - 1N HCl (64:26:10, v/v).

Developed at room temperature.

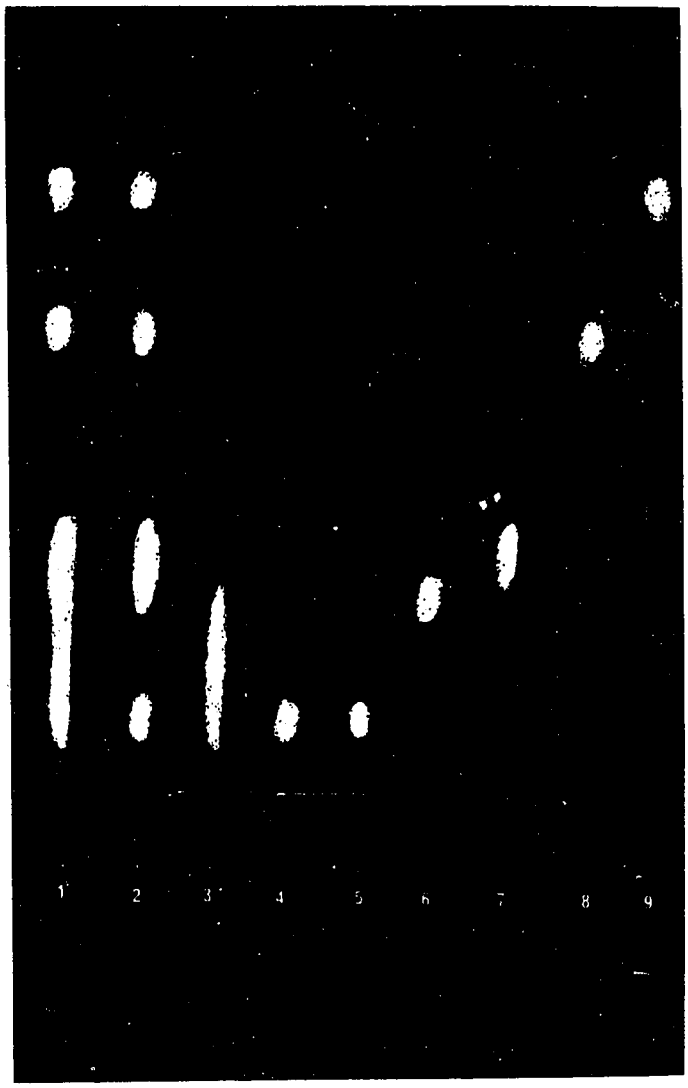


Figure 3

Drawing of thin-layer chromatogram of phosphorylated glycolytic intermediates. (1) = sample untreated; (2) = sample treated with KOH; (3)=GA-3P; (4) = G-1-P; (5) = G-6-P; (6) = F-6-P; (7) = FDP; (8) = 3PGA; (9) = PEP.

20 cm plate, coated with cellulose Macherey-Nagel Type MN 300.

Solvent: acetone-acetonitrile - 1N HCl (64:26:10, v/v).

Developed at room temperature.

by using long (41 cm) plates instead of the 20 cm plates. The solvent was allowed to rise 38-40 cm from the bottom. As shown in fig. 4, this longer development enables the separation of fructose-6-phosphate from fructose-1,6-diphosphate. Separation of the phosphorylated glycolytic intermediates isolated from the tissue extract was done on these long plates. Standards were applied simultaneously on one part of the plate; this portion of the plate was stained after the development, and the fractions in the test sample were cut out according to the position of the stained standards. Table I shows the position constants of the glycolytic phosphate esters on these long plates.

Another difficulty was to separate glucose-6-phosphate from glucose-1-phosphate. These two compounds occupied the same position whether the development was done on short plates (fig.2, 3), or long ones (fig.4). Also, no change in the solvent composition effected their separation. We have finally developed a method to separate these two compounds by prior hydrolysis of the glucose-1-phosphate followed by the separation of the free glucose from glucose-6-phosphate on DEAE cellulose plate. The spot from the first chromatograph on the long cellulose plates containing the mixture of G-1-P and G-6-P (see fig.4) was eluted in water, and the G-1-P was hydrolyzed (7 minutes, 1N HCl, 100°C) (357) without affecting the stable G-6-P. The sample was then freeze dried, dissolved in 50 µl water, and applied to small plates (microscopic slides, 7.5 x 2.5 cm), coated with DEAE cellulose. The development was done

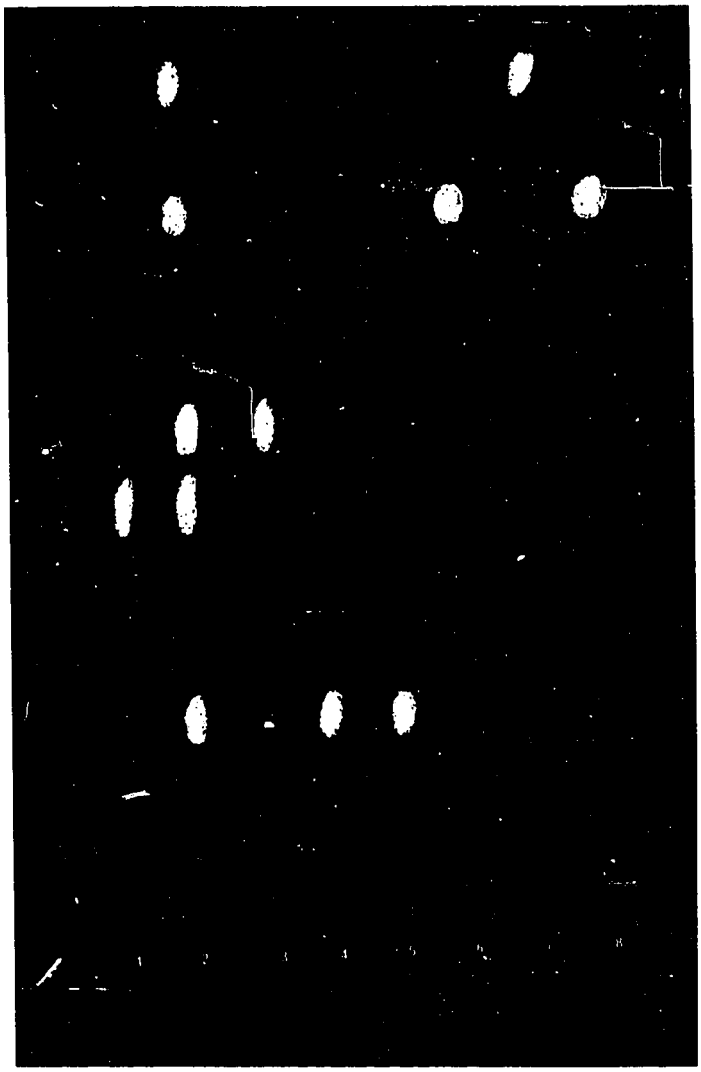


Figure 4

Drawing of thin-layer chromatogram of phosphorylated glycolytic intermediates. (1) = F-6-P; (2) = Mixture; (3) = FDP; (4) = G-6-P; (5) = G-1-P; (6) = 3PGA; (7) = PEP; (8) = 2PGA.

41 cm plate, coated with cellulose Macherey-Nagel Type MN 300.

Solvent: acetone-acetonitrile - 1N HCl (64:26:10, v/v).

Developed at room temperature.

TABLE I

Position Constants of Glycolytic Phosphate Esters (41 cm plates).

Phosphate ester	R_F
G-6-P	0.18
G-1-P	0.18
F-6-P	0.33
FDP	0.43
3PGA	0.62
2PGA	0.62
PEP	0.74

Solvent: acetone-acetonitrile - 1N HCl (64:26:10, v/v)

Chromatographed at room temperature.

Cellulose Macherey-Nagel Type MN 300.

at room temperature, in a small glass container saturated with the solvent isopropyl alcohol - formic acid - water, in the ratio 30:20:10 v/v (361). The sample or standards (16 µg) were applied in a spot at a distance of 0.7 cm from the bottom and the solvent was allowed to rise to a height of 6.5 cm from the bottom. The plates were dried in a stream of warm air, and run again in the solvent to the same height. The plates were then allowed to dry. The spots were detected using the spray reagent aniline hydrogen phthalate (0.1M aniline hydrogen phthalate in water-saturated n-butanol), which is a modification of the spray used by Lamkin et al. (362). The plates were dried and heated in an oven at 110°C for 15 minutes to develop the brown colour.

To prepare DEAE-cellulose plates, 7.5 g of DEAE cellulose (cellulose N,N-diethylaminoethyl ether) were shaken with 50 ml water. The mixture was then transferred to an electric blender. 7.5 g of cellulose (Macherey-Nagel Type MN 300) and 120 ml of distilled water were added and mixed in the blender for a few seconds. The small plates were coated with this slurry and allowed to dry overnight at room temperature.

Fig. 5 shows the separation of G-6-P from free glucose (derived from G-1-P after hydrolysis) on the DEAE cellulose plates. In our experiments we have measured only the G-6-P (fig. 5, lower spot).

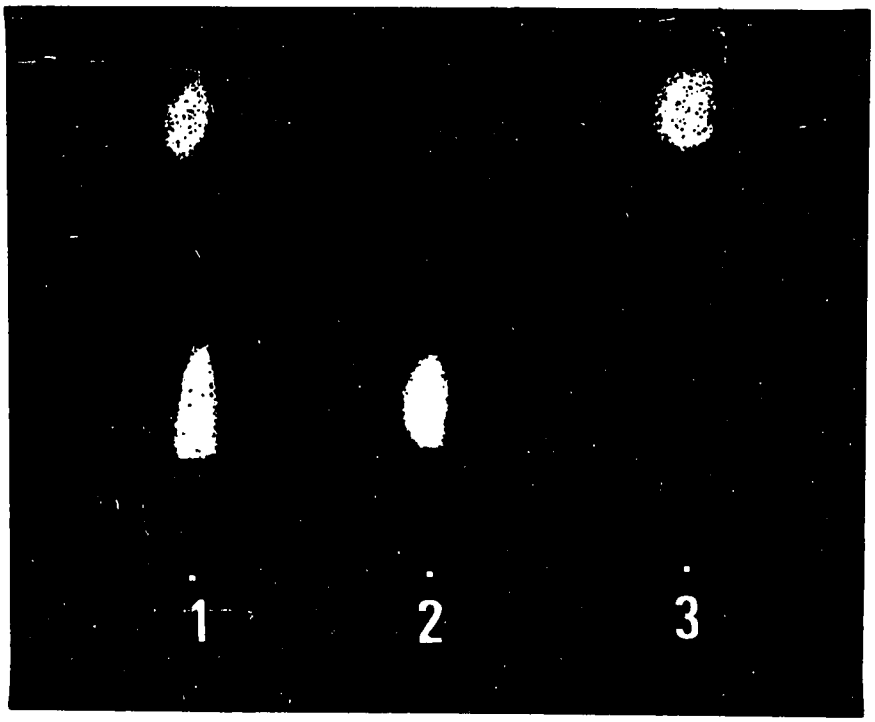


Figure 5

Drawing of thin-layer chromatogram showing separation of free glucose from G-6-P. (1) = Mixture of G-6-P and G-1-P after hydrolysis (1N HCl, 100°C, 7 min); (2) G-6-P; (3) = Glucose;
Plates (7.5 x 2.5 cm) coated with DEAE cellulose .
Solvent: isopropyl alcohol-formic acid - water (30:20:10,v/v).
Developed at room temperature.

4. Determination of radioactivity of the phosphorylated glycolytic intermediates.

The C^{14} in the separated glycolytic intermediates (G-6-P, F-6-P, FDP, mixture of 3PGA and 2PGA, and PEP) was measured as follows: the compounds, corresponding to stained standards, were cut out from the plate, eluted in 1.3 ml water, and the cellulose removed by centrifugation at 4°C. 50 µl of the solution of each separated compound was counted in liquid scintillation counter, in 10 ml of dioxane-naphthalene counting solution (351). The remaining solution was used for the estimation of the concentrations of the compounds. The dioxane-naphthalene counting solution consisted of 50 g naphthalene, 5 g of 2,5-diphenyloxazole (PPO) and 125 mg of 1,4-Bis- [2-(5-phenyloxazolyi)] -benzene (POPOP) per 500 ml of p-dioxane.

The samples were counted in a two-channel liquid scintillation counter. A commercial unquenched C^{14} standard, a set of C^{14} quenched standards and a blank for the background counts (obtained from Nuclear Chicago Corp.), were counted with the samples in each experiment. A blank containing 50 µl of water in 10 ml of dioxane-naphthalene counting solution was counted too and subtracted from the counts of each tested compound. The counting efficiency of each sample calculated by the channels-ratio method, ranged from 69 to 75 per cent.

5. Estimation of the concentrations of the phosphorylated glycolytic intermediates.

The concentrations of G-6-P, F-6-P, FDP, 3PGA, 2PGA, and PEP, were determined by enzymatic methods as described by Cartier et al. (355). The following modifications were introduced: (a) The concentration of the reduced diphosphopyridine nucleotide used in the measurements of FDP, 3PGA, 2PGA, and PEP, was lowered to 7.5 mM, as the original concentration (15 mM) was found to be too high (O.D. at 340 mμ over 1.000) to give accurate readings. The reduced concentration was still in sufficient excess to bring the reactions with each substrate to completion, as tested with known amounts of substrates. (b) The final volume of the reaction mixture was reduced from 3 ml to 1.5 ml by using half the volume of the solutions originally called for. This doubled the optical density changes (ΔE) and enabled the estimation of the intermediary compounds of glycolysis at very low concentration.

The standard curves obtained with known amounts of the glycolytic phosphate esters in a final reaction volume of 3 ml or 1.5 ml are shown in figures 6-11; a straight line relationship existed in the range of the compounds tested.

6. Recoveries.

A mixture of the phosphorylated glycolytic intermediates studied

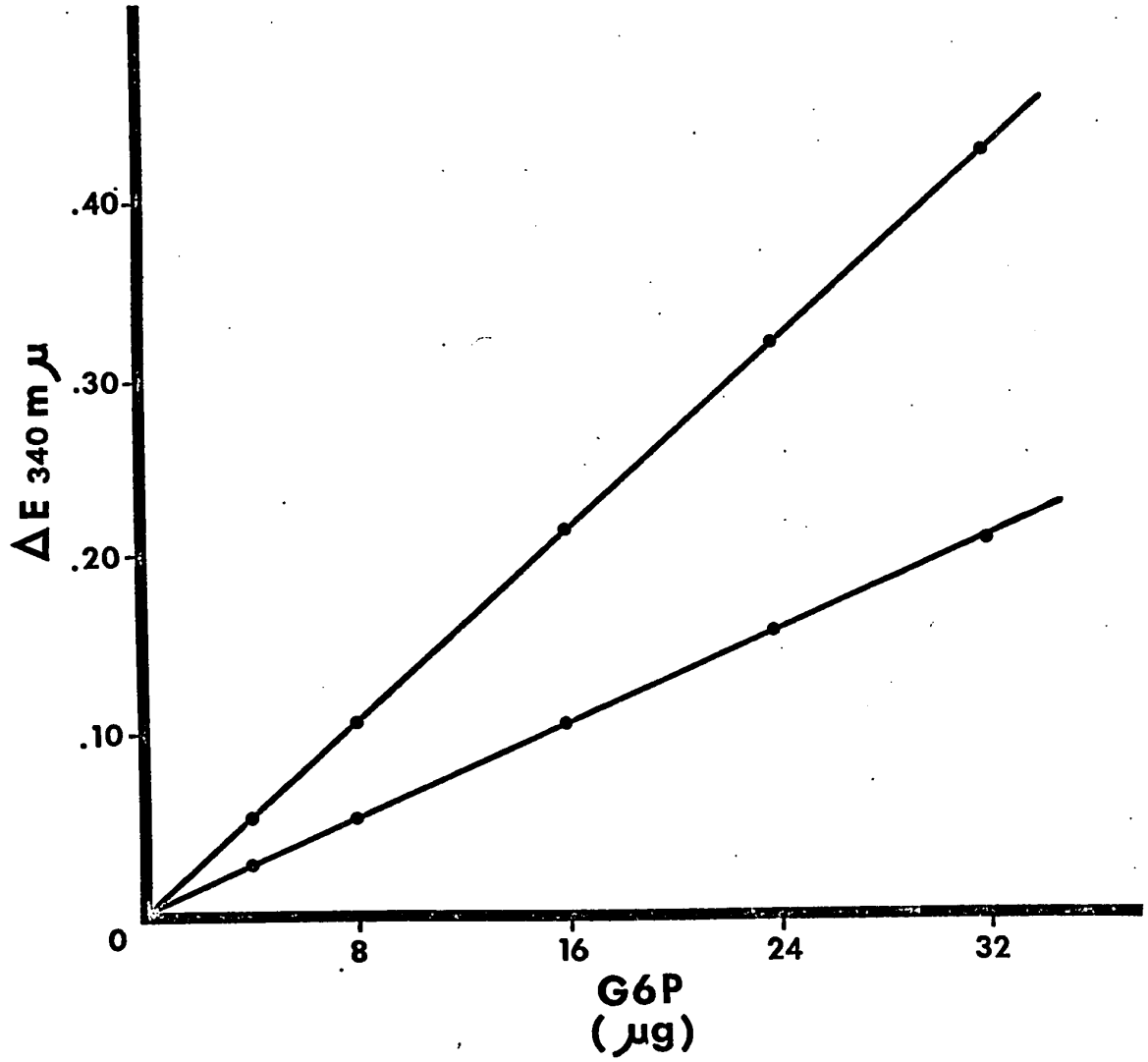


Figure 6

G-6-P standard curve. Upper curve: final reaction volume 1.5 ml. Each value plotted is the mean of 4 determinations.

Lower curve: final reaction volume 3.0 ml. Each value plotted is the mean of 6 determinations.

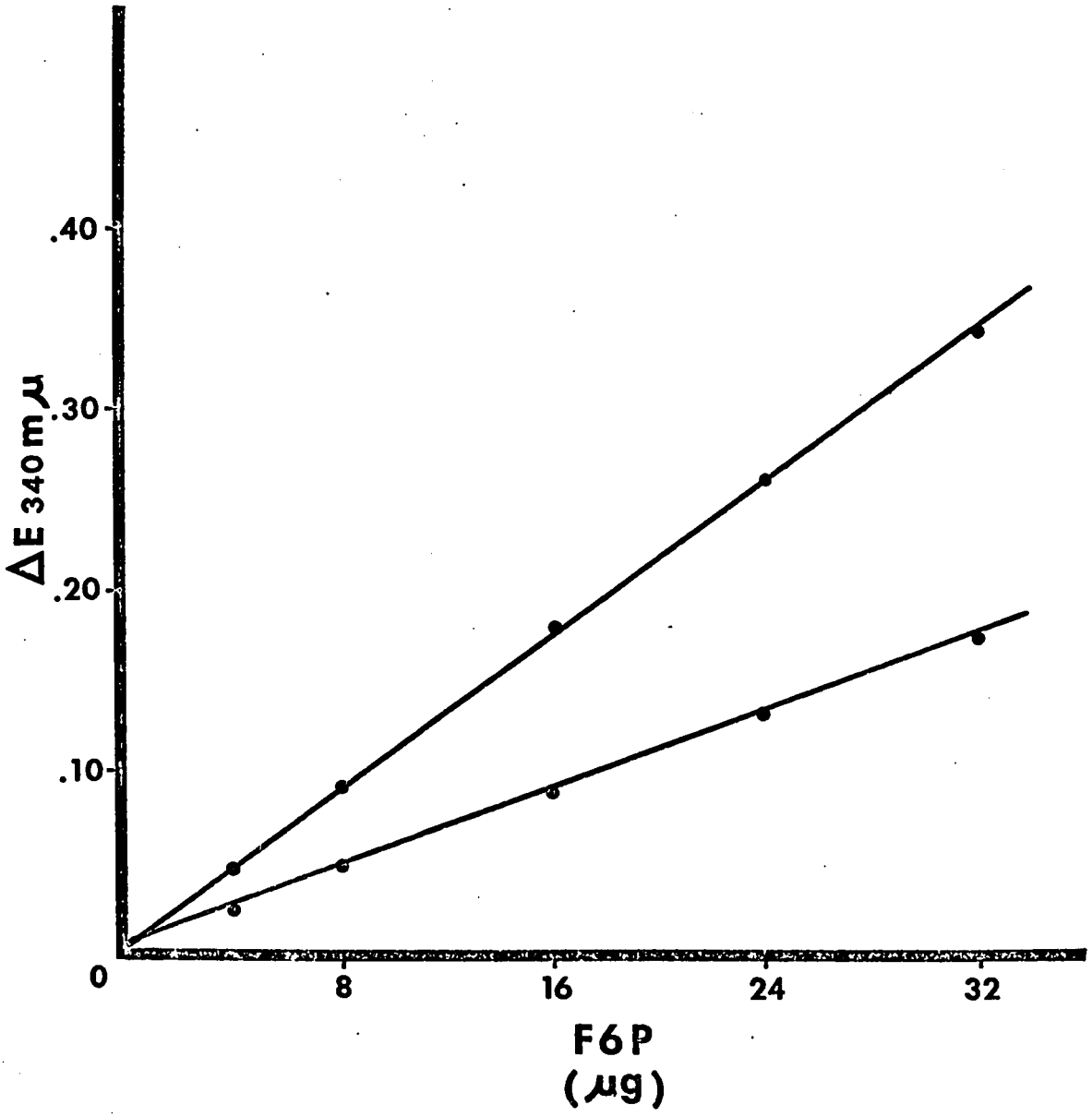


Figure 7

F-6-P standard curve. Upper curve: final reaction volume 1.5 ml. Each value plotted is the mean of 4 determinations.

Lower curve: final reaction volume 3.0 ml. Each value plotted is the mean of 6 determinations.

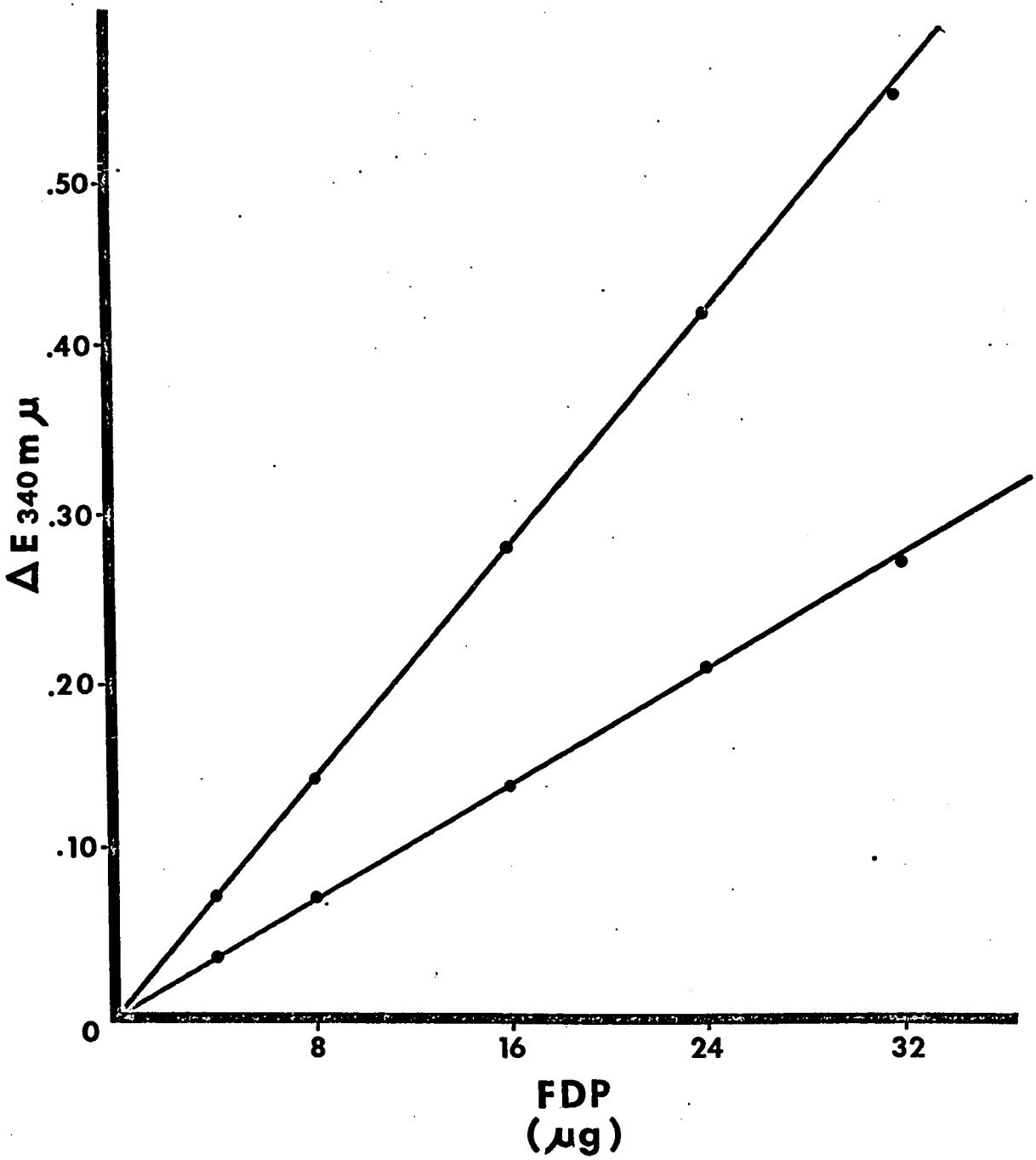


Figure 8

FDP standard curve. Upper curve: final reaction volume 1.5 ml. Each value plotted is the mean of 4 determinations.

Lower curve: final reaction volume 3.0 ml. Each value plotted is the mean of 6 determinations.

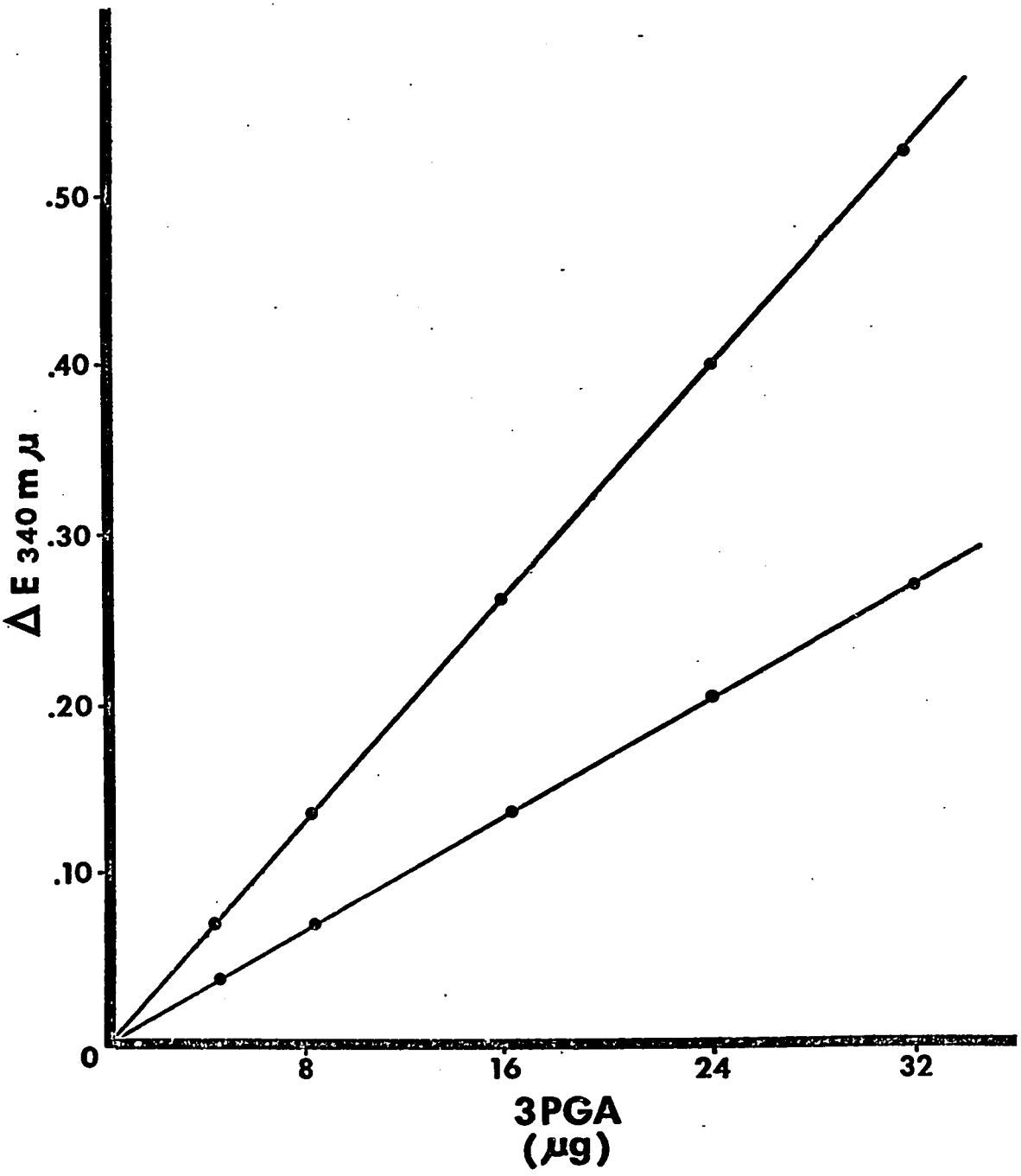


Figure 9

3PGA standard curve. Upper curve: final reaction volume 1.5 ml. Each value plotted is the mean of 4 determinations.

Lower curve: final reaction volume 3.0 ml. Each value plotted is the mean of 6 determinations.

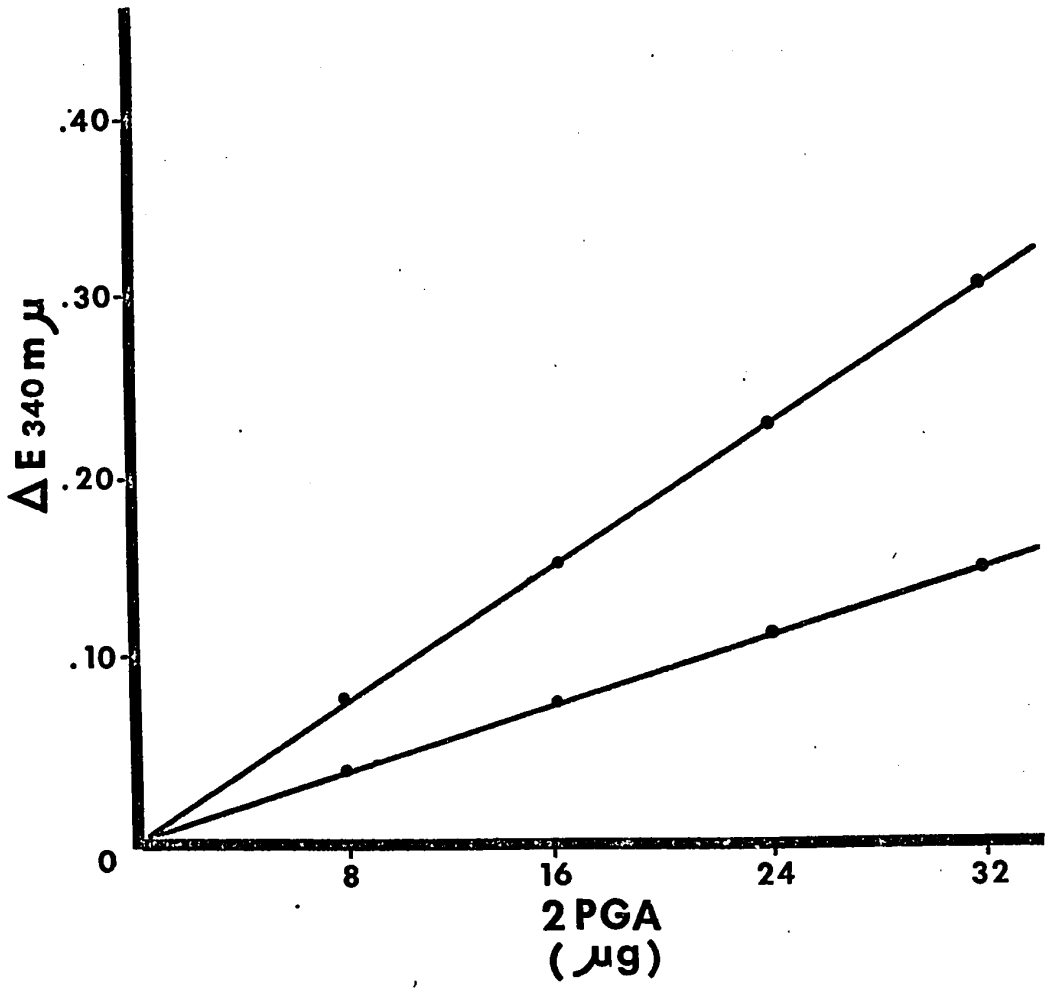


Figure 10

2PGA standard curve. Upper curve: final reaction volume 1.5 ml. Each value plotted is the mean of 4 determinations.

Lower curve: final reaction volume 3.0 ml. Each value plotted is the mean of 6 determinations.

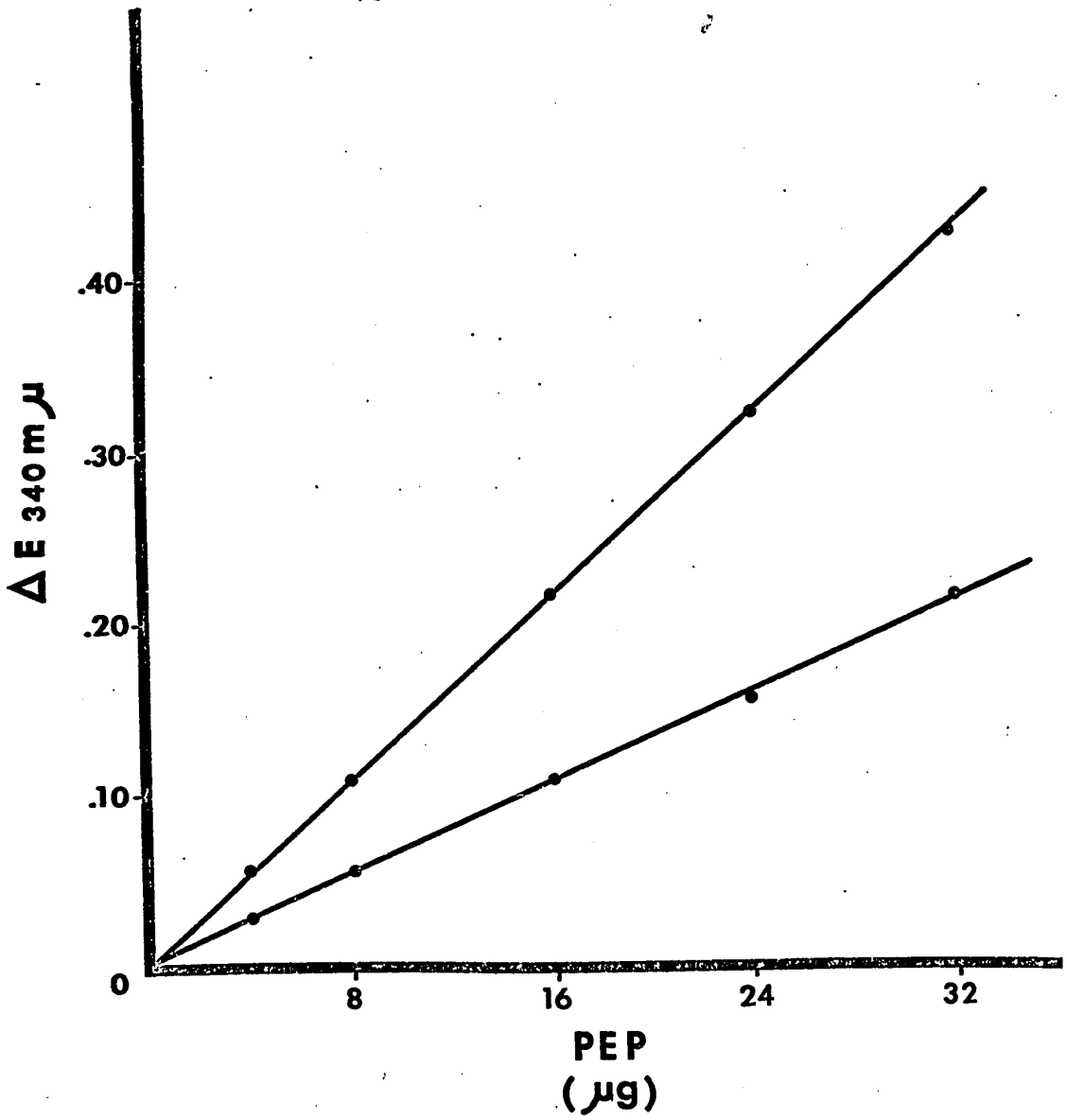


Figure 11

PEP standard curve. Upper curve: final reaction volume 1.5 ml. Each value plotted is the mean of 4 determinations.

Lower curve: final reaction volume 3.0 ml. Each value plotted is the mean of 6 determinations.

(G-6-P, F-6-P, FDP, 3PGA, 2PGA, and PEP) was passed through the whole procedure of isolation and thin layer chromatographic separation. The concentrations of the compounds tested were measured in the starting and the final solutions. Table II shows the percentage recovery obtained from a mixture containing 16 µg of each intermediate which was passed through the whole procedure. This amount was chosen as it is roughly close to that present per 14 hemidiaphragms (for each experiment 14 hemidiaphragms were pooled together to obtain measurable amounts of glycolytic intermediates). Preliminary determinations of the % recovery of the compounds from each step in the procedure, revealed that enormous losses can occur at the step of freeze drying, preceding the thin layer chromatographic separation, if the sample is allowed to stand under vacuum overnight. The freeze-drying process was therefore carefully watched not to be left longer than necessary for drying (about 2-3 hr). Taking this precaution, the final recovery of the intermediary compounds of glycolysis in 7 experiments was in the range of 89 ± 2 to 96 ± 3 per cent (Table II).

F. Materials.

D-Glucose-C^{14}(U) (specific activity 261 mc/mM), sodium DL-lactate-2-C^{14} (specific activity 35.6 mc/mM), sodium DL-lactate-1-C^{14} (specific activity 26.5 mc/mM), and sodium pyruvate-1-C^{14} (specific activity

TABLE II

Recoveries of the Phosphorylated Glycolytic Intermediates.

Compound	Recovery (%)
G-6-P	93 ± 1*
F-6-P	89 ± 2
FDP	95 ± 2
3PGA	96 ± 3
2PGA	90 ± 2
PEP	93 ± 3

* Mean ± S.E.M. in 7 experiments.

27.2 mc/mM) were obtained from Nuclear Chicago Co.

Recrystallized trypsin-heated beef insulin, Lot PJ-4609, was a gift from Eli Lilly and Co. Ltd.

The following enzymes were purchased from Boehringer Mannheim Corp.: Glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, glycerol-1-phosphate dehydrogenase, triose phosphate isomeras, aldolase, lactate dehydrogenase, pyruvate kinase, enolase, and phosphoglycerate mutase.

Reduced diphosphopyridine nucleotide, disodium and adenosine-5'-diphosphate, sodium, were purchased from P.L. Biochemicals Inc. Triphosphopyridine nucleotide, monosodium, was purchased from Calbiochem.

The glycolytic phosphate ester salts were purchased from Sigma Chemical Co.

The glucostat (for the enzymatic determination of glucose) was obtained from Worthington Biochemical Corp.

DL-sodium lactate was supplied as 60% syrup by Fisher Scientific Company. It was depolymerized by boiling and neutralized by addition of NaOH.

EXPERIMENTAL

INTRODUCTION

After the demonstration of Levine *et al.* (1, 2) of the action of insulin on cell membrane transport, the effects of insulin have usually been attributed to its ability to enhance the penetration of glucose into cells. In recent years, however, evidence has accumulated to indicate a variety of insulin effects independent of glucose transport (4-9, 134, 136-138, 301). These reports have encouraged us to study, whether insulin in the mammalian muscle acts directly on glucose utilization via glycolysis and oxidation in Krebs cycle, independently of its action on glucose transport and to localize the site(s) of its action.

A. Effect of insulin on glucose oxidation in rat diaphragm independent of its effect on glucose transport.

Two different experimental conditions were set up in which the effect of insulin on glucose transport has been eliminated:

1. Incubation in the absence of glucose in the medium.

Principle of the experiment.

The principle of this experimental design was similar to that used by Wool and Krahl (332) for studying the insulin effect on protein synthesis independent of amino acid transport. Rat hemidiaphragms were pre-loaded, in vivo, with glucose-u-C^{14} and then incubated in a glucose-free medium in the presence and absence of insulin. Under these conditions the insulin effect on $C^{14}O_2$ production from the intracellular glucose was studied. This experimental design eliminates the effect of insulin on glucose transport.

Procedure.

Rats were given an intraperitoneal injection of 2 μc of glucose-u-C^{14} in 0.25 ml saline (sp.ac. 261 mc/mmole). Ten minutes after injection, rats were decapitated; hemidiaphragms were prepared, weighed, then rinsed at 0°C in 10 ml of KRB for 5 minutes to allow equilibration of the extracellular glucose with the medium (Fig. 12). The concentration of radioactivity of this medium was measured; the total radioactivity present in the extracellular fluid of the tissue was calculated by assuming that the extracellular fluid

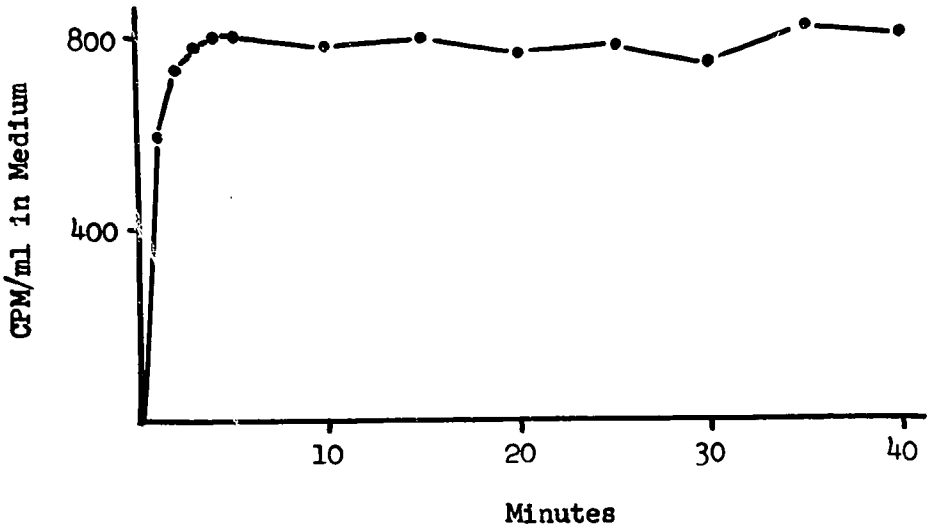


Figure 12

Rate of equilibration of glucose-C^{14} between extracellular fluid of rat hemidiaphragm and incubation fluid, at 0°C. Tissue from animal previously injected with glucose-C^{14} was rinsed in Krebs Ringer bicarbonate medium; aliquots of the medium were removed for assay of radioactivity at the times shown. Each point represents the mean of 6 determinations.

accounted for 35% of the wet weight (332, 363) and that the concentration of radioactivity in this fluid was the same as that in the rinsing medium.

Each hemidiaphragm was then gently blotted and transferred to a Warburg vessel containing 2 ml KRB. One flask of each pair also contained insulin (0.2 units/ml). Incorporation of radioactivity into CO_2 and glycogen was determined after incubation for various times.

Results.

Insulin produced an increase in $C^{14}O_2$ production of about 20% (Fig.13). The glycogen content of the muscle fell rapidly; insulin had no effect on the rate of fall (Fig. 13).

Prior treatment of the insulin with M/50 cysteine (49) completely abolished the effect on $C^{14}O_2$ production: in 8 experiments, the values were 384 $\pm$ 23 cpm (mean $\pm$ S.E.M.)/100 mg wet weight/h, and 350 $\pm$ 29 cpm/100 mg/h for control and cysteine-treated insulin, respectively. This established that the insulin effect noted previously was not a nonspecific protein effect.

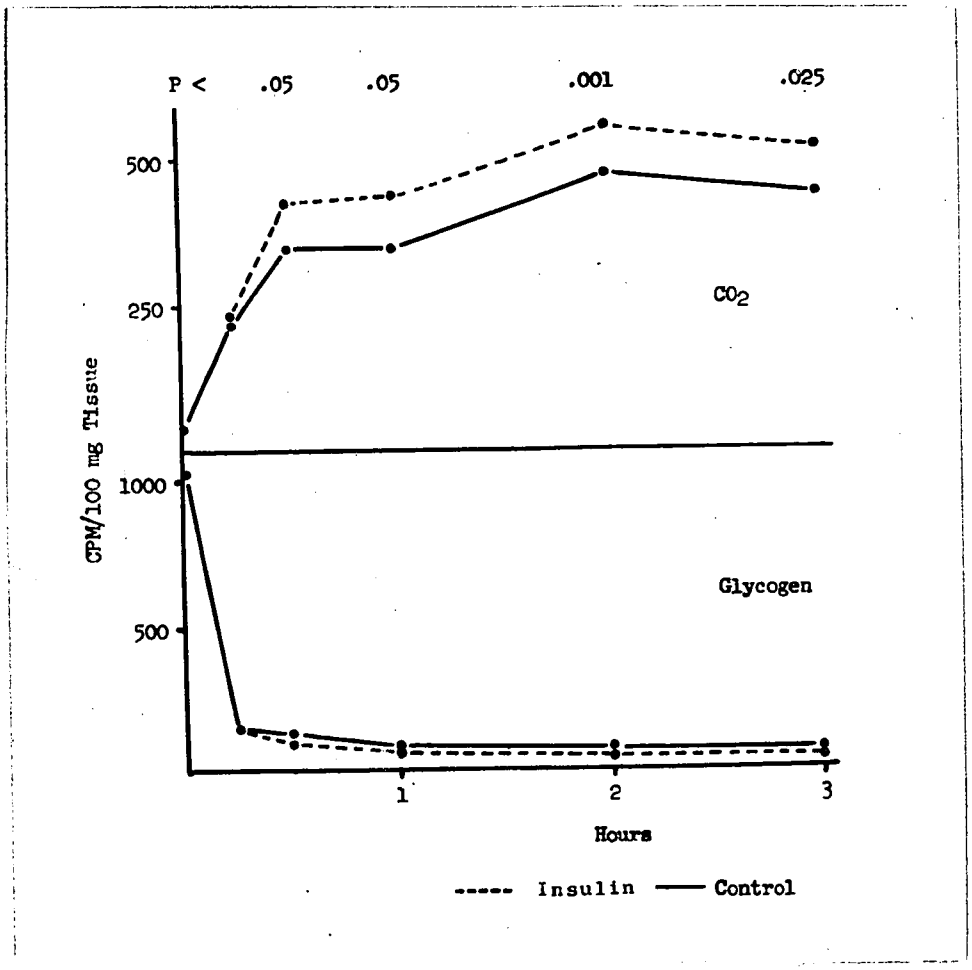


Figure 13

Production of $C^{14}O_2$ and loss of glycogen-C^{14} by hemidiaphragm obtained from rats previously injected with glucose-C^{14}, and incubated in Krebs-Ringer bicarbonate buffer, in the presence and absence of insulin (0.2 u/ml). P values for differences in $C^{14}O_2$ are based on values from paired hemidiaphgrgms; differences in glycogen-C^{14} are not significant. Values represent means of 18 experiments at 2 hours, and of 8 experiments at each of the other time intervals.

Discussion.

In these experiments, glucose-u-C^{14} entered the muscle cells, *in vivo*, and was rapidly utilized in the 10 minutes prior to removal of the diaphragm, since a significant amount had already been incorporated into glycogen and small amounts into $C^{14}O_2$ (Fig. 13, "0" time). Oxidation continued *in vitro*, and was enhanced by the addition of insulin. The mean radioactivity present in the extracellular fluid of the diaphragm at "0" time was 27.48 $\pm$ 0.78 (mean $\pm$ S.E.M.)cpm/100 mg tissue wet weight, while the mean increase in $C^{14}O_2$ produced by insulin in the subsequent 2-hour incubation was 84 cpm/100 mg tissue wet weight (Fig. 13). It is thus clear that transport and oxidation of all the radioactive glucose present in the extracellular fluid of the diaphragm at the beginning of the incubation could not account for all of the increase in $C^{14}O_2$ production brought about by insulin. The possibility that glucose leaked from the cell and that insulin increased the re-uptake of this glucose seems most unlikely since, as mentioned before, the injected glucose-u-C^{14} was rapidly metabolized *in vivo* in the 10 minutes prior to removal of the diaphragm (Fig. 13, "0" time), and so it appears that the glucose-u-C^{14} had already been "captured" in the cell as glucose-6-phosphate by the irreversible hexokinase reaction at the time when the tissue was incubated. We also know that free glucose accumulates

in the rat diaphragm only under special experimental conditions where transport exceeds the phosphorylating capacity of the hexokinase system, such as incubation in a medium containing a very high concentration of glucose or when the phosphorylating capacity of the hexokinase is reduced by low temperature (71). It is therefore probable that the insulin effect resulted from stimulation of glycolysis and oxidation by a direct intracellular action.

The results indicating a fall in glycogen content of the diaphragm (Fig. 13) incubated in the absence of glucose, and no effect of insulin on the rate of fall are compatible with those reported by Fisher and Williamson (364) in heart perfused with nutrient-free Krebs-bicarbonate medium.

2. Incubation in the presence of glucose in the medium.

Principle of the experiment.

Paired hemidiaphragms were incubated with glucose in the presence and absence of insulin; in the latter case the glucose concentration was increased so that glucose uptake was approximately equal for the paired tissues. Under these conditions, in which again the effect of insulin on glucose transport has been eliminated, insulin effect on glucose incorporation into CO_2, glycogen and lipid was studied.

Procedure.

Measurements were made of rates of glucose oxidation of paired hemidiaphragms, one incubated in KRB containing insulin and a glucose concentration of 5.56 mM, the other in KRB containing no insulin and a higher glucose concentration selected to provide a glucose uptake equal to or slightly greater than that of the first hemidiaphragm. All flasks also contained glucose-u-C^{14} sufficient to give a specific activity of 0.036 mc/mmole. Glucose uptake and CO_2 production were measured after incubation for 2 hours. Control vessels containing only the incubation media were included in each experiment.

Results.

The results (Table III) indicate that tissue incubated in the presence of insulin, with comparable or even lower levels of glucose uptake, had a CO_2 production greater than that of tissue incubated without insulin. The insulin effect diminished with decreasing concentration but it was still evident and statistically significant at concentrations within the physiological range. In all these experiments the concentrations of glucose were carefully selected so that the glucose uptake in the presence of insulin would never exceed that of the control. The glucose uptake by the control was in some cases much higher than those incubated with insulin, this can account for the fact that the percentage increase in $C^{14}O_2$ prod-

TABLE III

Insulin Effect on Glucose Incorporation into CO_2

Expt.	Glucose Conc.* (mM)	Insulin Conc. (units/ml)	Glucose Uptake (mg/g wet wt./2 h)	% Increase in $C^{14}O_2$ Production with Insulin
1	5.56	2×10^{-1}	6.45 ± 0.11 (16)**	53 ± 17
	8.61	—	6.69 ± 0.12 (16)	*** $p < 0.01$
2	5.56	10^{-2}	6.22 ± 0.13 (15)	19.0 ± 2.7
	8.33	—	6.62 ± 0.12 (15)	$p < 0.001$
3	5.56	10^{-3}	5.18 ± 0.23 (6)	20.0 ± 5.3
	8.33	—	6.57 ± 0.22 (6)	$p < 0.025$
4	5.56	10^{-4}	4.98 ± 0.21 (5)	15.2 ± 4.5
	6.94	—	5.64 ± 0.17 (5)	$p < 0.05$

* Each flask also contained .036 mc glucose-U-C^{14}/mole glucose.

** Mean, standard error, and number of determinations.

*** p values were calculated according to student's test on paired hemidiaphragms.

uction by insulin was not very linear; nevertheless it was clearly dependent on the dose of insulin.

In one experiment, the incorporation of label into glycogen and lipid was also determined (Table IV). The conditions were identical to those of Table III (Expt. 2). All the parameters indicated in the table were increased by insulin, although the glucose uptake was slightly lower in the insulin-containing flask.

A very small part of the triglyceride radioactivity was found in the fatty acid fraction, the larger part being in the glycerol fraction. Both were increased by insulin but only the increase in the glycerol radioactivity was statistically significant.

Discussion.

This is further evidence of a direct action of insulin on glycolysis and oxidation in muscle, not dependent on glucose transport. The same conclusion was reached by Lynn et al. (6) and by Autor and Lynn (7) for rat adipose tissue.

The increase by insulin of glucose incorporation into glycogen confirms the well-known direct intracellular effect of insulin on glycogenesis (4, 173).

The fact that insulin increased the incorporation of glucose into glyceride-glycerol gives additional information as to the site of insulin action which is probably in the glycolytic pathway on some

TABLE IV

Insulin Effect on Glucose-U-C^{14} Incorporation into CO_2, Glycogen and Lipid by Isolated Rat Hemidiaphragm.

Glucose uptake	(a)	6.11 ± 0.13*	
(mg/g wet wt./2 h)	(b)	6.67 ± 0.14	
$C^{14}O_2$	(a)	73,973 ± 2,200	20.5 ± 3.3**
(cpm/g wet wt./2 h)	(b)	61,437 ± 1,400	$p < 0.001$
C^{14}-Glycogen	(a)	93,936 ± 4,400	190 ± 37
(cpm/g wet wt./2 h)	(b)	35,814 ± 3,800	$p < 0.001$
C^{14}-Total Lipid	(a)	27,724 ± 1,200	22.1 ± 5.3
(cpm/g wet wt./2 h)	(b)	22,768 ± 710	$p < 0.005$
C^{14}-Triglyceride	(a)	25,969 ± 1,100	21.4 ± 6.9
(cpm/g wet wt./2 h)	(b)	21,589 ± 2,400	$p < 0.025$
C^{14}-Triglyceride Fatty Acids	(a)	2,548 ± 510	49.8 ± 29
(cpm/g wet wt./2 h)	(b)	1,817 ± 260	$p > 0.2$

(a) Glucose concentration 5.56 mM, insulin 10^{-2} u/ml.

(b) Glucose concentration 8.33 mM, no insulin.

* Mean ± standard error of mean of 9 experiments.

** % Increase produced by insulin.

step(s) above the formation of α-glycerophosphate.

Conclusions.

Both experiments provide evidence of a direct effect of insulin on glucose utilization via glycolysis and oxidation through the Krebs cycle in the mammalian muscle, the effect being independent of glucose transport.

B. Studies of the effect of insulin on oxidation of lactate in rat diaphragm.

Introduction.

The previous results showed that insulin increases glucose oxidation in the rat diaphragm independently of its effect on glucose transport. Our next attempts were to locate the site(s) of insulin action. The effect could be either on some step in the Embden-Meyerhof pathway or in the Krebs cycle (as the pentose cycle is not significant in muscle).

In the present experiments the effect of insulin on the

oxidation of lactate labeled in carbon 1 or carbon 2 was studied.

Procedure.

Paired hemidiaphragms were incubated in 2 ml of KRB medium containing sodium DL-lactate-1-C^{14}, or sodium DL-lactate-2-C^{14}, and unlabeled DL-sodium lactate. The final specific activity of each substrate in the incubation medium was 0.1 μc/μmol. One flask of each pair of hemidiaphragms contained insulin (0.1 u/ml). The hemidiaphragms were incubated at 37° for two hours.

The incorporation of C^{14} from lactate into CO_2 was measured.

Control vessels containing only the incubation media were included in each experiment.

Results.

The results are presented in Table V. More of lactate carbon 1 than of carbon atom 2 appeared as $C^{14}O_2$. Insulin caused a very small, but statistically significant increase of 8.7 $\pm$ 1.7 per cent in $C^{14}O_2$ production from lactate-1-C^{14}. There was no effect on lactate-2-C^{14} oxidation.

TABLE V

Effect of Insulin on Oxidation of Sodium Lactate in Isolated Rat Hemidiaphragm.

	Insulin**	$C^{14}O_2$ (cpm/g wet wt./2 h)	% Increase produced by insulin
DL-Lactate-1-C^{14}***	+	242,483 ± 21,876 (12)*	8.7 ± 1.7
	-	225,212 ± 21,560 (12)	p< 0.001
DL-Lactate-2-C^{14}***	+	153,845 ± 11,533 (10)	3.9 ± 3.7
	-	146,371 ± 6,836 (10)	p< 0.4

* Mean, standard error, and number of determinations.

** Insulin, 0.1 u/ml.

*** DL-Lactate-1-C^{14} or DL-Lactate-2-C^{14} s.a. = 0.1 μc/μmol.

Discussion.

The small effect of insulin on lactate-1-C^{14} oxidation, and the lack of effect on the oxidation of lactate-2-C^{14}, suggests that insulin has some effect on the reaction of decarboxylation of pyruvate catalyzed by pyruvate dehydrogenase.

Villee and Hastings (365, 366) found no effect of insulin on the oxidation of pyruvate-2-C^{14} to carbon dioxide in diaphragms from normal rats; our results with lactate-2-C^{14} are compatible.

No studies on oxidation of lactate-1-C^{14} or pyruvate-1-C^{14} to CO_2 by the rat diaphragm have been reported. In the frog sartorious muscle, the oxidation of lactate-1-C^{14} to CO_2 was found to be increased by insulin (367). In frog sartorious muscle in summer, but not in winter, insulin also stimulated the oxidation of pyruvate labeled in carbon atoms 1, 2 or 3 (368). In this respect frog muscle differs from the mammalian muscle.

The stimulation of lactate-1-C^{14} oxidation, indicates that insulin has some effect on the step of decarboxylation of pyruvate catalyzed by pyruvate dehydrogenase. This effect cannot, however, account for the much higher increase by insulin in CO_2 production from glucose (Table III). Also the fact that we got an increase by insulin of the incorporation of C^{14} from glucose into the glycerol fraction of lipid (Table IV) which means an increase in the α-glycerophosphate (as no glycerokinase was found in muscle (366)), led us to the conclusion that there must be an additional site(s) of insulin action in the Embden-Meyerhof pathway above the step of α-glycerophosphate formation.

C. Studies of the effect of insulin on glycolysis in the rat diaphragm.

Introduction.

We have concluded from the previous experiments: (a) that insulin acts on glucose oxidation independently of its effect on glucose transport, (b) that the site of its action is not the Krebs cycle as no effect could be detected on the oxidation of lactate-2-C^{14} to CO_2.

Our next goal was therefore to try to locate the site of insulin action on glycolysis in muscle, by studying the effect of insulin on the glycolytic intermediates under conditions identical to those of experiment A 2 (incubation in the presence of glucose and glucose-u-C^{14}, in circumstances where the effect of insulin on glucose transport is eliminated).

The first step was to develop a suitable method for the isolation, separation, and estimation of specific activity of the glycolytic intermediates (p.58 - 70).

Before starting our studies, certain control experiments were performed.

1. Control experiments.

The purpose of these experiments was two-fold:

(a) To study whether radioactivity is detected in the phosphorylated glycolytic intermediates of unincubated rat hemi-

diaphragms, from added glucose-u-C^{14}.

(b) To determine the unincubated levels of the phosphorylated glycolytic intermediates in the rat diaphragm.

Principle of the experiment.

Uniformly labeled C^{14}-glucose and unlabeled glucose were added to unincubated frozen diaphragms (the amount added was calculated to be approximately that which would be retained in the extracellular fluid of the hemidiaphragms from the incubation media). The phosphorylated glycolytic intermediates were extracted from these unincubated hemidiaphragms, isolated, and separated by thin layer chromatography. Each separated intermediate was tested for radioactivity. In the same experiment the concentration of the glycolytic intermediates was also measured to determine the steady state concentrations of the intermediates, in vivo.

The assumption was that if radioactivity were detected in the glycolytic intermediates of the unincubated rat diaphragm it would mean that,a)either the original glucose-u-C^{14} is contaminated by phosphorylated glycolytic intermediates, or b) that some metabolic changes (incorporation of the glucose-u-C^{14} into the phosphorylated glycolytic intermediates) can take place without incubation, at the temperature of liquid nitrogen. Each of these two possibilities would introduce an error in the results.

Procedure.

15 rats were given nembutal (5 mg/100 gm intraperitoneally), and slightly cooled with liquid nitrogen as soon as they reached light surgical anaesthesia (2-4 minutes) (357). The dissection was carried out in a cold room, the diaphragm was frozen, in situ, with liquid nitrogen and cut out while being frozen. The diaphragms were kept in a porcelain mortar immersed in liquid nitrogen. 525 µl of a solution containing 0.5 µc of glucose-u-C^{14} and 787 µg of glucose was added to the frozen hemidiaphragms, and ground together in the mortar. From these ground hemidiaphragms, the phosphorylated glycolytic intermediates were extracted, isolated and separated by methods described on p.58-70. Aliquots of each compound were measured for radioactivity and amount.

Results.

(1) Radioactivity in the glycolytic intermediates:

The results are presented in Table VI. As can be seen, radioactivity was detected in all of the indicated compounds except G-6-P in the unincubated rat diaphragms.

(2) Unincubated levels of the glycolytic intermediates of rat diaphragm:

The results are presented in Table VII. The values are close to the unincubated levels reported by Karl et al. (369) except for a

TABLE VI

Radioactivity in Phosphorylated Glycolytic Intermediates.

(Control Experiments)

Compound	Radioactivity (cpm/1.5 g wet wt.)	
	Exp. (1)	Exp. (2)
G-6-P	0	0
F-6-P	610	608
FDP	712	688
3PGA + 2PGA	640	628
PEP	1,322	1,264

TABLE VII

Unincubated Levels of the Phosphorylated Glycolytic Intermediates in Rat Hemidiaphragm.

Compound	Concentration (nmoles/g wet wt.)	
	Exp. (1)	Exp. (2)
G-6-P	151.0	154.0
F-6-P	23.1	23.3
FDP	19.9	19.6
3PGA	33.4	33.6
2PGA	8.7	8.9
PEP	22.7	22.5

higher level of G-6-P. This difference can be attributed to the nutritional state of the animals; the animals used by us were fed ad libitum, while Karl et al. (369) worked with rats fasted overnight; Kipnis (370) has shown that phosphorylation of glucose in diaphragm muscle is impaired by starvation. Segal and Lopez (371) have reported that liver of fed normal animals contains a much higher G-6-P level than liver of rats deprived of food for 17 hours. Similar results were reported by Steiner and Williams (372).

Conclusions.

The fact that we have detected radioactivity in the phosphorylated glycolytic intermediates of the unincubated rat hemidiaphragms from added glucose-u-C^{14} even at the temperature of liquid nitrogen indicated either that the original glucose-u-C^{14} is contaminated, or that some metabolic changes take place in the diaphragm without incubation. We had therefore to test these two possibilities.

(c) Test of purity of glucose-u-C^{14}.

Principle of the experiment.

The position of glucose on cellulose thin layer chromatography (p.60) was found to be very close to that of G-6-P (Fig. 14). All the other phosphorylated glycolytic intermediates migrate faster

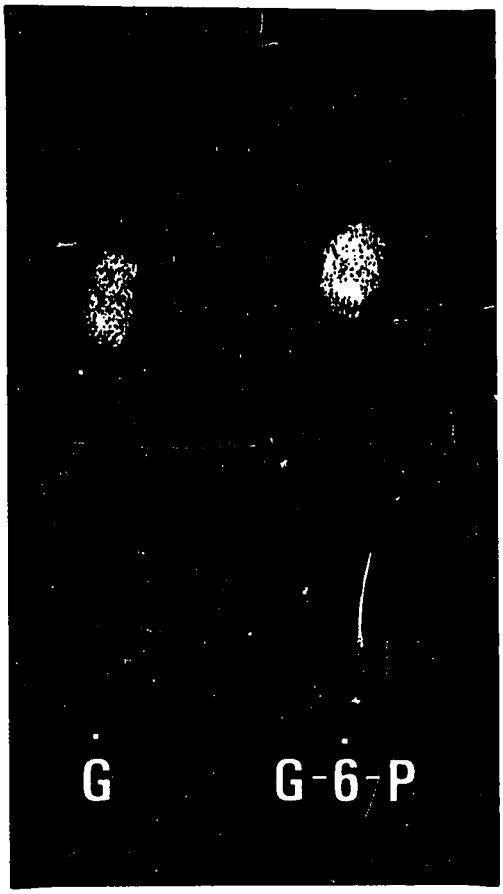


Fig. 14

Drawing of thin layer chromatogram of glucose and glucose-6-phosphate on cellulose Macherey-Nagel MN 300.
Solvent: acetone-acetonitrite-1N HCl(64:26:10, v/v)
Plate size: 41 cm.
Development at room temperature.

(see Fig. 4). As we have seen, no radioactivity was detected in the G-6-P fraction (Table VI). These findings enabled us to test for the purity of the original glucose-u-C^{14}, by running the glucose-u-C^{14} on the thin layer cellulose plate and testing whether radioactivity is detected in the area above the spot of glucose (this area corresponds to F-6-P, FDP, 3PGA + 2PGA, and PEP, Fig. 4).

Procedure.

250 μc of glucose-u-C^{14} were applied (after reducing the volume by freeze drying) to cellulose plates (Machery-Nagel MN 300). 2 μl of standard solution of glucose (4 mg/ml) was run simultaneously. Chromatograms were developed in acetone-acetonitrile-1N hydrochloric acid (64:26:10, v/v). The part of the plate containing the standard glucose was sprayed with aniline-hydrogen phthalate, and the color developed (110°C, 15 minutes). According to this standard the glucose-u-C^{14} spot was cut out and eluted in water, and an aliquot was counted. All the area above this spot was also eluted and counted. This area corresponds to F-6-P, FDP, 3PGA, 2PGA and PEP.

Results.

1.8% of radioactivity of glucose was detected in the above mentioned area, revealing that the original glucose-u-C^{14} is con-

taminated by the compounds tested. This degree of contamination can seriously interfere with the estimate of specific activity in the proposed experiments.

When diluted solutions of glucose-u-C^{14}, which had been stored for several weeks in the freezer, were tested by the same method, the contamination was found to be higher (3-4.8%), perhaps due to microbial action.

We then knew that the original glucose-u-C^{14} had to be purified before each experiment. Since the eluted glucose-u-C^{14} (corresponding to the glucose standard) was now free from contamination with the phosphorylated compounds under study, thin layer chromatography was used thereafter as a method for purification of glucose-u-C^{14}.

(d) To test whether glucose-u-C^{14} can be incorporated into the glycolytic intermediates of unincubated rat diaphragm.

Procedure.

To be completely sure that no metabolic changes of the glucose by the unincubated rat hemidiaphragms kept at liquid nitrogen temperature can occur, the following control experiment was performed: 0.5 μc of the purified glucose-u-C^{14}, and 787 μg of unlabeled glucose were added again to 15 frozen hemidiaphragms, as in the starting experiment. The hemidiaphragms were ground, and the glycolytic intermediates were extracted, isolated and separated.

Now again the separated compounds were counted to test whether we still found any radioactivity in these fractions.

Results.

No radioactivity appeared now in any of the glycolytic intermediates.

Conclusions.

We could now be sure that all the radioactivity in the glycolytic intermediates detected before (Table VI), was due to contamination of the original glucose-u-C^{14}, and not due to any metabolic changes of the glucose by the unincubated hemidiaphragms.

The glucose-u-C^{14} was therefore purified before each experiment by thin layer chromatography in the same way as in the experiment for testing its purity.

2. Effect of Insulin on Radioactivity and Concentration of Phosphorylated Glycolytic Intermediates.

Principle of the experiment.

Hemidiaphragms were incubated under conditions similar to those described in experiment A 2; paired hemidiaphragms were incubated with glucose in the presence and absence of insulin; in the latter case the glucose concentration was increased so that glucose uptake by the paired tissues was approximately equal. Under these circumstances, in which the effect of insulin on glucose transport was eliminated, its effect on the concentration and radioactivity of the phosphorylated glycolytic intermediates was studied.

Procedure.

Paired hemidiaphragms from each rat were incubated in 2 ml KRB media, one containing insulin (10^{-2} unit/ml) and a glucose concentration of 5.56 mM, the other containing no insulin and a glucose concentration of 8.33 mM. This concentration was selected to provide a glucose uptake equal to or slightly greater than that of the first hemidiaphragm. All flasks contained purified glucose-u-C^{14} (p.102), sufficient to give specific activity of 240-248 cpm/nmole. Control flasks with the two different concentrations of glucose, containing

no tissue, were incubated simultaneously in each experiment to enable measurements of glucose uptake. Incubations were carried out at 37°C for two hours, with agitation. For each experiment paired hemidiaphragms from 14 rats were incubated individually, then washed and combined into two pools (insulin, no insulin) for extraction of glycolytic intermediates. This experimental design permitted a minimum and constant time interval between excision of diaphragm and start of incubation, while pooling the tissue was necessary to obtain reliable measurements of concentration of metabolites. The phosphorylated glycolytic intermediates were extracted, isolated and separated by methods described on p.58-70. The radioactivity and the concentration of the separated glycolytic intermediates from each of the two samples (insulin, no insulin) were then measured. The radioactivity of the glucose in the starting media, and the glucose content in all the media were also measured.

Results.

The results of 4 experiments are presented individually in Tables VIIIa - VIIId, and are summarized in Table VIIIe. Tables VIIIa - VIIId record the amount, the radioactivity content and the specific activity of the phosphorylated intermediates of the

TABLE VIII a

Effect of Insulin on the Radioactivity and Concentration of Phosphorylated Glycolytic Intermediates in the Rat Hemidiaphragms.

Compound	(a) Radioactivity cpm/g wet wt.	(a) Concentration nmoles/g wet wt.	(a) s.a. cpm/nmole	(b) Radioactivity cpm/g wet wt.	(b) Concentration nmoles/g wet wt.	(b) s.a. cpm/nmole
G-6-P	993	28.3	35	1,036	31.5	33
F-6-P	1,449	6.3	230	1,728	7.2	240
FDP	1,792	8.3	216	1,290	5.7	226
3PGA)	2,878	28.3	89	1,227	19.0	55
2PGA)		4.0			3.5	
PEP	5,233	22.8	229	3,952	18.2	217
Glucose in medium	cpm/ml 1,342,200	nmoles/ml 5,555	cpm/nmole 242	cpm/ml 2,027,520	nmoles/ml 8,333	cpm/nmole 243
Glucose uptake	mg/g wet wt/2 h 5.67 ± 0.05 (14)*			mg/g wet wt/2 h 6.37 ± 0.12 (14)		

(a) Glucose concentration 5.56 mM, insulin 10^{-2} u/ml.

(b) Glucose concentration 8.33 mM, no insulin

* Mean, standard error, and number of determinations.

TABLE VIII b

Effect of Insulin on the Radioactivity and Concentration of Phosphorylated Glycolytic Intermediates in the Rat Hemidiaphragms.

Compound	(a) Radioactivity cpm/g wet wt.	(a) Concentration nmoles/g wet wt.	(a) s.a. cpm/ nmole	(b) Radioactivity cpm/g wet wt.	(b) Concentration nmoles/g wet wt.	(b) s.a. cpm/ nmole
G-6-P	867	28.7	30	1,260	32.3	39
F-6-P	1,307	5.9	221	1,622	6.7	242
FDP	2,020	10.2	198	1,344	6.0	224
3PGA)		20.9			17.8	
)	1,496		62	1,152		55
2PGA)		3.3			3.0	
PEP	4,069	19.6	208	1,953	9.3	210
	cpm/ml	nmoles/ml	cpm/ nmole	cpm/ml	nmoles/ ml	cpm/ nmole
Glucose in medium	1,350,940	5,555	243	1,997,120	8,333	240
	mg/g wet wt/2h			mg/g wet wt/2 h		
Glucose uptake	6.18 ± 0.14 (14)			6.74 ± 0.12 (14)		

Composition of media as given in Table VIII a.

TABLE VIII c

Effect of Insulin on the Radioactivity and Concentration of Phosphorylated Glycolytic Intermediates in the Rat Hemidiaphragms.

Compound	(a) Radioactivity cpm/g wet wt.	(a) Concentration nmoles/g wet wt.	(a) s.a. cpm/nmole	(b) Radioactivity cpm/g wet wt.	(b) Concentration nmoles/g wet wt.	(b) s.a. cpm/nmole
G-6-P	1,271	36.2	35	1,404	36.0	39
F-6-P	997	4.9	203	1,123	5.4	208
FDP	4,970	21.1	235	3,152	12.9	244
3PGA)		26.3			16.3	
)	1,780		55	1,065		49
2PGA)		5.8			5.2	
PEP	4,398	19.8	222	2,699	11.0	245
	cpm/ml	nmoles/ml	cpm/nmole	cpm/ml	nmoles/ml	cpm/nmole
Glucose in medium	1,374,260	5,555	247	2,070,580	8,333	248
	mg/g wet wt/2 h			mg/g wet wt/2 h		
Glucose uptake	5.98 ± 0.13 (14)			6.46 ± 0.14 (14)		

Composition of media are as given in Table VIII a.

TABLE VIII d

Effect of Insulin on the Radioactivity and Concentration of Phosphorylated Glycolytic Intermediates in the Rat Hemidiaphragms.

Compound	(a) Radioactivity cpm/g wet wt.	(a) Concentration nmoles/g wet wt.	(a) s.a. cpm/nmole	(b) Radioactivity cpm/g wet wt.	(b) Concentration nmoles/g wet wt.	(b) s.a. cpm/nmole
G-6-P	855	33.5	26	840	36.6	30
F-6-P	1,275	5.5	232	1,659	7.0	237
FDP	2,002	9.4	213	1,241	6.1	203
3PGA)	2,879	37.4	66	1,998	22.6	72
2PGA)		6.0			5.3	
PEP	4,070	19.2	212	3,545	N.M.	
Glucose in medium	cpm/ml 1,361,760	nmoles/ml 5,555	cpm/nmole 245	cpm/ml 2,058,580	nmoles/ml 8,333	cpm/nmole 247
Glucose uptake	mg/g wet wt/2 h 5.63 ± 0.13 (14)			mg/g wet wt/2h 6.27 ± 0.13 (14)		

Composition of media as given in Table VIII a.

N.M. = not measured.

TABLE VIII e

Insulin Induced Changes in Radioactivity and Concentration of Phosphorylated Glycolytic Intermediates in the Rat Hemidiaphragms.

Compound	Radioactivity cpm/g wet wt.		p	Concentration nmoles/g wet wt.		p	s.a. cpm/nmole	p
G-6-P	-139±90		>0.2	-2.43±0.88		<0.1	-3.75±1.85	>0.1
F-6-P	-276±54	(-17.5%)	<0.025	-0.93±0.20	(-13.8%)	<0.025	-10.25±3.80	<0.1
FDP	+939±290	(+52.0%)	<0.05	+4.58±1.25	(+58.3%)	<0.05	-8.75±7.40	>0.2
3PGA)	+898±275	(+68.9%)	<0.05	+9.30±2.39	(+48.3%)	<0.05	+10.25±8.45	<0.4
2PGA)				+0.53±0.08	(+12.2%)	<0.01		
PEP	+1405±339	(+54.6%)	<0.05	+7.90±1.69	(+72.0%)	<0.05	-4.30±10.30	>0.5

Values are mean ± S.E. of changes produced by insulin, based on results presented in Tables VIIIa - VIIId.

rat hemidiaphragms. Each value is a single observation on 14 hemidiaphragms. The tables also show the radioactivity, concentration and specific activity of the glucose in the medium, and the glucose uptake by the hemidiaphragms incubated in the two different media.

The radioactivity and concentration of FDP, 3PGA and 2PGA, and PEP were significantly increased by insulin, while there were consistent decreases in the radioactivity and concentration of F-6-P. The decrease in the concentration and radioactivity of G-6-P was not statistically significant. There was no statistically significant effect of insulin on the specific activities of the intermediates.

The specific activity of G-6-P, 3PGA and 2PGA was much lower than that of F-6-P, FDP and PEP. The specific activities of the latter intermediates were very close to that of the glucose in the medium. Table IX shows the ratio of the specific activities of the phosphorylated glycolytic intermediates to that of the glucose in the medium in the presence and absence of insulin. Insulin had no effect on these ratios.

TABLE IX

Ratio of Specific Activities of Phosphorylated Glycolytic Intermediates to Specific Activity of Glucose in Medium.

Compound	Insulin	Ratio of s.a.*	p (insulin)
G-6-P/glucose	+	0.129 ± 0.009	N.S. (p> 0.4)
	-	0.144 ± 0.009	
F-6-P/glucose	+	0.907 ± 0.030	N.S. (p> 0.5)
	-	0.946 ± 0.038	
FDP/glucose	+	0.882 ± 0.027	N.S. (p> 0.5)
	-	0.917 ± 0.034	
3PGA+2PGA/glucose	+	0.279 ± 0.030	N.S. (p > 0.4)
	-	0.236 ± 0.020	
PEP/glucose	+	0.891 ± 0.020	N.S. (p> 0.5)
	-	** 0.918 ± 0.034	

* The mean ratio of specific activities of phosphorylated glycolytic intermediates to that of glucose in medium of hemidiaphragms incubated with and without insulin.

Each value is a mean of 4 determinations.

The values were calculated from data presented in Tables VIIIa - VIIId).

** Mean of 3 determinations.

Discussion.

The results in Tables VIIIa - VIIIe , which show that insulin consistently decreased the concentration and radioactivity of F-6-P and increased those of FDP, 3PGA and 2PGA, and PEP in rat diaphragm, suggest that insulin increased the rate of conversion of F-6-P to FDP, i.e., it stimulated the phosphofructokinase reaction which is normally the rate-limiting step of glycolysis. The stimulation of this rate-limiting step by insulin can also account for the increases in C^{14}-triglyceride glycerol, and in $C^{14}O_2$ observed in the previous experiments (in addition to the small stimulatory effect of insulin on pyruvate dehydrogenase reaction).

The concentrations of the phosphorylated glycolytic intermediates in the incubated hemidiaphragms (Tables VIIIa - VIIId) are lower than those in the unincubated tissue (Table VII). A related observation was made by Karl et al. (369) who incubated hemidiaphragms with or without glucose in the medium; the concentrations of the glycolytic intermediates in samples incubated without glucose present in the medium, were lower than those in the unincubated samples. In the presence of high concentration of glucose (250 mg%) in media they found that there was a tendency to restore the values of the intermediates to the unincubated levels. These results indicate that the concentration of the intermediates is dependent on the rate of glucose utilization. In the present

experiments the glucose concentration in the medium was only 150 mg%; presumably the rate of glucose uptake was lower than that at 250 mg% and thus the concentrations of glycolytic intermediates were lower than in the work of Karl et al.

It may be pointed out incidentally that the concentration of these intermediates may have been lower in the present work than in that of Karl et al., not only because of the lower glucose concentration but also because of differences in the nutritional state of the animals; Newsholme and Randle (248) have found that increasing the time of prior starvation increases significantly the concentration of G-6-P and F-6-P of the incubated rat diaphragm or the perfused rat heart. Karl et al. (369) used fasted rats, whereas the rats used by us were fed ad libitum.

The fact that the specific activities of F-6-P, FDP and PEP are very close to that of the glucose in the medium, indicates that equilibrium had virtually been reached. From the precursor-product relationships in the presence of a continuous source of label we would expect the specific activities of all compounds between glucose and PEP to be equal to equilibrium, yet the specific activities of G-6-P and of the phosphoglyceric acids were much lower than those of their precursors and their products. These results suggest the existence of more than one pool of G-6-P and phosphoglyceric acids

in the diaphragm muscle; one pool of each compound, labeled from its precursor and having a specific activity at least equal to that of its product, and one or more pools either not labeled, or labeled to a much smaller extent. The procedure used for estimation of specific activity is based on the total amount of the compound in the tissue and therefore gives an average value for all the hypothetical pools. Consequently the existence of such unlabeled pools would explain the fact that specific activities of G-6-P and PGA are lower than those of their products.

The existence of more than one pool of G-6-P has been similarly suggested for the liver by London (373), Threlfall (374), and recently Threlfall and Heath (375). Threlfall's suggestion (374) was based on experiments in which he injected rats with C^{14}-glucose and found that the specific activity of UDPG was much higher than those of G-6-P, glycerophosphate and lactic acid. He did not measure the other glycolytic intermediates.

Shaw and Stadie (376) have postulated the existence of two Embden-Meyerhof pathways in rat diaphragm differing in cytological location. They derived their conclusion from experiments in which they compared the metabolic pattern and response to insulin of rat diaphragms incubated in two different buffers: phosphate-saline or bicarbonate-phosphate-saline medium containing isotopic glucose or phosphorylated metabolites either alone or in combination. In the phosphate-saline medium phosphofructokinase participating in Embden-

Meyerhof pathway No.1 which they regard to be intracellular, was shown to be inactive; the medium glucose was never incorporated into intracellular FDP obtained by extraction from the washed diaphragm. Lactic acid, however, was formed according to their suggestion by an Embden-Meyerhof pathway No.2. The latter pathway is presumably located on the cell surface as phosphorylated intermediates added to the medium freely interchange with their analogous compounds formed in this pathway. This pathway is insulin nonresponsive and it leads to the formation of lactic acid but not to glycogen. In bicarbonate-phosphate-saline medium, phosphofructokinase of the "intracellular" Embden-Meyerhof pathway No.1 is active (medium labeled glucose is incorporated into intracellular FDP obtained by extraction from washed diaphragm) the lactic acid formed by this pathway is increased by insulin. Glycogen is formed in both media only by the insulin responsive Embden-Meyerhof pathway No.1. The Embden-Meyerhof pathway No.1 is regarded to be intracellular since in contrast to the Embden-Meyerhof pathway No.2 (surface), the intermediary phosphate esters cannot be washed out of the diaphragm and, moreover, the phosphorylated intermediates in the medium do not interchange with their analogues obtained by extraction from washed diaphragms.

It seems unlikely that the source for dilution of the label in G-6-P and phosphoglyceric acids in the present experiments was their analogues of the postulated Embden-Meyerhof pathway No.2 lo-

cated on cell surface since the intermediates of the latter pathway were removed by washing after incubation.

Dully et al. (377) reached a similar conclusion to that of Shaw and Stadie (376), for the voluntary skeletal muscle; they compared the ratios of incorporation of label from glucose-1-C^{14} to that from glucose-6-phosphate-1-C^{14} into glycogen, lactate and CO_2, and found a higher ratio of incorporation (glucose-1-C^{14}/glucose-6-phosphate-1-C^{14}) into glycogen than into lactate and CO_2, suggesting the presence of a minimum of two glucose-6-phosphate pools.

Antony et al. (378) incubated rat diaphragms with a mixture of C^{14}-labeled glucose and C^{14}-pyruvate and compared the distribution of C^{14} from these substrates in glycogen and glucose-6-phosphate; they found a greater incorporation of glucose carbon, relative to pyruvate carbon, into glycogen than into glucose-6-phosphate. This result suggests the existence in muscle of more than one pool of G-6-P or of a pathway from glucose to glycogen without G-6-P as an intermediate. Our results showing that the specific activity of G-6-P was much lower than that of F-6-P and FDP clearly indicate the existence of two or more G-6-P pools in the diaphragm, unless we assume the existence of a direct pathway from glucose to F-6-P without G-6-P as an intermediate, an assumption which has no factual basis at all. The present results also suggest the existence of more than one pool of phosphoglyceric acids in the diaphragm muscle.

D. Studies of gluconeogenesis in rat diaphragm.

Introduction.

In these experiments we attempted to identify the unknown pool(s) which cause the dilution of the radioactivity in G-6-P and phosphoglyceric acids. Threlfall and Heath (375) have found that in the liver of rats injected with [U-C^{14}]-fructose, the specific activity of G-6-P and glycerol-1-phosphate was lower than that of glucose and UDPG. Based on these experiments and from kinetic analysis of the results, they arrived at the conclusion that the G-6-P pool is compartmented into gluconeogenic and glycolytic components and that the triose phosphates are similarly compartmented. We were interested to study whether similar compartmentation between glycolytic and gluconeogenic pathways exists in muscle as well.

In the same experiments we have studied the effect of insulin on the phosphorylated intermediates in the gluconeogenic pathway from pyruvate. There are no reported comparable studies on the effect of insulin on gluconeogenic process in the mammalian muscle; most studies were performed on liver probably due to the fact that the gluconeogenic process in this tissue is quantitatively much more important than that in muscle. The main gluconeogenic pathway in the liver involves the pyruvate carboxylase and phosphoenolpyruvate carboxykinase reactions. In muscle there

is no pyruvate carboxylase (213, 239); gluconeogenesis, however, proceeds through the reversal of the pyruvate kinase reaction (240-242). Insulin, in vitro, was shown to suppress glucose output by the liver (178, 289). Insulin in the liver acts also as a suppressor of the biosynthesis of the key enzymes of gluconeogenesis (294-296). It seemed important to study whether insulin has any effect, in vitro, on the gluconeogenic intermediates in muscle.

Procedure.

Paired hemidiaphragms were incubated in 2 ml KRB media containing sodium pyruvate-1-C^{14} and unlabeled pyruvate (25 mM) with no glucose present. The final specific activity of the pyruvate in the medium was 359 cpm/nmole. One flask of each pair of hemidiaphragms contained insulin (10^{-2} units/ml). Incubations were carried out at 37°C for two hours with agitation. The phosphorylated intermediates were extracted from 14 pooled hemidiaphragms, isolated, separated, and their radioactivity and concentration were measured as in the preceding experiments.

Results.

The results are presented in Tables Xa and Xb. The tables record the amount, the radioactivity content and the specific activity of the phosphorylated intermediates of the rat hemidiaphragms, in the presence and absence of insulin. Each value is a single observation on 14 hemidiaphragms.

It can be clearly seen that the radioactivity, concentration and specific activities of all the intermediates were unaffected by the presence of insulin.

The specific activities of the G-6-P and phosphoglyceric acids were again lower than those of the other intermediates whose specific activities were very close to that of the pyruvate in medium. Comparison was made (Table XI) between the ratios of specific activities of phosphorylated intermediates to that of pyruvate in medium in the gluconeogenic pathway and to that of glucose in medium in the glycolytic pathway studied in the preceding experiments; the ratios of the specific activities of G-6-P and of phosphoglyceric acids to the glucose in the medium were found to be significantly different from the ratios of the specific activities of these intermediates to the pyruvate in medium. The differences between the corresponding ratios of specific activities of F-6-P, FDP and PEP were not significant.

TABLE X a

Effect of Insulin on the Radioactivity and Concentration of Phosphorylated Intermediates in Rat Hemidiaphragms Incubated with Pyruvate-1-C^{14}.

Compound	With insulin			No insulin		
	Radioactivity cpm/g wet wt.	Concentration nmoles/g wet wt.	s.a. cpm/ nmole	Radioactivity cpm/g wet wt.	Concentration nmoles/g wet wt.	s.a. cpm/ nmole
G-6-P	1,125	14.8	76	1,095	14.6	75
F-6-P	2,001	5.8	345	2,024	5.9	343
FDP	1,835	5.7	322	1,754	5.5	319
3PGA)		45.5			46.0	
)	2,929		58	3,084		60
2PGA)		5.0			5.4	
PEP	7,791	24.5	318	8,000	25.0	320

Paired hemidiaphragms were incubated for 2 hr in KRB media containing sodium pyruvate-1-C^{14} and unlabeled pyruvate (final s.a. 359 cpm/nmole).

One flask of each pair contained insulin (10^{-2} u/ml).

Each value is a single observation for 14 hemidiaphragms.

TABLE X b

Effect of Insulin on the Radioactivity and Concentration of Phosphorylated Intermediates in Rat Hemidiaphragms Incubated with Pyruvate-1-C^{14}.

Compound	With insulin			No insulin		
	Radioactivity cpm/g wet wt.	Concentration nmoles/g wet wt.	s.a. cpm/ nmole	Radioactivity cpm/g wet wt.	Concentration nmoles/g wet wt.	s.a. cpm/ nmole
G-6-P	1,086	10.5	103	1,071	10.6	101
F-6-P	2,238	6.5	344	2,072	6.0	345
FDP	1,911	5.4	354	1,800	5.2	346
3PGA)		40.6			41.0	
)	2,047		39	2,275		41
2PGA)		11.9			11.3	
PEP	8,520	23.8	358	7,892	23.0	343

Composition of media and conditions of incubation are as given in Table X a.

TABLE XI

Comparison of Ratios of Specific Activities of Phosphorylated Intermediates to that of Glucose in Medium (Glycolytic Pathway) and to that of Pyruvate in Medium (Gluconeogenic Pathway).

Compound	Ratio of s.a. of the compound to that of glucose in medium*	Ratio of s.a. of the compound to that of pyruvate in medium **	p
G-6-P	0.136 ± 0.006	0.247 ± 0.020	$p < 0.001$
F-6-P	0.927 ± 0.023	0.959 ± 0.009	N.S. ***
FDP	0.900 ± 0.021	0.934 ± 0.025	N.S. ***
3PGA+2PGA	0.258 ± 0.018	0.137 ± 0.016	$p < 0.005$
PEP	0.904 ± 0.018 (7)	0.932 ± 0.027	N.S. ***

* Each value is a mean ± S.E. of 8 determinations. The values were calculated from data presented in Tables VIIIa - VIIId.

** Each value is mean ± S.E. of 4 determinations. The values were calculated from data presented in Tables Xa, Xb.

*** Not significant, ($p > 0.4$).

Values for both insulin treated and untreated tissues were studied together as there were no statistically significant differences between the two.

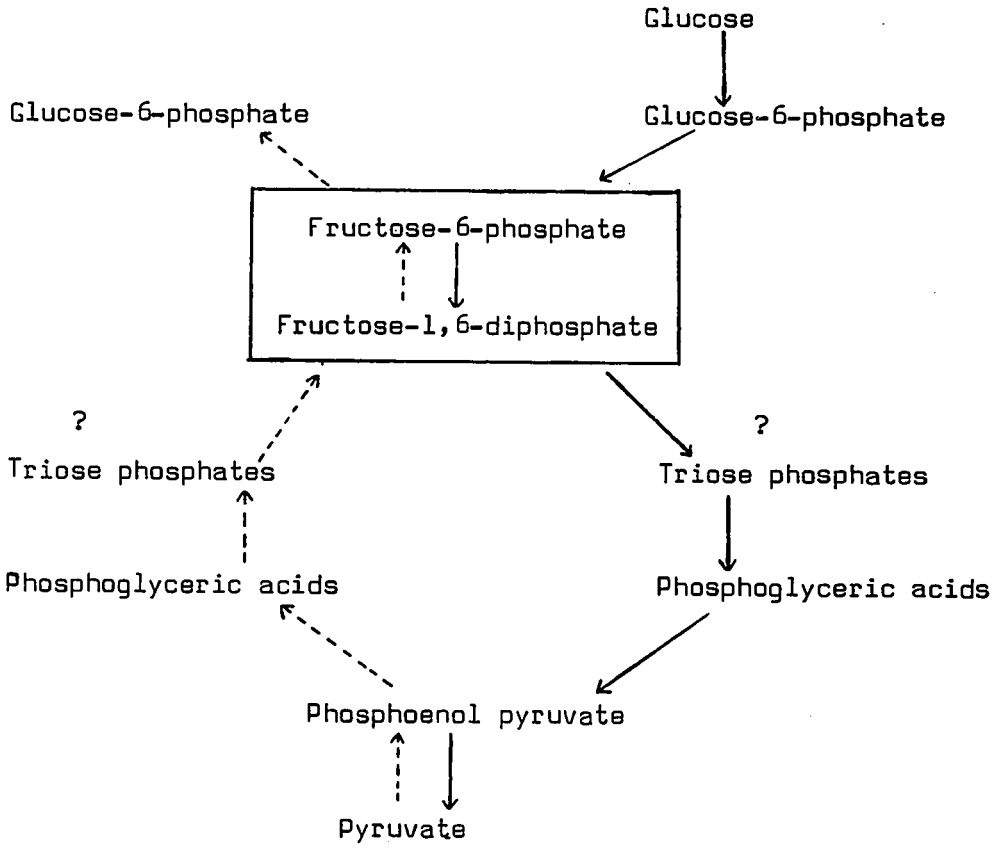
Discussion.

It is evident that insulin added, *in vitro*, has no effect on the concentration, radioactivity or specific activities of the intermediates in the gluconeogenic pathway from pyruvate in the rat hemidiaphragm. These results are not comparable to those reported by Weber *et al.* (294-296) for the liver where a suppressive effect on the biosynthesis of the key enzymes of gluconeogenesis was demonstrated by the administration of the hormone, *in vivo*. Of the four key gluconeogenic enzymes acting in the liver only the fructose-diphosphatase has been demonstrated in muscle (224). The present results would indicate that insulin, *in vitro*, has no effect on this enzyme in the rat diaphragm.

From the results presented in Table XI indicating that the relative ratios of the specific activities of G-6-P and phosphoglyceric acids in the glycolytic pathway differ from those of the gluconeogenic pathway, it is concluded that the G-6-P and phosphoglyceric acids of the glycolytic and gluconeogenic pathways are compartmented. This is in agreement with the conslusion of Threlfall and Heath (375) for the liver.

By adopting the scheme of interaction of glycolysis and gluconeogenesis proposed by Threlfall and Heath (375) for the liver, and adding the information we have obtained for the other phosphorylated intermediates not studied by them, the interaction of the glycolytic and the gluconeogenic chains in the muscle can be represented as

shown in scheme 1; based on the results of Table XI it is suggested that the pools of G-6-P and phosphoglyceric acids involved in glycolysis are separated from those involved in gluconeogenesis. The F-6-P, FDP and PEP appear to be in the same pool (or if separated, it would be to a very small extent). The triose phosphates were not studied by us; according to Threlfall and Heath (375) the triose phosphates of the glycolytic and gluconeogenic pathways are also compartmented.



Scheme 1

Interaction of glycolysis and gluconeogenesis.

GENERAL DISCUSSION

Under two different experimental conditions it has been demonstrated in the present study that insulin acts directly on glucose oxidation in the rat diaphragm independently of its action on glucose transport. These results are in agreement with the finding reported for rat adipose tissue by Lynn et al. (6) who showed that when adipose tissue was treated with digitonin and hyaluronidase in the presence of large amounts of glucose, a condition in which glucose completely equilibrates with tissue water, the addition of insulin caused an increased production of CO_2 and triglyceride (aerobically) or lactate (anaerobically) from glucose.

Insulin consistently decreased the concentration and radioactivity of F-6-P and increased the concentration and radioactivity of FDP, 3PGA and 2PGA, and PEP in the rat hemidiaphgrams incubated with glucose-u-C^{14} under conditions in which the effect of the hormone on the transport of glucose had been eliminated. These results indicate that insulin stimulates the phosphofructokinase reaction, which is the rate-limiting step of glycolysis in muscle. A related observation was made by Özand and Narahara for the isolated frog sartorious muscle incubated in the absence of glucose (301); insulin lowered the concentration of glucose-6-phosphate during incubation in the presence of epinephrine. Under anaerobic conditions insulin also increased the formation of lactate. The decrease in the con-

centration of glucose-6-phosphate produced by insulin could only be demonstrated in the presence of epinephrine.

Insulin augmented $C^{14}O_2$ production from lactate-1-C^{14} by the rat hemidiaphragm, but it did not increase $C^{14}O_2$ production from lactate-2-C^{14}. From these results it is concluded that insulin has a stimulatory effect on pyruvate dehydrogenase reaction. This is a second site of insulin's intracellular action detected in the present work; however, it seems to be quantitatively less important than the augmentation of the phosphofructokinase reaction.

In the frog sartorious muscle (in summer) but not in the normal hemidiaphragm, insulin also seems to have a direct effect on the Krebs cycle as it stimulates the production of $C^{14}O_2$ from pyruvate labeled in any one of the three carbon atoms (368). The metabolic regulation in the isolated frog sartorious muscle differs from the mammalian muscle in other aspects as well, e.g. anoxia, which causes augmentation of the activity of phosphofructokinase in the mammalian diaphragm (248) and heart muscle (244), does not affect the activity of phosphofructokinase in the frog sartorious muscle (301). Epinephrine leads to activation of phosphofructokinase in mammalian heart muscle (252) but does not activate the phosphofructokinase of the frog sartorious muscle (301).

The present studies of the specific activities of phosphorylated glycolytic intermediates in rat hemidiaphragms incubated with glucose-u-C^{14}, indicate the existence of more than one pool of glucose-6-phosphate and phosphoglyceric acids in the diaphragm muscle. The possibil-

ity of compartmentation of glucose-6-phosphate in the diaphragm muscle has been recently suggested by Antony et al. (378). Their experiments could not, however, differentiate between such a possibility and that of a pathway for the conversion of glucose into glycogen without glucose-6-phosphate as an intermediate.

The ratios of specific activities of glucose-6-phosphate and of phosphoglyceric acids to that of the substrate in the medium during glycolysis differed significantly from those during gluconeogenesis, indicating that in the rat diaphragm the pools of glucose-6-phosphate and phosphoglyceric acids involved in glycolysis are separated from those involved in gluconeogenesis.

Possible mechanisms of insulin's stimulatory action on phosphofructokinase reaction.

It is possible that insulin acts directly on the enzyme itself, or it may cause the activation of the enzyme by some indirect action since as we know the enzymatic activity of phosphofructokinase is regulated by a variety of agents (188 - 200). The possibility exists that this effect may be mediated by an action on potassium, since potassium ions activate phosphofructokinase (200); however, this seems unlikely, since Zierler (379) and Zierler et al. (380) demonstrated that though insulin alters potassium flux, it produces no change in intracellular potassium concentration in muscle. Furthermore, potassium ion has a relatively small effect on the phospho - fructokinase reaction (200).

The possibility that the phosphofructokinase may be activated by increased transport of phosphate ions by insulin, seems also unlikely, since although insulin has been shown to stimulate the transport of phosphate ion in the perfused rat heart (381), Volfin et al. (382) have reported that insulin does not increase the rate of penetration of radioactive phosphorus in the rat diaphragm.

ATP (180 - 193) and citrate (194 - 197) are potent inhibitors of phosphofructokinase. Decrease in either the ATP:ADP (AMP) ratio or the level of citrate would indirectly lead to activation of phosphofructokinase. It has been demonstrated by Regen et al. (383) and Garland et al. (254) that in hearts from alloxan diabetic rats phosphofructokinase is markedly inhibited. This depression was not accompanied by any change in the level of adenine nucleotides or inorganic orthophosphate. However, citrate concentration was found to be increased in alloxan diabetes (254); this suggests very indirectly that insulin may be involved in regulating citrate levels. It is unlikely that insulin decreases citrate concentration by increasing citrate oxidation, since we would then anticipate increased lactate-2-C^{14} oxidation to $C^{14}O_2$ but we did not find such an effect. It is possible that insulin decreases citrate concentration by activating citrate cleavage enzyme; this enzyme is increased in the liver following the injection of insulin (384). However, a similar elevation in citrate cleavage enzyme was obtained by administration of glucose or fructose, suggesting that insulin does not act by exerting a direct effect on

citrate cleavage enzyme but by increasing glucose utilization (384). In any case it would be interesting to study whether insulin in the normal rat diaphragm muscle, under conditions in which its effect on glucose transport has been eliminated, has any effect on the concentration of citrate.

Margreth (185) and Margreth et al. (186, 187) have found that 25-30% of frog muscle phosphofructokinase is associated with the microsomal fraction of the muscle (the specific activity of the microsomal phosphofructokinase was found to be 6 times as high as that of the soluble enzyme (185). Glycolysis in cytoplasm of the muscle was found to be stimulated by addition of the microsomal fraction (185). The stimulation was evident on the process of conversion of glucose-6-phosphate to lactate but not on that of fructose-1,6-diphosphate to lactate; it was concluded that the stimulating effect on glycolysis by the microsomal fraction is accounted for largely by the phosphofructokinase associated with the microsomes. Similarly, in the pigeon breast muscle, addition of endoplasmic reticulum was shown to have no influence on the process of conversion of fructose-1,6-diphosphate to lactic acid, but considerably activated phosphofructokinase reaction (385). Keeping in mind the recent findings that insulin causes changes in the ribosome itself (386), one may speculate that insulin by some action on the ribosome, either leads to the activation of the ribosomal phosphofructokinase, or affects the localization of the enzyme in a way which would bring it closer to its site of action. In view of the recent finding of

Borrebaek and Spydevold (138) that insulin in the absence of glucose has an influence on the localization of hexokinase in the rat epididymal fat pads, the latter suggestion seems like a resonable possibility.

SUMMARY

When rat hemidiaphragms, pre-loaded *in vivo* with glucose-C^{14}, were incubated in a glucose-free medium, insulin added *in vitro* increased the production of $C^{14}O_2$ from the intracellular glucose. In another experiment, paired hemidiaphragms were incubated with glucose in the presence and absence of insulin; in the latter case, the glucose concentration was increased so that glucose uptake was approximately equal for the paired tissues. Under these circumstances, CO_2 production, as well as glyceride-glycerol production, was higher in the presence of insulin. These results are evidence of a direct action of insulin on glucose oxidation in the rat diaphragm independent of its effect on glucose transport.

Insulin increased $C^{14}O_2$ production from lactate labeled in carbon 1 by the rat diaphragm whereas it did not augment $C^{14}O_2$ production from lactate labeled in carbon 2. From these results it is concluded that insulin has a stimulatory effect on the step of decarboxylation of pyruvate, catalyzed by pyruvate dehydrogenase.

Insulin decreased the concentration and radioactivity of F-6-P and increased those of FDP, 3PGA and 2PGA, and PEP in rat hemidiaphragms incubated with glucose-u-C^{14} under circumstances in which the effect of insulin on the transport of glucose had been eliminated. These results are evidence of a stimulatory action of insulin on the phosphofructokinase reaction.

Measurements of specific activities of the phosphorylated glyco-

lytic intermediates in rat hemidiaphragms incubated with glucose-u-C^{14}, reveal the existence of more than one pool of glucose-6-phosphate and of phosphoglyceric acids in the diaphragm muscle.

Insulin, added in vitro, had no effect on the radioactivity, concentration and specific activities of phosphorylated glycolytic intermediates of the rat hemidiaphragms incubated with pyruvate-1-C^{14}.

In this tissue, the ratios of specific activities of glucose-6-phosphate and of phosphoglyceric acids to that of the substrate in the medium during glycolysis differed significantly from those during gluconeogenesis, indicating that the pools of glucose-6-phosphate and phosphoglyceric acids involved in glycolysis are seperate from those involved in gluconeogenesis.

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