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Studies on regulatory factors in carbohydrate metabolism with especial reference to the mechanism of insulin action

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Dissertation

STUDIES ON REGULATORY FACTORS IN CARBOHYDRATE METABOLISM, WITH ESPECIAL REFERENCE TO THE MECHANISM OF INSULIN ACTION

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CHAPTER I

INTRODUCTION

A. Statement of Problem

This study involves a consideration of physiological factors which influence sugar transfer into muscle cells. As a consequence of recent studies, which will be reviewed in detail in this introductory chapter, it now appears that one primary action of insulin is to facilitate the transfer of glucose and certain other sugars through a cytostructural barrier, thus making the sugar available for the cellular enzymatic machinery.

Since sugar transfer can best be studied by using a sugar which is not metabolized by muscle tissue enzymes according to the accepted scheme of intermediary carbohydrate metabolism, the transfer of galactose into isolated rat diaphragm muscle was studied. Galactose, an isomer of glucose, was selected for study because it has been reported that muscle tissue does not contain any galactokinase (154). This means that the muscle cannot transform galactose into glucose, which then could enter the scheme of intermediary carbohydrate metabolism. Such a galactokinase has been shown to exist in the liver and kidney of the mammalian organism (178, 17, 18).

Therefore, in vitro experiments were performed using the isolated rat diaphragm muscle incubated in Krebs Ringer phosphate (KRP) medium, pH 7.4. The influence of various conditions and substances upon the transfer of C^{14}-labelled galactose was studied and will be reported.

B. Evaluation of Literature

As a point of reference for the work which follows, a very simplified scheme of intermediary metabolism, as it is thought to occur in mammals, is presented. See Figure 1. (68).

For many years now, research in various laboratories has revolved

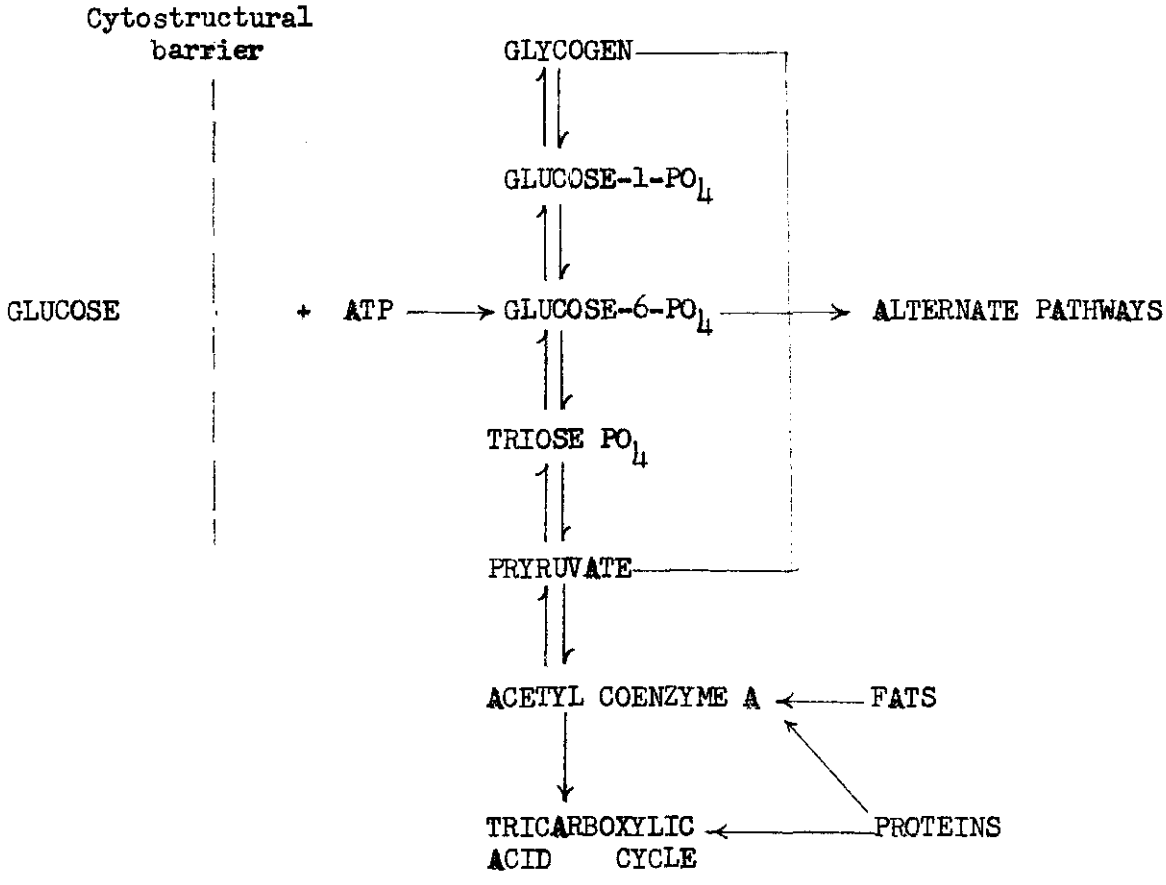


Fig. 1 An abbreviated scheme of current concepts of the intermediary metabolism of glucose (After Hechter,68)

around the question of the mechanism of insulin action in intermediary metabolism. It can be seen from Figure 1 that a block at various loci could result in marked aberrations from normal metabolism. It is possible that the abnormal metabolic picture, as seen in diabetes mellitus, could be due to a key block at a single locus in intermediary metabolism. On the other hand, it is possible to consider that several blocks occur at various loci resulting in the abnormal metabolic pattern of diabetes mellitus.

Therefore, it is possible to look upon the mechanism of insulin action as follows:

(1) insulin acts at a single definite locus, and all of the known effects of insulin on intermediary metabolism may be ascribed to this single action;

(2) insulin acts at several loci in which case there may be several mechanisms of insulin action.

An interesting outgrowth of the tremendous amount of work concerning the mechanism of insulin action has been the concept that carbohydrate metabolism occurs in the complete absence of insulin. This will be discussed in greater detail in the section which concerns itself with the effect of other hormones on insulin and carbohydrate metabolism. Suffice it to say here that carbohydrate metabolism is demonstrable in the adrenalectomized-depancreatized or hypophysectomized-depancreatized animal. To be sure, this basic metabolism is very unstable and cannot readily adapt itself to changes in the metabolic requirements of the organism. To achieve this, a hormonal system of balances and counterbalances has evolved.

In this connection, it is interesting to note that brain tissue which can undergo normal carbohydrate metabolism either in the presence of small amounts of insulin or in the complete absence of insulin, does not combine with significant quantities of labeled insulin (167).

C. Influence of Insulin on Hexokinase

From Figure 1, it can be seen that the first step in mammalian glucose utilization is as follows:

Glucose plus adenosine triphosphate (ATP) yields glucose-6-phosphate plus adenosine diphosphate (ADP).

This first step is referred to as the phosphorylation of glucose, which occurs in the presence of the enzyme, hexokinase. When referring to glucose phosphorylation, it is better to use the term glucokinase or glucohexokinase; since other hexokinases, such as galactokinase, have been shown to exist in mammalian liver and kidney (178, 17, 18).

In 1929, Cori and Cori (29) determined the effect of insulin upon the utilization of glucose, fructose, and mannose. These sugars were injected into eviscerated rats and the carcasses analyzed for residual sugar at various time intervals following injection. Under these experimental conditions, they found that insulin stimulated the utilization of glucose, but not that of fructose or mannose. Since muscle possesses two phosphorylating enzymes, glucokinase and fructokinase (150), Cori makes the following interpretation of such results (34): "The localization of the insulin effect is also fairly clear. It must be at a point before the metabolic pathway of glucose and fructose are joined and this leaves practically only the first reaction of phosphorylation of glucose by ATP as the point of action of insulin."

In confirmation of this opinion, one could cite the work of Gameltoft et al (50). These workers demonstrated an increased utilization of glucose by the perfused cat hind leg in the presence of insulin. This was not the case with fructose.

Therefore, it would seem that insulin acts primarily at one locus. Also, it might be possible to consider that insulin specifically controls the rate of glucose phosphorylation through an action on the specific enzyme, glucohexokinase. All of the metabolic derangements accompanying diabetes mellitus could thus be ascribed to the failure of this first reaction in glucose metabolism, thus effectively interfering with the uptake and metabolic fate of glucose. In addition, insulin action not only results in an increased utilization of glucose but also in an increased formation of CO_2 and glycogen from such glucose. This has been shown by means of experiments using non-radioactive glucose or C^{14}-labelled glucose (181, 190, 156, 52). Historically, this hexokinase-regulatory action of insulin is one of the oldest of the theories advanced to explain the mechanism of insulin action.

The work of Chernick and Chaikoff (21) seems also to indicate that the mechanism of insulin action is at the level of the hexokinase reaction. They incubated liver slices from normal and alloxan-diabetic rats with C^{14}-labelled fructose or glucose in the medium. Liver slices from the diabetic rats showed an impaired ability to oxidize glucose to CO_2 or incorporate glucose into fatty acids. This was in contrast to the diaphragms from the normal rats. The formation of CO_2 from fructose was, however, unaltered in the liver slices from alloxan-diabetic rats, although fructose was not incorporated into fatty acids. From these results, the authors assume that the conversion of fructose to fructose-6-

phosphate is unimpaired in the liver from the alloxan-diabetic rat, but that the conversion of glucose to fructose-6-phosphate is impaired. They conclude that since both the tricarboxylic acid cycle and glycolytic cycle appear to be normal in the alloxan-diabetic liver, the block must be at the level of the glucokinase reaction (21).

To strengthen further the argument that the impairment of glucose utilization of the diabetic liver is at the glucokinase level, this group (22) studied the rate of conversion of fructose to glucose by liver from alloxan-diabetic rats. The results showed once again, that the oxidation of fructose to CO_2 was unaltered in alloxan-diabetic liver slices. Of greater significance, however, was the fact that the rate of conversion of fructose to glucose was also unimpaired. Therefore, one could reason from these results that fructokinase, mutase, isomerase, glucose-6-phosphatase must be unaltered in the alloxan-diabetic liver; thus indicating the impairment to be at the glucokinase level.

An indirect offshoot from the work of this group was the finding that while fructose is freely oxidizable to CO_2 by the alloxan-diabetic liver of the rat, it failed to be incorporated in the fatty acids. This suggested to the authors that a second defect exists in the metabolism of the alloxan-diabetic liver, i.e., a block in the conversion of 2-carbon-compounds to fatty acids. For example, it has been reported that the conversion of acetate labelled with C^{14} to fatty acids by liver slices is lower in the liver of the diabetic rat than in the liver from a normal rat (12, 21).

A large segment of experimentation in the field of intermediary metabolism is performed by using excised tissue, i.e., <u>in vitro</u> studies.

For example, the glucose uptake by isolated intact muscle has been utilized as a measure of the rate of the hexokinase reaction; i.e., glucose plus adenosine triphosphate (ATP yields glucose-6-phosphate plus adenosine diphosphate (ADP). Several questions must be answered before such a procedure may be considered valid. The first requires the demonstration that the glucose phosphorylated to glucose-6-phosphate by the muscle does not appear again as glucose in the medium. Villee and White (185) demonstrated that radioactive C^{14} added to isolated diaphragm muslce as carbonyl labelled pyruvate can be converted to glycogen but not to glucose, thus establishing that glucose liberation does not occur in isolated intact muscle. Also, the absence of glucose-6-phosphatase in intact muscle precludes indirect liberation of glucose from glycogen or glucose-6-phosphate. However, the liver does possess a glucose-6-phosphatase (174).

The second requirement would seem to be the demonstration that the ATP concentration in the muscle is not a limiting factor. Several experiments seem to indicate that the ATP concentration is not a limiting factor: (a) Slein et al (150) demonstrated that the concentration of ATP in normal muscle is many times the concentration necessary to saturate muscle hexokinase; (b) Walaas (187) demonstrated that, under anaerobic conditions, the uptake of glucose by the diaphragm muscle could be increased from 2.3 mg./gm./hr. to 4.2 mg./gm./hr. simply by raising the concentration of glucose in the incubation medium from 140 mg. % to 420 mg. %. Even though under such anaerobic conditions the concentration of acid-labile phosphorus of ATP is only 2-3 mg. %, as compared to 17-22 mg. % in normal diaphragm muscle, there was sufficient ATP present to

double the uptake of glucose when the concentration of glucose in the incubation medium was increased; (c) Krahl (85) demonstrated that the values for the acid-labile phosphorus of ATP for normal diaphragms was 17-22 mg. %, for diabetic diaphragms, 18 mg. %.

D. Action of Insulin on Transfer of Certain Sugars Across Cell Barriers

In recent years evidence has accumulated showing that it is possible to demonstrate an increased uptake of various sugars, in addition to glucose, by the action of insulin. Such an action of insulin has been demonstrated on galactose (98, 57, 97, 59, 60, 192, 39, 62), fructose (39, 62, 106), mannose (41), l-arabinose (59), and d-xylose (59). Such evidence is taken to mean, by many workers in the field, that the Hexokinase Regulatory Theory of insulin action is no longer tenable, since the theory is based on the specificity of insulin influence on the glucohexokinase enzyme. As a result, an alternative theory for the mechanism of insulin action has been formulated and states that insulin facilitates the transfer of certain sugars into the cells of certain tissues and organs. Essentially this theory could be called the Transfer Mechanism Theory. Levine, Goldstein and coworkers at the Michael Reese Hospital in Chicago are primarily responsible for this theory (98, 57, 97, 59, 60).

This mechanism of insulin action might involve the physical processes of permeability of certain free sugars across cytostructural interfaces, chemical processes responsible for such transfer, or both.

The procedure employed by the workers at Michael Reese Hospital was as follows: (a) the experiments were performed on eviscerated-nephrectomized dogs or, occasionally rats; the blood glucose level of these

animals was maintained by intravenous injections of glucose; when required, insulin was added to the infusion mixture. The substances to be tested were administered intravenously in a saline solution. The action of insulin, if any, was determined by measuring the rate of disappearance of the substance from the blood in the presence and in the absence of insulin. Levine et al (98) observed that following the administration of glucose the blood glucose level fell continuously, indicating a continuous utilization of glucose by the animal preparation. They then tested galactose, urea, sucrose, and creatinine. Galactose, an isomer of glucose, was selected for study because it had been reported that muscle does not contain a galactokinase (154). This means that the muscle cannot convert galactose to glucose, which then could enter the metabolic scheme. Such a galactokinase has been shown to exist in the liver and kidney of the mammalian organism (178, 17, 18). However, these organs were removed from the experimental animals.

Following the administration of galactose, a constant blood galactose level was attained in about one and one-half hours after injection and maintained for approximately four hours. Calculation based on the initial and final blood galactose concentrations and the weight of the preparation indicated that the volume of distribution of galactose was approximately 45% of the body weight. When insulin was injected together with the galactose, a constant blood galactose level was again reached. However, the volume of galactose distribution was now approximately 70% of the body weight, which is interpreted to mean a distribution in total body water.

Following the administration of urea, sucrose, or creatinine constant

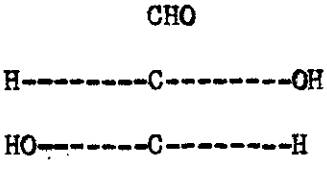
blood levels were also attained within one and one-half hours following injection. Urea, being a freely permeable substance, attained a volume of distribution which was approximately 70% of the body weight. Sucrose and creatinine attained a volume of distribution which was approximately 40-45% of body weight. Insulin had no effect on the volume of distribution of these substances.

These workers (59) then tested the following sugars: l-arabinose, d-xylose, d-arabinose, d-mannose, l-sorbose, d-fructose, l-rhamnose, and d-sorbitol. The results showed that d-xylose and l-arabinose responded to the action of insulin in a manner identical with the galactose response. The volume of distribution for these three substances in the absence of insulin was approximately 40-45% of body weight; in the presence of insulin, approximately 65-70% of body weight. On the other hand, under these experimental conditions, d-arabinose, d-mannose, l-sorbose, d-fructose, l-rhamnose, and d-sorbitol did not respond to the presence of insulin. The non-responsive sugars underwent a volume of distribution of approximately 35-45% of body weight, which was unaffected by the presence of insulin.

This response or lack of response of the various sugars tested was found not to be related to the utilizability of the sugar. For example, galactose, l-arabinose, and d-xylose respond to the presence of insulin but are nevertheless nonutilizable by the eviscerated-nephrectomized preparation. Of the sugars that were found not to respond to the presence of insulin, d-arabinose, l-rhamnose, and d-sorbitol are not utilizable by the preparations; however, d-mannose, d-fructose, and l-sorbose are.

As a result of such work, the authors conclude that a possible action

of insulin might be to facilitate the transfer of certain sugars from the extracellular to the intracellular phase of certain organs and tissues. For a better understanding of the overall physiological action of insulin, it will be necessary to ascertain whether the action of insulin in the liver is similar to that observed in the extrahepatic tissues. The authors also concluded (59) that this insulin-controlled transfer system shows specificity of action with respect to the chemical configuration of the insulin-responsive sugars. Since they found that insulin influences glucose, galactose, d-xylose, and l-arabinose, they concluded that insulin affects only those sugars which possess the following configuration on carbons one, two and three:



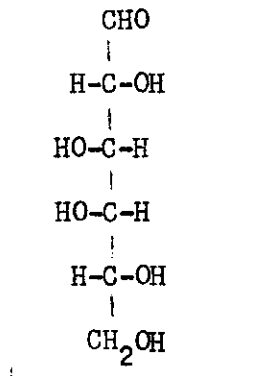
See Table 1. (after Goldstein et al 59).

This theory of the specificity of the insulin-responsive transfer system becomes untenable in the light of present-day knowledge. For instance, it is now known that d-fructose does respond to the action of insulin (39, 62, 106). It will be recalled that the work of Cori and Cori (29), Gameltoft et al (50), and Goldstein et al (59) shows d-fructose to be unaffected by the presence of insulin. How may this paradox be resolved?

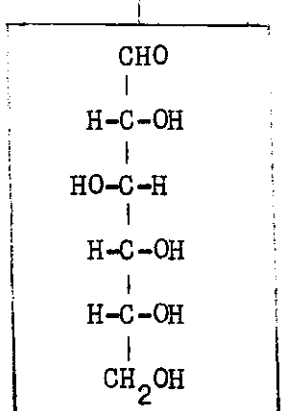
Mackler and Guest (106) demonstrated that both hexokinase and fructokinase are present in rat diaphragm muscle. Two pathways of fructose phosphorylation are possible: one, via hexokinase; the other,

Table 1. Relation of Chemical Structures of Sugars to the Action of Insulin (after Goldstein, et al., 24)

1. Responsive to Insulin

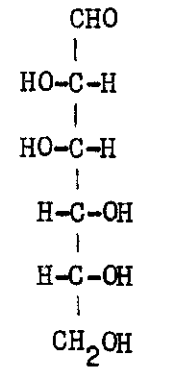


d-galactose



d-glucose

2. Not Responsive to Insulin



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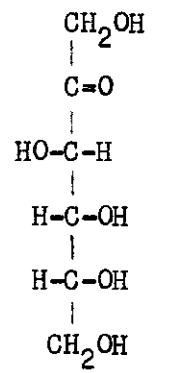
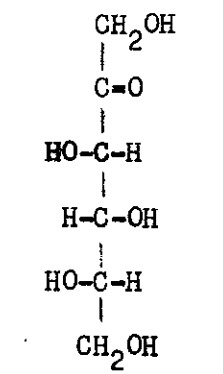
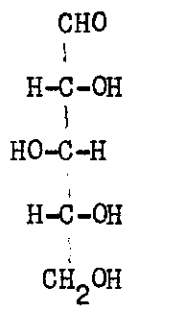


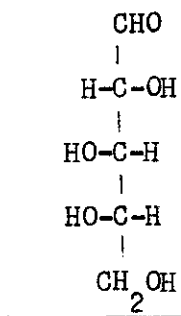
d-fructose



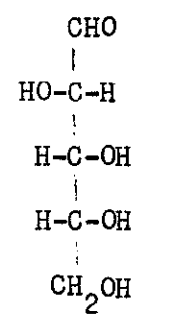
l-sorbose



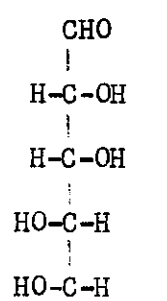
d-xylose



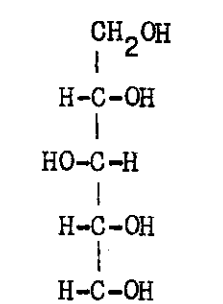
l-arabinose



d-arabinose



l-rhamnose



d-sorbitol

via fructokinase. They incubated isolated rat diaphragms in a physiological salt solution containing fructose. After a period of incubation, the uptake of fructose was determined by measuring the rate of disappearance of fructose from the medium. Upon addition of insulin, the uptake of fructose by the diaphragm muscle during the period of incubation was increased. If the incubation is now carried out in a medium containing equimolar concentrations of glucose and fructose, insulin increased the uptake of glucose, but not of fructose, by the diaphragm muscle. One must conclude from this that in the presence of an equimolar concentration of glucose, fructose phosphorylation occurs almost entirely via the fructokinase pathway which is unaffected by insulin (150). On the other hand, in the absence of glucose, fructose phosphorylation can occur via the hexokinase pathway; which, we have seen, is affected by insulin.

Therefore, fructose metabolism in intact animals or in eviscerated animals, whose blood glucose levels are maintained, occurs primarily via the fructokinase pathway. Hence, Cori and Cori (29), Goldstein et al (59), and others (21, 96, 117) were unable to demonstrate an insulin action on d-fructose.

A somewhat similar picture is seen with respect to the influence of insulin on mannose. Goldstein et al (59) observed that when the eviscerated-nephrectomized animal is kept aglycemic, i.e., its blood glucose level not maintained by glucose infusion, mannose will show a greater rate of disappearance from the blood in response to the presence of insulin. They postulated that this action might be explained by assuming the conversion of mannose to glucose in the blood, as first suggested by Mann and Magath (110). In this way, the action of insulin

could be explained as acting directly on glucose. Drury and Wick (41), however, observed that very little, if any, mannose is converted to glucose in the blood. These authors also demonstrated, using eviscerated-nephrectomized rabbits, that insulin accelerates the rate of transfer of mannose from the extracellular to the intracellular spaces of the extrahepatic tissue. In this respect, mannose resembles glucose and galactose. The presence of glucose, however, reduces the action of insulin on mannose indicating a competition for insulin action. The extrahepatic tissues under insulin action oxidize considerable amounts of mannose to CO_2 (41).

Wick and Drury (192) also demonstrated that a high concentration (800 mg. %) of glucose interferes with the transfer of galactose into the cells of the extrahepatic tissues of the rabbit.

Haft et al (62) incubated rat diaphragms in a physiological salt solution containing one of a series of sugars. They demonstrated an increased uptake in the presence of insulin for d-glucose, d-fructose, d-galactose, l-arabinose (after two hours of incubation), but not for d-arabinose nor d-xylose. The authors support the hypothesis that insulin acts by facilitating the rate of entry of some sugars into the cells. In addition, one could consider that glucose competes successfully against fructose, galactose and mannose for a carrier or transfer system, which is insulin-responsive. In the absence of glucose, fructose, galactose and mannose can become part of the carrier system and exhibit an insulin response.

Based upon the clinical fact that intense muscular work or exercise can lead to a state of insulin hypersensitivity in a diabetic patient

(77, 135), Goldstein et al (60) studied the effect of muscular work on the transfer of sugars. They employed eviscerated-nephrectomized dogs, eviscerated-nephrectomized-depancreatized dogs, and eviscerated-nephrectomized rats with complete section of the lumbar spinal cord. One limb of each animal preparation was made to do work. Using the same technique as described above (59), they tested the action of muscular work on the same series of sugars. In all three types of animal preparations, they observed that those sugars which were found to be insulin-responsive, responded to muscular work by means of an increased volume of distribution. Those sugars which were found to be non-responsive to insulin, were unaffected by muscular work. The authors, therefore, state: "It is thus postulated that both insulin and a humoral product of contracting skeletal muscle can activate a transfer mechanism for certain types of sugars across cell surface barriers."

Hechter, in his recent review on hormone action (68), states: "The isolation of this postulated humoral agent, should serve to clarify the nature of the transfer mechanism upon which insulin acts; it is not necessary to add that this postulated humoral agent, if established by direct experimentation, should posses practical importance as a therapeutic agent in diabetes if the working hypothesis of Levine and Goldstein is correct (i.e., that insulin acts at a single point of glucose metabolism, to facilitate penetration into the cell)."

E. The Effect of Various Hormones on Carbohydrate Metabolism

Let us now review some of the actions of other hormones on carbohydrate metabolism. As a result of the work of Houssay and colleagues (71, 72, 73, 74) in Argentina, it was established that the anterior pituitary

influences carbohydrate metabolism. They observed that the removal of the pituitary from a pancreatectomized animal resulted in an amelioration of the diabetic state. Conversely, the administration of extracts of crude anterior pituitary to depancreatized-hypophysectomized animals resulted in an intensification of the diabetic state. Bennett and Li (6) observed that purified pituitary products, such as growth hormone and adrenocorticotropic hormone (ACTH) intensified the glycosuria in partially diabetic rats. It was also demonstrated (40, 46) that, in the hypophysectomized animal, the proportion of carbohydrate metabolized is greater than that in the normal animal. These, and many other experiments, seem to indicate that certain factors from the anterior pituitary depress carbohydrate utilization. The absence of these anterior pituitary factors as in hypophysectomy results in an enhanced utilization of carbohydrate by animal tissues.

Soon thereafter, it became evident that the adrenal cortex is also implicated in carbohydrate metabolism. Long and Lukens (102) observed that the removal of the adrenals from a diabetic animal resulted in an amelioration of the diabetic state. Ingle et al (75) found a reduced uptake of glucose by eviscerated-normal or eviscerated-adrenalectomized rats following injection of C-11 oxysteroids. Conversely, Ingle et al (76) found that glucose disappearance was greater in eviscerated-adrenalectomized rats than that for the eviscerated-normal animal. These, and many other experiments, seem to indicate that certain factors from the adrenal cortex depress carbohydrate utilization. The absence of these adrenal cortical factors, as in adrenalectomy, results in an enhanced utilization of carbohydrate by animal tissue.

The experiments cited above, which indicate a relationship between carbohydrate metabolism and certain factors from the anterior pituitary and adrenal cortex, were performed *in vivo*. Let us determine whether this relationship has also been found to exist *in vitro*. When one employs the technique of incubating isolated rat diaphragm muscle in a physiological salt solution containing glucose, it is possible to demonstrate a greater glucose uptake by diaphragm muscle obtained from hypophysectomized rats than that taken up by diaphragm muscle obtained from normal rats (182, 87, 129). In reviewing the literature it becomes evident that in order to demonstrate this effect of hypophysectomy on glucose uptake, diaphragms must not be removed from the rats until at least ten days after hypophysectomy. This was demonstrated by Li *et al* (99) using diaphragms removed from rats ten days following hypophysectomy and by Perlmutter and Greep (133) using diaphragms removed from rats four days after hypophysectomy. In both cases, no effect of hypophysectomy on glucose uptake was detected. Krahl and Park (87) also observed that the effect of hypophysectomy in rats does not become fully evident until about ten days after the operation, at which time it is possible to observe that the adrenal cortex has atrophied to a large extent. They also observed that the optimum time for demonstrating the effects of hypophysectomy on carbohydrate metabolism is around fifteen to twenty days after the operation, with these effects disappearing around one hundred and twenty days following the operation.

Some *in vivo* experiments which demonstrate a relationship between carbohydrate metabolism and certain factors from the adrenal cortex have already been mentioned. Also the *in vitro* work with hypophysectomized animals indicates a relationship between the factors from the anterior

pituitary and the adrenal cortex on carbohydrate metabolism. Using the same in vitro technique as described in the preceding paragraph, it was found that the uptake of glucose by diaphragm muscle obtained from adrenalectomized rats was greater than that of diaphragm muscle obtained from normal rats (182, 88). The effects of adrenalectomy alone, however, are not great enough to account for the increased glucose uptake of diaphragms obtained from hypophysectomized animals.

Even though the isolated diaphragm obtained from hypophysectomized, adrenalectomized or hypophysectomized-adrenalectomized rats shows, in vitro, an increased glucose uptake as compared to normal diaphragm muscle, addition of insulin to the incubation medium results in a still further increase in glucose uptake (182, 87, 129, 133). In other words, such a diaphragm muscle is still capable of responding to insulin. This has been interpreted by various authors to mean (87, 133), that normally the glucose uptake of muscle is under an inhibitory influence exerted by factors from the anterior pituitary and the adrenal cortex. It is also agreed that although removal of the anterior pituitary and adrenal cortices results in an increased glucose uptake by muscle, this muscle can still respond to insulin by a further increase in glucose uptake. This suggests the presence of one or more additional inhibitory factors. These inhibitory influences are reversed by insulin. It can be stated therefore that one of the actions of insulin on carbohydrate metabolism is to counteract the inhibition on glucose uptake by muscle exerted by factors from the anterior pituitary, adrenal cortices, and other unknown factors.

Let us now consider what is known about the nature of the factors

from the anterior pituitary which influence glucose uptake by the mammalian organism. Park et al (131) studied the effect of highly purified growth hormone on the uptake of glucose by the isolated rat diaphragm, incubated in a physiological salt solution containing glucose. The authors employed both the Armour growth hormone preparation and electrophoretically homogeneous samples of growth hormone which were crystallized one to four times, according to the method of Wilhelmi et al (193). Hypophysectomized rats were injected intraperitoneally, eighteen to twenty-four hours before removal of the diaphragm, with both preparations of growth hormone, in dosages of two hundred, twenty, or two micrograms per one hundred grams body weight. A significant inhibition of the glucose uptake by the isolated diaphragm muscle was produced at each dosage level. This inhibition of glucose uptake lasts for at least twenty-four hours following the injection of the growth hormone and may be demonstrated both in hypophysectomized and normal rats. This inhibition can be completely reversed by insulin.

The two preparations of purified growth hormone used were similar in their ability to depress the uptake of glucose by the isolated diaphragms in vitro and in their ability to stimulate growth when injected into hypophysectomized rats for four days before removing their diaphragms (131). It was also observed (131) that when crystalline growth hormone is subjected to various physical and chemical procedures, the ability to depress glucose uptake and the ability to stimulate growth are lost or retained to equal degrees. These authors, therefore, postulate that since a dose of two micrograms of growth hormone is nearly minimal for the production of growth in rats, it is possible that growth hormone may play

a role physiologically in controlling glucose uptake by the mammalian organism.

Park and Daughaday (129) observed rats injected, three to six hours before removal of the diaphragm, with Armour growth hormone or with twice-crystallized growth hormone in dosages of ten to four hundred micrograms. No consistent depression of the glucose uptake by the isolated diaphragm muscle could be detected. Employing these same conditions, these workers also demonstrated that the administration of 400 micrograms of ACTH alone or together with 400 micrograms of growth hormone resulted in no consistent depression of the glucose uptake by the isolated diaphragm muscle. On the other hand, Park and Krahl (130) observed normal or hypophysectomized rats injected, 3 to 6 hours before removal of the diaphragms, with 10 mg. of Wilhelmi Fraction A or 3 mg. once-crystallized growth hormone. An inhibition of the glucose uptake by the isolated diaphragm could be demonstrated. Park and Daughaday (129) injected rats with 10 micrograms of Fraction A or Fraction A-1 and removed the diaphragms three hours later. They were also able to demonstrate an inhibition of the glucose uptake by the isolated diaphragm muscle.

From these data, Park et al (131) postulate that since highly purified, crystalline growth hormone preparations do not influence the uptake of glucose by the isolated diaphragms 3 hours after injection, but depress the uptake of glucose by these diaphragms 24 hours after injection, these crystalline growth hormone proteins may be transformed in the body into inhibitor substances capable of depressing glucose uptake. Apparently, these inhibitor substances are present in crude pituitary extracts and in Wilhelmi Fraction A, which is a partially purified pituitary extract

containing the growth hormone. Repeated precipitations and crystallizations during the process of purifying the growth hormone apparently reduce the concentration of the glucose inhibitor(s).

Other types of experiments seem to confirm the role of growth hormone on glucose metabolism. For example, Cotes et al (35) injected a total of 4 mg. per day of various highly purified growth hormone preparations into cats. After 5 days of such repeated injections, they observed that the cats developed glycosuria. This was confirmed by Campbell et al (16) using dogs for their experiments.

In the preceding experiments, which were performed to test the influence of growth hormone or crude pituitary extracts on the uptake of glucose by the isolated diaphragm muscle, the substances were injected into the animals rather than added to the medium directly. Park et al (131) incubated diaphragms from hypophysectomized rats in a physiological salt solution containing glucose and to which were added various preparations of highly purified growth hormone or Fraction A. An increase in the glucose uptake of the diaphragms was observed. This increase was not produced by diaphragms from normal rats, except when the incubation medium contained a high concentration of glucose. It was also observed by these workers that the injection of crystalline growth hormone into hypophysectomized rats produced a drop in blood sugar. If the diaphragm is removed from the animal and tested in vitro, it will show an increased rate of glucose uptake. This increase becomes evident approximately one hour after injection and then tends to disappear.

This insulin-like action of various pituitary fractions may explain the hypoglycemia which follows the administration of large doses of growth

hormone to normal or adrenalectomized rats (118) or to hypophysectomized dogs (38). The hypoglycemia and increased glucose uptake cannot be considered as due to a secretion of insulin as a consequence of the injection of the various pituitary fractions, since they are observed in eviscerated rats (131), in diabetic dogs (38), and in vitro (131). The hypoglycemia wears off in a few hours and is followed by the development of relative insulin resistance which is evidence for the onset of the action of the inhibitor(s) (131). As a consequence of this, in vivo experiments of approximately 18-24 hours duration can be performed without interference from the stimulatory effects of various pituitary fractions.

The relation of the adrenals to glucose utilization, as observed both in vivo and in vitro has already been discussed. As to the nature of the substances involved, both Upjohn's lipo-adrenal extract and cortisone have been shown to be active in this respect (129). For example, when diaphragms from hypophysectomized-adrenalectomized rats are incubated in a physiological salt solution containing glucose, prior injection of growth hormone alone, 18 to 24 hours before removal of the diaphragm, resulted in only a slight depression of glucose uptake. When Upjohn's lipo-adrenal extract or cortisone were then injected intramuscularly in divided doses, 24 and 6 hours before removal of the diaphragm, the depression of the glucose uptake of the isolated diaphragms was greatly increased. Administration of the lipo-adrenal extract or cortisone, by themselves, had no effect on the glucose uptake. When the adrenal steroids are added directly to the incubation medium, it is difficult to obtain decisive results on glucose uptake. Perhaps this is due to the

difficulty in dissolving the adrenal steroids in aqueous media. As a consequence of this, Verzar et al (95, 180) dissolved a number of steroids in 0.5% ethanol. These solutions were then added directly to a physiological salt solution containing glucose, in which isolated diaphragms from normal rats were incubated. The authors observed a decreased glucose uptake and a decreased glycogen formation by the isolated normal diaphragms as a result of some steroids, whereas others had no effect.

How may the influence of the anterior pituitary and adrenal cortex on carbohydrate metabolism be correlated with the known actions of insulin and with the several theories of the mechanism of insulin action?

An attempt was made by workers in Cori's laboratory to study these phenomena in cell-free systems. About ten years ago, these workers reported that they were able to demonstrate an influence of insulin and other hormones on a cell-free hexokinase system. This was widely acclaimed as a milestone, being the first definitive demonstration of an influence of hormones on an enzyme-system contained in a cell-free preparation. It has already been mentioned that Cori and his colleagues believe that insulin acts specifically to control the rate of glucose phosphorylation by the specific enzyme, glucohexokinase. When the influence of the anterior pituitary and adrenal cortex on carbohydrate metabolism was being worked out, these workers extended their original theory. They now suggested that the anterior pituitary, adrenal cortex, and as yet unknown factors, influence carbohydrate metabolism by inhibiting the activity of the glucokinase. This results in a decreased glucose uptake with a consequent decreased glucose utilization. Since it has been shown that such a depression can usually be reversed by the action of

insulin, then one primary action of insulin must be the release of the glucohexokinase from the various inhibitory influences. This would result in a reactivation of the glucohexokinase, with a consequent return to normal glucose uptake and utilization. It is this theory which they set out to test, using cell-free hexokinase systems.

The rate of the hexokinase reaction (glucose plus ATP——G-6-PO_4 plus ADP) in cell-free extracts was determined by measuring the rate of glucose disappearance from the incubation medium containing glucose, or by measuring the evolution of CO_2 in a bicarbonate medium containing glucose. It was found (138, 139) that in muscle extracts from alloxan-diabetic rats, the addition of adrenal cortical extract (ACE) markedly inhibited the hexokinase reaction. The addition of ACE to muscle extracts from normal rats, however, resulted in no inhibition of the hexokinase reaction. When certain anterior pituitary fractions were added to muscle or brain extracts from normal rats, an inhibition of the hexokinase reaction resulted, especially if ACE was also added. Whenever inhibition was observed, insulin resulted in a release of such inhibition. Insulin by itself had no effect on the hexokinase reaction. It was also demonstrated (139) that inhibition produced by ACE could not be duplicated by using the crystalline compounds A, B and E. Therefore, the activity must be associated with one or more unidentified substances present in ACE.

Colowick et al (25) observed that when muscle extracts from normal rats or beef-brain extracts are incubated in the presence of ACE, no effect upon the hexokinase reaction could be detected. If anterior pituitary extract (APE) is then added, the hexokinase reaction is inhibited 30 to 75%. They also demonstrated that when muscle extracts

from alloxan-diabetic animals are incubated in the presence of ACE alone, a marked inhibition of the hexokinase reaction could be observed. When insulin was present in the incubation mixture, significant increases in the hexokinase reaction in about 50% of the cases could be observed; i.e., insulin produced a release of ACE inhibition in about 50% of the cases. They also postulate that since ACE alone has an inhibitory effect on muscle extracts from alloxan-diabetic rats, then these extracts must already contain an inhibitor substance, presumably of pituitary origin. The inhibitor substance in APE is as yet unidentified, since ACTH, growth hormone, and prolactin when tested were found not to be inhibitory.

Reid et al (140) also demonstrated that crude APE can inhibit the hexokinase activity of extracts of various tissues. This inhibition is reversed by insulin. They also found that the ability of APE to inhibit the hexokinase reaction in vitro showed no relationship to the diabetogenic activity of the APE in vivo.

On the other hand, Broh-Kahn and Mirsky (14) demonstrated that the hexokinase activity of muscle extracts from alloxan-diabetic rats corresponded to that of muscle extracts from normal rats and that insulin produced no effect in either case. Also, they could occasionally observe an inhibition of hexokinase activity by the diabetogenic factors of the anterior pituitary, which was reversed by insulin. However, they could also obtain inhibitory effects on hexokinase activity by non-specific extracts, e.g., spleen extracts.

Stadie et al (159) also concluded that they could not demonstrate any difference in the hexokinase activity of muscle or kidney extracts from diabetic rats when compared to normal controls. Stadie et al (161)

also obtained muscle extracts from completely depancreatized rats. The hexokinase activity corresponded to that of normal controls and was not influenced by ACE, insulin, or ACE plus insulin.

Christensen et al (23) studied the hexokinase activity of red blood cell hemolysates of the rat. They state: "The activity of the enzyme as obtained from normal rats is not effected by the addition of insulin or adrenal cortical extract. Enzyme preparations from diabetic or hypophysectomized animals show no essential variation in activity from those of normal animals and are also uninfluenced by hormone additions."

Reiss and Rees (141) studied suspensions of the grey matter of rat brain. They observed that the hexokinase activity was greater after hypophysectomy and adrenalectomy.

It can be seen from the work just reviewed that the demonstration of hormone action on cell-free systems is very inconsistent and very difficult to control. The workers in Cori's laboratory feel, when all of the in vivo and in vitro experiments are considered, that their theory of the interaction of hormones on the rate of the hexokinase reaction is justifiable. Nevertheless, Cori, (33) in discussing the work on cell-free systems, admits that the evidence could be construed so as to indicate that insulin can have a stimulatory effect upon other enzyme systems in addition to that on the hexokinase reaction.

How can the contra-insulin effects of the inhibitor substances from the anterior pituitary and adrenal cortex be explained in the light of the transfer mechanism theory of Levine et al? With this in mind, Goldstein et al (58) injected dogs with dosages of cortisone sufficient to make the animals unresponsive to intravenously administered insulin.

After 3 days of cortisone administration (10-25 mg./day), they studied the volume of infused galactose, in the presence and absence of insulin, in these dogs which were made into eviscerated-nephrectomized preparations. The volume of distribution of galactose, with and without insulin, was shown to be the same as that observed in the normal eviscerated-nephrectomized preparation, without previous cortisone treatment. Stated differently, this means that although enough cortisone was administered in vivo to make the dog unresponsive to insulin, it did not inhibit the action of insulin on galactose uptake by the extrahepatic tissues of the eviscerated-nephrectomized preparation. They postulated, therefore, that their findings indicate that the contra-insulin action of the adrenal cortex is not located in the extrahepatic tissues. Such a conclusion poses a difficult problem, since there is evidence in the literature to indicate that muscle tissue of adrenalectomized animals responds to insulin differently from muscle obtained from a normal animal; also, that muscle from normal animals, in the presence of excess cortisone, responds differently from untreated muscle with respect to insulin action.

This section on the effects of hormones and other factors, both identifiable and unknown, on carbohydrate metabolism would not be complete without a review of the work, which indicates the presence in blood of substances inhibitory to the hexokinase reaction.

Weil-Malherbe (188) studied the effects of plasma from diabetic and normal human subjects on the hexokinase reaction of rat brain homogenates. The influence of plasma from 14 diabetic patients was studied. The plasma of 7 of these diabetic patients was shown to inhibit the hexokinase reaction of the rat brain homogenates. This inhibition could be reversed

by injection of insulin into the diabetic patient prior to the removal of the blood. Addition of insulin to the assay system had no effect. Serum from non-diabetic patients seemed to favor the hexokinase activity of the brain homogenate. The author also states that the inhibitor substance is unstable, losing most of the activity in about 12 hours even when stored at 0°C. This is an interesting observation in view of the statement by Cori (33) that the hexokinase inhibitor substance of ACE is extremely unstable. He found that storage of the ACE for 1 to 2 hours at 0°C frequently resulted in a loss of inhibitory activity, when tested on cell-free systems.

In 1953, Bornstein and Park (10) reported experiments in which isolated diaphragms from normal rats were incubated in a physiological salt solution containing glucose, with and without insulin. To this medium was added an equal volume of serum obtained from normal, alloxan-diabetic, hypophysectomized and adrenalectomized rats. The uptake of glucose from the medium by the incubated diaphragms was then determined by measuring the disappearance of glucose from the medium at the end of the incubation. The incubation was carried out under aerobic conditions at mammalian body temperature (37°C). The results are expressed as mg. of glucose uptake/gram diaphragm wet weight/hour incubation. Table 2 with the results of their experiments is reproduced from their paper (10). The serum was stored at 5°C. and used as soon as possible.

It can be seen from this table that when isolated, normal rat diaphragms are incubated in medium plus serum obtained from alloxan-diabetic rats, the uptake of glucose from the medium by the diaphragm muscle is less than that of isolated diaphragms incubated in medium plus

Table 2

Effect of Sera From Various Types Of Donor Rats On Glucose Uptake Of Normal Rat Diaphragm (After Bornstein, et al., 74)

Exper. No.	Type of Rat Used As Serum Donor	Medium	Glucose Uptake, mg./gm./hr.	
			No added Insulin	With added Insulin
1	No serum in medium	Buffer, 3%	2.2 ± 0.2	6.2 ± 0.35
2	No serum in medium	Albumin in buffer	3.1 ± 0.2	6.4 ± 0.30
3	Normal	Serum	4.2 ± 0.2	6.0 ± 0.25
4	Diabetic	Serum	3.0 ± 0.13	5.3 ± 0.12
5	Diabetic-Adrenalectomized	Serum	4.1 ± 0.23	6.1 ± 0.11
6	Diabetic-hypophysectomized	Serum	4.1 ± 0.09	6.0 ± 0.19
7	Diabetic-hypophysectomized after injection of growth hormone and cortisone	Serum	2.8 ± 0.12	5.3 ± 0.14
8	Diabetic-hypophysectomized after injection of growth hormone	Serum	4.2 ± 0.15	5.9 ± 0.20
9	Diabetic-hypophysectomized after injection of cortisone	Serum	4.0 ± 0.20	5.8 ± 0.30
10	Diabetic-hypophysectomized 168 hrs. after last injection of growth hormone and cortisone	Serum	2.9 ± 0.16	

Where indicated, 0.2 mg. growth hormone (Armour 22 KRa) and 0.1 mg. of cortisone (Merck) were injected i.p. daily for 2 days prior to the taking of the blood.

serum obtained from normal rats. The authors thus speak of this as a demonstration of the inhibitory action of diabetic serum, using the uptake of glucose by the isolated rat diaphragm muscle as a test system. The presence of insulin resulted consistently in an increased uptake of glucose by the isolated diaphragm, whether the incubation occurred in Krebs-Ringer solution, in medium plus alloxan-diabetic serum or in medium plus normal serum. This, they postulate, denotes a counteraction of the inhibitory action of diabetic serum.

When isolated normal diaphragms were incubated in medium plus serum obtained from alloxan-diabetic rats previously adrenalectomized or hypophysectomized, the uptake of glucose was comparable to that when incubation was carried out in medium plus normal rat serum. Thus, serum obtained from alloxan-diabetic-adrenalectomized or from alloxan-diabetic-hypophysectomized rats does not show an inhibitory action on glucose uptake by muscle under these test conditions. If the diaphragms were incubated in medium plus serum obtained from diabetic-hypophysectomized rats previously injected with growth hormone and cortisone, the uptake of glucose by the diaphragms was again depressed indicating the presence of the inhibitor(s). This depression of glucose uptake was also found to be reversed upon addition of insulin to the incubation medium. If the diaphragms were incubated in medium plus serum obtained from diabetic-hypophysectomized rats previously injected with growth hormone or cortisone, the uptake of glucose by the diaphragms was comparable to that when incubation was carried out in medium plus normal rat serum.

Bornstein and Park (10) repeated the experiments outlined in Table 2. In this set of experiments, however, the diaphragms were obtained from

diabetic, hypophysectomized, or diabetic-hypophysectomized rats. Essentially the same results were obtained as when diaphragms from normal rats were used. Also, these workers incubated normal rat diaphragms in medium to which growth hormone and/or cortisone were added. The results showed no effects of these hormones on glucose uptake by diaphragms when added *in vitro*. This was taken by the authors to mean that bodily passage is necessary for these hormones to act, in a way as yet unknown, to enable the serum to depress glucose uptake *in vitro*. The authors state in the summary: "The insulin-reversible inhibitor of glucose uptake in the blood of diabetic rats is formed as a result of endogenous pituitary and adrenal activity."

Bornstein (11) considered the possibility that the inhibitor substance found in alloxan-diabetic serum might be a protein-steroid complex, which may be associated with the lipoprotein fraction of serum. It had been found previously by Oncley *et al* (126) that some of the steroids circulating in the blood are shown to be present in the lipoprotein-rich fractions of plasma, especially in the β-lipoprotein. Therefore, Bornstein (11) tested lipoprotein fractions of human and rat blood to ascertain whether an inhibition of glucose uptake by isolated diaphragm could be demonstrated. When lipoprotein fractions from serum of alloxan-diabetic rats were tested, an inhibition of glucose uptake by the isolated diaphragm was observed. This inhibition was reversed by insulin. This inhibitory action was found to be associated with the β-lipoprotein fraction (Cohn's Fraction III_0), which could be inactivated by storage in an ice bath or by freezing. The best stability was achieved by storing the lipoprotein between 4-7°C. I would like to point out the similarity

with respect to stability, between this inhibitor substance and that discussed by Cori (33) and Weil-Malherbe (188). This has already been reviewed above.

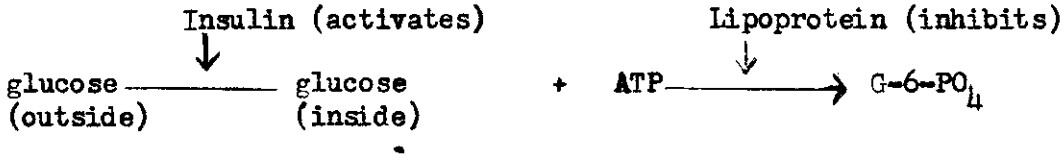
When the lipoproteins were removed by flotation from the serum of alloxan-diabetic rats, no inhibition was found to occur when the lipoprotein free serum was tested. Also, when the lipoprotein fractions obtained from normal rat serum, normal human serum, or serum from hypophysectomized-diabetic rats were tested, no inhibitory effects on the glucose uptake by isolated diaphragms could be observed.

In a more recent communication, Krahl and Bornstein (90) tested the effects of lipoproteins obtained from plasma, anterior pituitary, liver, and muscle on the glucose uptake of cell-free, saline muscle extracts. All of these lipoproteins were found to inhibit the glucose uptake by the muscle extracts. The effect of insulin was then studied to ascertain whether a reversal of the lipoprotein inhibition occurred. Insulin was added in 42 experiments; in 12 of these experiments, a reversal of the lipoprotein inhibition was detected. If insulin inactivated by alkali, serum albumin, hyaluronidase, or glucagon were added not one case of reversal of lipoprotein inhibition was observed.

It will be remembered that after considering the literature on the effect of inhibitors and other factors on glucose utilization in cell-free extracts, the conclusion was reached that work of this nature must be interpreted with utmost caution. The test system is not easy to control and does not give consistent results. Therefore, the fact that insulin reversed the lipoprotein inhibition in 12 out of 42 experiments may or may not be considered as a demonstration for the insulin-reversible

nature of lipoprotein inhibition. In preparing the lipoproteins tested, the authors found that precipitating the lipoproteins with zinc allowed for longer storage and better keeping properties. The authors, however, were aware of the possibility of the zinc ions influencing the system to produce false or meaningless results. They therefore removed the zinc by means of versene, a chelating agent, before dialysis of the lipoprotein. They then determined that the maximum concentration of zinc in the purified lipoprotein was less than 1×10^{-7} M, which in their estimation would have no effect on the test system. It will be mentioned later that Krebs et al (91) found that insulin resulted in an increased O_2-consumption in a muscle mince of pigeon breast muscle in the presence of boiled muscle extract and components of the Krebs cycle. This did not occur when zinc insulin was used, and the authors attributed this to the effect of the zinc contained by the insulin on the test system.

In discussing the work on lipoprotein inhibitors of glucose uptake, Hechter (68) states: "It seems likely that the site of pituitary-adrenocortical influence upon glucose uptake is exerted at the level of the glucokinase reaction, but that insulin action is not directly involved in this step. If we can accept this premise, the picture which emerges would be the following:



On this view, insulin overcomes the lipoprotein inhibition observed in intact cells by increasing the rate-limiting step involved in transfer so that the intercellular glucose is increased and made available for

that portion of the glucokinase system which is not inhibited by lipoprotein."

Considerable space has been allotted to a consideration of the effects of various hormones on carbohydrate metabolism. This discussion is by no means complete. However, the problem of the interaction of hormones in influencing metabolism, the presence of inhibitors and stimulators, etc., is of extreme potential import clinically, as well as for a more complete understanding of metabolism. I have already mentioned the potential clinical implications if one were successful in isolating the humoral product, postulated by Goldstein et al (60) to be liberated as a result of muscular contraction and shown to facilitate sugar uptake.

Before leaving this section, I should like briefly to discuss some of the effects of epinephrine on carbohydrate metabolism. Walaas and Walaas (186) incubated isolated diaphragms obtained from normal rats in a physiological salt solution containing glucose and determined the glucose uptake under the conditions of the experiment. When epinephrine was added directly to the incubation medium (1 microgram/ml.), there was a significant decrease in the glucose uptake by the isolated diaphragm. Thus, it was demonstrated that epinephrine decreases glucose utilization in vitro. These workers did not test the effect of insulin on this inhibition of glucose uptake. It is of interest at this point to cite the work of Meyerhof and Randall (116), who observed that adrenochrome inhibits hexokinase action in brain extract.

Cori et al (30) injected a small amount of epinephrine (0.03 mg./kg. body wt.) into normal rabbits. Within three minutes after the injection, the blood sugar and blood lactic acid rose precipitously. This may be

the result of an increased glycogenolysis. This results in a rapid breakdwon of glycogen stored in the muscle and reflected as a rise in blood lactic acid, and a rapid breakdown of glycogen stored in the liver and reflected as a rise in blood glucose.

It is also important to mention that a decreased peripheral utilization of glucose after injection of epinephrine has been observed in rats (26), man (27, 153), rabbits (28), and dogs (32).

It has also been shown (1, 134) that during epinephrine action the concentration of blood inorganic phosphate decreased, as did the urinary excretion. Cori (31) then demonstrated that during epinephrine action hexose phosphate accumulated in muscle, which could account for the decrease in blood inorganic phosphate. Walaas et al (186) demonstrated that isolated rat diaphragms incubated in a physiological salt solution containing glucose plus epinephrine show a decreased glucose uptake, a loss of glycogen, an increased formation of lactic acid, and an accumulation of intermediary products, such as hexose-6-phosphate.

How may the influence of epinephrine on carbohydrate metabolism be interpreted in the light of what has already been said concerning this subject? It can be seen from the work of Walaas et al (186) that epinephrine can have a direct effect on muscle. The effects of epinephrine injection into the whole animal have also been discussed. It has been shown by McDermott et al (112) that direct application of epinephrine to pituitary grafts may result in a release of pituitary factors. We have also seen that factors from the pituitary and adrenal cortex decrease the peripheral utilization of glucose. Might this not explain the decreased peripheral glucose utilization observed in animals

following epinephrine administration? While such a possibility looks attractive, it does not explain the fact that epinephrine has a direct effect on muscle, as shown by in vitro experiments.

An alternative explanation would be to consider the action of epinephrine as accelerating liver and muscle phosphorylase. This would account for the decrease in liver and muscle glycogen, the increase in lactic acid formation, and the accumulation of intermediary products of glycolysis, such as glucose-6-phosphate. The decreased peripheral utilization of glucose might be explained as due to such an accumulation blocking the phosphorylation of glucose: G plus ATP $\xrightarrow{\text{Hexokinase}}$ G-6-PO_4 plus ADP. In other words, the law of mass action might be invoked and the reaction depressed due to an accumulation of the end-product. This would then be reflected by a decreased peripheral glucose uptake. It has already been shown that the concentration of ATP is not a limiting factor.

It is interesting to note in this connection, that Welt et al (189) by infusing C^{14}-labelled glucose into anesthetized rats observed that after treatment with cortisone there was a 7-fold increase in gluconeogenesis as compared to the normal rats. A 2-fold increase in gluconeogenesis was also observed in alloxan-diabetic animals. On the other hand, the rate of glucose oxidation to CO_2 did not seem to be affected by cortisone treatment, and a smaller proportion of liver glycogen was formed from body glucose than in normal rats. The authors, therefore, feel that "these results argue that under these conditions a major disturbance in glucose metabolism in the rat treated with cortisone is a striking increase in the rate of glucose production with little, if any, effect upon glucose oxidation."

It has already been indicated that ACE or cortisone in vivo depresses

glucose utilization in the mammalian organism. It has also been noted that the influence of the adrenal cortex on carbohydrate metabolism is greatly enhanced by the administration in vivo of APE or growth hormone. However, administration of these factors in vitro does not seem to influence carbohydrate metabolism. Perhaps the law of mass action may be applied here, as well. Since we see from the work of Welt et al (189), and Long et al (103) that cortisone and 11-oxygenated-steroids administered to animals result in an increased rate of glucose formation from non-carbohydrate precursors (gluconeogenesis), might this not result in a decreased peripheral uptake of glucose? Stated differently, one might say that the initial step of phosphorylation of glucose by hexokinase is partially by-passed; nevertheless, the glycolytic and tricarboxylic cycles can function normally, the source of glucose-6-phosphate now being from non-carbohydrate precursors rather than from blood glucose phosphorylated by hexokinase.

While such an explanation seems attractive, I am aware that this does not necessarily account for the presence or action of inhibitors found in serum obtained from diabetic rats or diabetic-hypophysectomized rats after injection of growth hormone and cortisone.

Such a view, however, might also explain the in vivo action of APE or growth hormone in suppressing peripheral glucose utilization. The fact that both APE or growth hormone and ACE or cortisone in vivo can produce their effects on peripheral glucose utilization only after a certain minimum time following administration, could be taken to mean that time is necessary for the stimulation of gluconeogenesis with its consequent effects on peripheral glucose utilization.

As a consequence of such reasoning, it is possible to consider the actions of the anterior pituitary and adrenal cortex (and possibly the action of epinephrine and other inhibitor substances) on carbohydrate metabolism as occurring at loci in the scheme of carbohydrate metabolism other than the hexokinase reaction. However, overtly it may appear as if such effects were due to inhibition of the hexokinase reaction. We then could relegate to insulin the primary role of facilitating, in a manner as yet unknown, the entry of glucose and other insulin-responsive sugars into the cell. This hypothesis might explain the work of Goldstein et al (58) which we have already discussed in detail. They injected normal dogs with dosages of cortisone sufficient to make animals unresponsive to intravenously administered insulin. After 3 days, they eviscerated-nephrectomized these dogs and studied the volume of distribution of infused galactose in the presence and absence of insulin. They found that insulin facilitated the uptake of galactose by the extrahepatic tissues in a manner comparable to that observed in eviscerated-nephrectomized dogs not previously treated with cortisone. We have already cited evidence that no galactokinase is present in tissues other than liver, kidney, and intestines. Since these organs were removed in the experimental animals, then the galactose could not be converted to glucose and thus enter the carbohydrate metabolic scheme. As a result, the entry of galactose into the cells of the extrahepatic tissues was facilitated by the action of insulin, despite previous treatment of the whole animals with cortisone in amounts sufficient to render the whole animals resistant to insulin action. Stated differently, it is possible to interpret Goldstein's results by assuming that cortisone in vivo rendered the whole animal resistant to insulin action, but allowed insulin

to act on galactose in the eviscerated-nephrectomized preparations since no galactokinase was present in such preparations to convert it to glucose. Presumably, if whole animals had been used to test the effects of cortisone administration on the influence of insulin on galactose uptake, the authors might have found the whole animals to be resistant to insulin with respect to galactose as well. This result could be expected, since galactose would be changed in the whole animal to glucose and the utilization of glucose depressed as a result of cortisone pretreatment.

We have already seen that the depression of peripheral glucose uptake resulting from the in vivo action of the anterior pituitary or adrenal cortex can be reversed by the action of a sufficient amount of insulin. How may this be interpreted in the light of the possible hypothesis that I have put forth? This could be explained by considering that the locus of insulin action is on the transfer system responsible for the entry of certain sugars into the cells. This concept was proposed by Levine and colleagues and has already been discussed at great length. Therefore, the presence of sufficient insulin, either in vivo or in vitro, would increase the rate of entry of the insulin-responsive sugars into the cells of the tissues. Such a response to insulin could occur uninfluenced by the in vivo actions of the anterior pituitary or adrenal cortex. Overtly, however, this action of insulin could appear as if insulin counteracts the action of the anterior pituitary and adrenal cortex on the carbohydrate metabolism. This hypothesis is to be considered as an attempt to integrate the interactions of certain hormones on carbohydrate metabolism.

F. The Influence of Insulin on Fat and Protein Metabolism

If we turn our attention once again to Figure 1, it becomes apparent that normal fat and protein metabolism are dependent upon normal carbohydrate metabolism.

Let us very briefly summarize some of the work on the relation of insulin to fat metabolism. It has been shown (169, 171, 170) that lipogenesis from glucose or its intermediary products is markedly impaired in alloxan-diabetic animals. In addition, it was shown (171) that insulin administration increases lipogenesis in normal rabbits. These workers thus conclude (171) that "the insulin level affects the rate of utilization of glucose in all of its manifestations and that lipogenesis is simply one of the several processes in which products derived from glucose are utilized."

From these experiments, one may conclude that this action of insulin may be considered as specific upon fatty acid synthesis (21, 22) or as a consequence of insulin action on carbohydrate metabolism (171). We have already discussed the former possibility in our review of the hexokinase theory. While this controversy concerning the mechanism of insulin action on fat metabolism is still unsettled, confirmation of the stimulatory effects of insulin in fatty acid synthesis is very plentiful (9, 21, 22, 20, 4, 12).

From the above it is evident that synthesis of fatty acids from glucose, lactic acid, and pyruvate is impaired in diabetic animals. It is also well-known from clinical experience that the diabetic state is accompanied by an increased production of ketone bodies, i.e. ketosis. Could such a condition be the result of decreased lipogenesis by the

liver and hence a favoring of oxidative formation of ketone bodies?

Osborn et al (127) studied the metabolic fate of C^{14}-labelled pyruvate by livers, obtained from alloxan-diabetic rats. They found a decreased rate of lipogenesis from C^{14}-labelled pyruvate with an associated increase in oxidation. If the rats were treated with insulin prior to removal of the liver, they found a great increase in fatty acid synthesis from the C^{14}-labelled pyruvate with a corresponding decrease in oxidation. The authors thus conclude that insulin may shift the metabolic fate of the C_2 fragments arising from such a glucose intermediate as pyruvate.

It is interesting to note that while insulin favors fatty acid synthesis from the C_2 fragments by the liver with a corresponding decrease in ketone formation, crude pituitary extracts, growth hormone, and cortisone have been shown to favor ketone body formation by the liver. It has been shown that injection of crude pituitary extracts in animals can produce ketonemia and ketonuria (146, 7). It was also established that the liver was essential for the production of ketonemia by crude pituitary extracts (119). Later, it was shown (147) that when liver slices were incubated in a medium to which crude pituitary extract had been added, the production of ketone bodies took place at an accelerated rate. This could then be construed as a direct action of the ketogenic principle(s) of crude pituitary extract on the liver cells, rather than due to a mobilization of fat from the fat deposits with a consequent increase in fat metabolism.

Tepperman and DeWitt (177) measured the rate of ketone body formation

by surviving rat liver slices. It was observed that ketogenesis by liver slices obtained from hypophysectomized rats was much less than in those from the normal rats. If growth hormone was injected into the hypophysectomized rats within 15 days following the removal of the pituitary, and the liver slices then incubated, a normal rate of ketogenesis could be observed. Injection of growth hormone later than 30 days following hypophysectomy has no effect on the decreased ketogenesis by the isolated liver. If, however, the long-term hypophysectomized rats had been maintained with cortisone, then growth hormone administration results in a normal rate of ketogenesis by the isolated liver slice. Cortisone injection by itself resulted in a slightly increased rate of ketogenesis.

It was also shown (177) that when liver slices obtained from normal rats were incubated in a medium containing growth hormone, an increased ketogenesis could be determined. This was observed with two preparations of growth hormones.

In concluding the discussion of the relationship of insulin action to fat metabolism, it can be seen that there is a loss in the ability to synthesize fatty acids by the diabetic liver. This is associated with an increased ketone formation, which reflects an increased oxidative metabolism by the diabetic liver. It was also demonstrated that hypophysectomy results in a restoration of ketogenesis to normal by the diabetic liver (13, 155). This indicates that hypophysectomy in diabetic rats results in a normal pattern of fatty acid metabolism (13, 155). This is strikingly reminiscent of the Houssay phenomenon, which we have already discussed in detail, in which it was observed that removal of the pituitary from a diabetic animal resulted in an amelioration of the

diabetic state. Conversely the administration of crude anterior pituitary extracts to an Houssay animal (hypophysectomized-depancreatized) resulted in an intensification of the diabetic state.

There is also ample evidence to indicate that the synthesis of protein is enhanced by insulin action. Krahl (89) incubated liver slices from normal and diabetic rats in a medium containing C^{14}-labelled glycine. He then determined the incorporation of the added glycine-I-C^{14} into liver glutathione and protein fractions of the liver. Krahl found that such an incorporation was reduced in liver slices obtained from diabetic rats. When glucose plus insulin were added to the medium, the incorporation was restored to normal. This led the author to consider that insulin exerts an influence on peptide synthesis through its effects on glucose metabolism. This was indicated by the observation that in the absence of added glucose, insulin had no effect on the incorporation of glycine-I-C^{14} by the diabetic rat. Furthermore, when glucose alone was added to the incubation medium, there was a rise in the incorporation of glycine-I-C^{14}. Glucose plus insulin restored the rate of incorporation to normal.

Sinex et al (149) observed that insulin increased the incorporation of C^{14}-alanine into protein by isolated diaphragm muscle obtained from normal, fed rats. This was found to be the case only when no substrates were added to the incubation medium. When pyruvate was added to the incubation medium, the incorporation of C^{14}-alanine into diaphragm protein was decreased. A slight decrease was also produced when glucose was added to the medium. These authors found that insulin had only slight effects on C^{14}-alanine incorporation in the presence of the added substrates. They thus conclude that "insulin may exert a stimulatory

effect on the formation of peptides or proteins either directly or through an effect on high energy phosphate bond formation."

The demonstration that insulin influences protein synthesis has been confirmed many times, both in vivo and in vitro. Forker et al (47) observed that the rate of incorporation of methionine-S^{35}, injected intravenously into eviscerated dogs, into muscle protein was significantly reduced in diabetes. Administration of insulin corrected this defect.

Lotspeich (105) demonstrated a fall in the concentration of the amino acids of the blood following injection of insulin into normal dogs.

These are but a few of the many papers demonstrating such an effect of insulin on protein metabolism.

G. The Influence of Insulin on Phosphate Metabolism and on Oxidative Systems

Up to this point in our introduction, we have discussed several theories concerning the mechanism of insulin action as well as the locus or loci of such action(s). One must also include a discussion of additional theories which have been introduced on this subject.

One such theory is concerned with the relation of insulin to phosphate metabolism. It is generally accepted that certain oxidative reactions which occur in the cell may be concerned with ATP synthesis or regeneration. As a result of this a theory has been advanced, which considers the influence of insulin to be on the oxidative reactions involving the Krebs Cycle (the tricarboxylic acid cycle). If we again examine Figure 1, it can be seen that the Krebs Cycle may also be involved in the generation of high energy phosphates, such as ATP, etc. Let us consider some of the experimental evidence which could argue for the validity of such a theory.

Krebs et al (91), in 1938, demonstrated an effect of insulin on

oxidative metabolism. They studied respiration in pigeon breast muscle mince in the presence of boiled muscle extract containing heat-stable enzymes and various components of the tricarboxylic acid cycle (Krebs Cycle). It was found that the presence of insulin, in this system, resulted in an increased O_2 consumption. There was no significant increase in the rate of O_2 uptake initially. However, over a period of 3 or 4 hours, the presence of insulin often resulted in a two-fold increase in the O_2 consumption, as compared to that in the absence of insulin. Since both glucose and glycogen rapidly break down to lactic acid during homogenation of the muscle in the process of preparing the mince, it was argued that the action of insulin must be on systems other than the hexokinase reaction. The effect of insulin in increasing O_2 consumption occurred only when components of the Krebs Cycle were added to the preparation. Therefore, it was concluded that insulin influences reactions concerned with the Krebs Cycle (see Figure 1).

Rice and Evans (142) incubated pigeon breast muscle, in the presence and absence of insulin, and then added pyruvate to the medium. During a subsequent period of incubation, it was observed that the pyruvate utilized by the muscle previously incubated with insulin was greater than that utilized by the muscle not exposed to insulin. Hence, the authors concluded that insulin is associated with pyruvate metabolism and the Krebs Cycle.

Stadie et al (157) also demonstrated that the presence of insulin increases pyruvate utilization by the pigeon muscle mince. This was also shown to occur when slices of pigeon breast muscle were incubated instead of minces. These authors concluded that insulin influences pyruvate

metabolism and the Krebs Cycle. However, when rat muscle minces were used insulin showed no effects on pyruvate utilization.

Shorr and Barker (148) confirmed the work of Krebs et al (91). These authors likewise found that insulin maintained O_2 consumption in minced pigeon breast muscle. However, no effect of insulin on the O_2 consumption of minced chicken breast muscle, minced skeletal muscle of the cat, dog, and rabbit, or minced dog cardiac muscle could be demonstrated. In addition, it was observed that the disruption of the cellular organization of a muscle by mincing results in a stimulation of carbohydrate oxidation, even in the absence of insulin.

Stare and Baumann (168) confirmed the stimulatory effect of insulin on the O_2-consumption of pigeon breast muscle. This stimulatory effect was even greater following pancreatectomy. They also demonstrated that the di- and tricarboxylic acids, cocarboxylase and substances in the muscle juice are necessary for the maintenance of respiration. The stimulatory effect of insulin on respiration in pigeon breast muscle actually can be considered as a prolongation or preservation of respiration for long periods of time. In the absence of insulin, respiration practically ceased after 2 to 4 hours. With added insulin, respiration was maintained. This was also observed by Krebs (91).

Stadie et al (155) demonstrated that when a muscle mince of normal pigeon breast was incubated with boiled muscle extract, the presence of citrate increased the O_2 consumption. Added insulin also increased the oxygen consumption. This stimulatory effect of insulin was not enhanced when muscle mince of diabetic cats or pigeons was used. It thus becomes apparent that it is very difficult to interpret the action of insulin on O_2 consumption by muscle mince preparations.

Villee and Hastings (183) studied the relation of insulin action to pyruvate metabolism in the rat. The test system employed was the isolated diaphragm obtained from normal and diabetic rats and incubated in a physiological salt solution containing isotopic pyruvate in the presence and absence of insulin. The isolated diaphragm obtained from alloxan-diabetic rats, when incubated as described above, showed both pyruvate utilization and oxidation to be decreased. When insulin was added to the medium, the authors observed an increase in the pyruvate oxidized, an increase in pyruvate utilization and an increase in the O_2 uptake and respiratory CO_2 from pyruvate. Insulin, on the other hand, showed no effect on the pyruvate metabolism of isolated diaphragms obtained from normal rats. It is thus concluded that the utilization of pyruvate and its oxidation to CO_2 are impaired in diaphragm muscle obtained from alloxan-diabetic rats. Addition of insulin to the incubation medium restores pyruvate metabolism to normal.

Frohman et al (48) measured the concentration of the various organic acids comprising the Krebs Cycle and of lactic and pyruvic acids in the liver and kidney of the rat by means of column chromatography. They demonstrated that the concentration of lactic and pyruvic acids in the liver and kidney of diabetic rats is essentially normal. The concentration of the organic acids comprising the Krebs Cycle was found to be significantly decreased in the tissues from the diabetic rats. The authors interpret this to mean that there is a metabolic block between pyruvic acid and the Krebs Cycle. Such a metabolic block could interfere with the oxidative phosphorylation mechanism of the cell, which in turn could be responsible for many of the metabolic derangements which

accompany diabetes mellitus. When insulin was administered to these diabetic rats, the concentration of the organic acids of the Krebs Cycle was restored to normal.

In conclusion it becomes apparent to the reader that the possible action of insulin on the oxidative reactions involving the Krebs Cycle must be accepted with reservation until more decisive experiments are forthcoming.

H. The Effect of Insulin on Carbohydrate Dissimilation

Perhaps it would be timely, at this point in our discussion, to present evidence which seems to indicate that insulin is concerned with carbohydrate oxidation. Wick et al (191) injected C^{14}-labelled glucose into eviscerated rabbits, and a constant blood glucose level was maintained. Expired CO_2 and urinary CO_2 were determined at various time intervals. At the end of the experiments, the animals were killed, their tissues frozen, and various fractions analyzed for C^{14}-radioactivity. The authors observed that when insulin was administered, four times as much expired CO_2 was derived from the labelled glucose, as compared with the non-insulinized group. This action of insulin was found not to be immediate, in that a time lag for the appearance of C^{14} in the expired CO_2 was observed. During this time lag, glucose disappears from the blood and is either oxidized or disposed of as water-soluble compounds, glycogen, protein, or fat. The authors conclude from this that insulin increases the complete oxidation of glucose by the extrahepatic tissues.

Stetten et al (172) employed intact, anesthetized rats in which constant levels of specific activity of circulating and urinary glucose were achieved by means of a continuous intravenous injection of C^{14}-

glucose. The rats were either alloxan-diabetic, phlorhizinized or normal. When the authors determined the specific activity of the respiratory CO_2 from these animals, they observed a decreased total oxidation of glucose in the alloxan-diabetic and phlorhizinized rats.

Feller et al (44) studied the rate of oxidation of C^{14}-glucose in normal and alloxan-diabetic rats. These workers found that the rate of glucose oxidation to CO_2 was slightly reduced in the alloxan-diabetic rat, although they do not consider this as the major metabolic defect of the alloxan-diabetic rat.

Feller et al (45) studied the rate of oxidation of C^{14}-glucose in the depancreatized dog. They found a marked reduction in the rate of oxidation of glucose to CO_2 in dogs, in which diabetes was induced by total pancreatectomy.

I. Main Problems Emerging From A Review of the Literature

It can be seen from a review of the literature that insulin affects the metabolism of carbohydrates, proteins and fats, as well as mineral metabolism. The possible mechanism of insulin action still remains a highly controversial problem. Many questions have been raised which have not yet been definitively resolved by the various contending theories for the mechanism of insulin action. For example:

(a) does insulin act primarily at a single locus or at several loci?

(b) is the marked derangement of metabolism, as seen in diabetes mellitus, to be considered as the resultant of a key block at a single locus or of many blocks at several loci?

(c) of the many metabolic reactions shown to be influenced by insulin, which are primarily influenced and which are secondarily influenced by

insulin?

The major contending theories of insulin action have been discussed. In brief, they may be formulated as follows:

(a) insulin may influence the transfer of glucose and certain other sugars into the cells of certain tissues, such as muscle; (b) insulin influences glucose metabolism by controlling the rate of formation of hexose-6-phosphate through the action of gluco-hexokinase; (c) insulin influences oxidative reactions involving the Krebs Cycle and the formation or regeneration of adenosinetriphosphate and other high-energy containing organic compounds.

It is apparent from this review of the literature that much more definitive evidence is necessary before it becomes possible to reconcile the various theories elaborated to explain the mechanism of insulin action.

CHAPTER II

METHODS FOR PROPOSED STUDY

Methods

Numerous experiments were undertaken originally with non-radioactive galactose. This reducing sugar was estimated by means of the Somogyi (151) modification of the Nelson (123) method. The uptake of non-radioactive galactose by isolated rat diaphragms was determined after incubating the tissue in Krebs Ringer phosphate (KRP) medium, pH 7.4, containing 100 mg. % galactose. Following incubation, the tissue was blotted, weighed, and homogenized with alumina in a known volume of water. The proteins were then precipitated with barium hydroxide and zinc sulfate (152) or with copper sulphate and sodium tungstate (84). The clear filtrate was then analyzed by the Nelson method (123) as modified by Somogyi (151). It was observed that the determination of muscle galactose by this method was not completely satisfactory as a routine procedure. It was felt that interfering reducing substances in the muscle tissue, capable of reacting with the Somogyi and Nelson reagents, were not uniformly or completely removed during the precipitation. This resulted in higher or non-reproducible values for the galactose taken up by the muscle tissue during incubation. Accordingly a more accurate method was sought and achieved by using C^{14}-labelled galactose in place of non-radioactive galactose.

It is possible to use isotopes as tracers in biological investigations because all the isotopes of a particular chemical element have identical chemical properties. This statement applies both to the naturally occurring stable isotopes and to the radioactive isotopes produced by nuclear reactions. This is due to the fact that the chemical properties of an element are dependent on the number and configuration of the planetary electrons, which are identical for all of the isotopes of a particular element.

The number of planetary electrons of an element is equal to the number of protons in the nucleus, which is taken as the atomic number of the element. The planetary electrons are negatively charged and the protons of the nucleus are positively charged. The nucleus also contains neutrons which have no electrical charge but have mass. Elements possessing the same number of protons, and hence the same number of planetary electrons, but different numbers of neutrons are isotopes. Therefore, isotopes of a particular element all have the same electrical charge but differ in mass. The isotopes of an element may be stable or radioactive and may be naturally occurring or produced by man by means of nuclear reactions. The latter has resulted in the production of many readily-available isotopes, which have been used in all fields of physical and biological research with tremendous success. For example, the two stable carbon isotopes are ${}_6C^{12}$ and ${}_6C^{13}$, and the radioactive isotope is ${}_6C^{14}$. This indicates that all three carbon isotopes have an atomic number of 6, which represents the number of protons in the nucleus or the number of planetary electrons. The three isotopes, however, vary in the number of neutrons in the nuclei; namely, 6, 7, and 8. Hence, they vary in mass. Isotopes may be identified by means of a mass spectrometer. If a radioisotope is used, it is easier to detect its radioactivity. Therefore, an isotope can act as a "label" which allows the fate of the element, or of a compound containing this element, to be traced through a series of physical and chemical changes. It is the mass or the radioactivity of an isotope which is really the label or tag. Therefore, in biological work, one refers to the labelled element as a tracer element.

It has been shown that when isotopes are used, differences in reaction rate may appear under nonequilibrium conditions. This is due to differences in isotopic mass. However, this difference in reaction rate is ordinarily too small to be significant in a tracer experiment using C^{14} (8).

When radioisotopes began to be used as tracers in biological work, it was necessary to ascertain whether the effects of radiation on tissues might produce alterations in cell physiology. This had to be ascertained before the results of tracer experiments with isotopes could be considered as valid. Experience soon showed that the amounts of isotopes used in most tracer studies were so small that radiation effects on biological systems could be discounted. For example, the effect of radioactivity in the medium on the permeability of the cell membrane of Nitella was studied. No deleterious effects could be observed at concentrations below one millicurie per liter, which is many times the amount necessary for most tracer studies (121).

The studies which were undertaken at the Worcester Foundation were performed using galactose labelled with a radioisotope of carbon, namely, C^{14}. The C^{14} isotope is long-lived, possessing a half-life of 5360 years. The half-life is the time required for half the nuclei present to undergo disintegration. It expels only soft beta particles of low penetrating power, no gamma rays being given off (56). Since only beta rays are expelled by radioisotope C^{14} and since only small amounts of C^{14}-labelled galactose were used in these experiments, it was assumed that the radiation effects in the incubating rat diaphragm muscle could be considered negligible. This assumption was further strengthened by the fact

that the uptake of galactose by isolated diaphragms was essentially the same whether non-radioactive galactose or non-radioactive galactose plus a tracer amount of C^{14}-labelled galactose were present in the medium.

Since the C^{14}-isotope expels only soft beta particles, which can penetrate only a few millimeters of tissue, careful handling and disposal of the C^{14}-labelled galactose is sufficient to safeguard the laboratory personnel.

Gemmill et al (52, 54, 53) demonstrated that glucose utilization and glycogen formation by the isolated rat diaphragm could be increased by the addition of insulin to the incubation medium. Thus, the action of insulin was demonstrated under in vitro conditions. The procedure used by Gemmill et al was as follows: the diaphragms were isolated, blotted lightly to remove adhering blood, and placed in a physiological salt solution. Aliquots of the medium and portions of the diaphragm were analyzed, at the beginning and the end of the period of incubation, for glucose, lactic acid, glycogen, etc.

The procedure employed in these studies is based, in principle, on that formulated by Gemmill et al. White rats obtained from the Charles River Breeding Laboratories, are fasted for 24 hours and killed by decapitation. Male rats, weighing between 100 and 200 grams, were used for these experiments. Diaphragms removed from rats of this size range are thin enough to allow for maximum diffusion when properly shaken. Following decapitation, as much blood as possible is drained from the carcass of the animal and the diaphragm removed immediately. The isolated diaphragms are placed on several thicknesses of filter paper moistened with the incubation medium. The central tendon is removed and the diaphragm is

trimmed free of adhering fat, connective tissue, and blood vessels. The diaphragm muscle is then sectioned into the right and left hemidiaphragms, blotted very lightly to remove adhering blood and placed in Warburg flasks containing 1.8 ml. of Krebs Ringer phosphate as the incubation medium.

The incubation medium is prepared fresh for each experiment, and the individual solutions are stored in the cold room. The component solutions used to make up the Krebs Ringer phosphate incubation medium are (179):

(1) 0.9 % NaCl (0.154 Molar)

(2) 1.15% KCl (0.154 Molar)

(3) 1.22% $CaCl_2$ (0.11 Molar)

(4) 2.11% KH_2PO_4 (0.154 Molar)

(5) 3.82% $MgSO_4 \cdot 7H_2O$ (0.154 Molar)

(6) 0.1 Molar phosphate buffer, pH 7.4 (17.8 grams of $Na_2HPO_4 \cdot 2H_2O$ plus 20 ml. I Normal HCL, dilute to one liter)

It will be noted that all of the solutions are isotonic with rat serum. To prepare the Krebs Ringer phosphate medium, the following amounts of the above are mixed (179):

100 parts of solution (1)

4 parts of solution (2)

3 parts of solution (3)

1 part of solution (4)

1 part of solution (5)

12 parts of solution (6)

The side arm of each Warburg flask contains a tracer quantity of C^{14}-labelled galactose plus non-radioactive carrier galactose in 0.2 ml. Krebs Ringer phosphate medium. One hemidiaphragm of each pair obtained from a single animal serves as the control tissue and the other hemidiaphragm as the experimental tissue. For example, one hemidiaphragm is placed in a vessel containing only Krebs Ringer phosphate in its main compartment, and the other hemidiaphragm is placed in a vessel containing Krebs Ringer phosphate plus zinc insulin or some other substance whose action is to be tested. In this manner, each animal serves as its own control and individual variations in metabolism are avoided. The concentration of insulin in the incubation mixture, when present, is 0.27 Units per ml. of incubation medium.

After all of the substances are placed in the Warburg flasks and their side arms, one hemidiaphragm is placed in the main compartment of each Warburg flask. The flask is attached to a manometer and placed in a constant temperature bath. In most of the experiments, the temperature was kept constant at $38^{\circ}C$. The whole assembly is then shaken to insure adequate diffusion of oxygen and other substances into the tissues, to allow the tissue to be surrounded by the medium at all times, and to prevent the tissue from becoming lodged in the flask. Adequate shaking is of prime importance when the O_2 consumption of the incubating tissue is being determined. Such studies will be reported in a later section.

When incubation of the diaphragm under aerobic conditions was desired, oxygen was flushed through the whole assembly and allowed to dissolve in the incubation medium. A length of rubber tubing leading from a cylinder containing 100% oxygen is connected to the open end of the

manometer, containing a 3-way stop-cock. The vent of the flask side-arm stopper is opened to permit communication with the exterior. The oxygen is then allowed to bubble through water contained in a glass jacket, which is immersed in the water bath. The oxygen is then allowed to flush through the flask, and the amount of oxygen absorbed by the medium depends upon the temperature and pressure. At the end of 5 minutes of gassing with oxygen, the rubber tubing is disconnected and the Warburg assembly made air-tight by means of the 3-way stop-cock of the manometer and the side-arm stopper of the flask. When a Krebs Ringer medium with a phosphate buffer is employed, 100% oxygen must be used. If a bicarbonate buffer had been used, then 95% O_2 - 5% CO_2 would have been necessary (179). Other gases may be flushed through the system, using the same apparatus. For example, in the experiments studying the effects of anaerobiosis on insulin action, nitrogen was flushed through the assembly.

After oxygenation, the 0.2 ml. Krebs Ringer phosphate containing a tracer quantity of C^{14}-labelled galactose plus non-radioactive carrier galactose is tipped into the main compartment, which contains 1.8 ml. Krebs Ringer phosphate medium with or without insulin or other substances, to form 2.0 ml. of the incubation medium containing 100 mg. % galactose. Incubation, with shaking, is carried out for a definite period of time at a constant temperature.

Following incubation, the tissue is removed, blotted between filter papers, and weighed on a torsion balance (Roller-Smith, number 298161). The tissue is then transferred to a tube containing a known amount of water. The tube is placed in a boiling water bath for 10 minutes. After cooling, the aqueous extract of the diaphragm muscle is filtered through

washed glass wool. A known volume of the filtrate is then planchetted to infinite thickness on stainless steel planchets and the radioactivity determined by means of a flow-gas Geiger-Muller counter, obtained from Nuclear Instrument and Chemical Corp., Chicago, Ill. The flow-gas counter is attached to a scaler which records the number of particles which produce ionization of the Q-gas, which is continuously being flushed through the ionization chamber of the flow-gas counter. The scaler was obtained from the Atomic Instrument Co., Cambridge, Mass. and is model 1030A. The Q-gas was obtained from Nuclear Instrument and Chemical Corp., Chicago, Ill. and has the following composition: helium, 99%, isobutane, 1%. The same volume of muscle filtrate is planchetted in all of the experiments, and is always planchetted to infinite thickness. In this way, the self-absorption factor is constant throughout the experiments and can therefore be neglected.

Aliquots of standard aqueous solutions containing C^{14}-labelled galactose were planchetted in the same manner. They were used as standards for calculating the amount of C^{14}-labelled galactose taken up by the isolated rat diaphragm during the period of incubation. Results are expressed as mg. of C^{14}-labelled galactose per gram of wet weight of diaphragm for the period of incubation.

The aqueous extract of diaphragm muscle planchetted contains salts extracted from the muscle during the 10 minutes of boiling. The salts could absorb the radioactive emissions and thus produce an error in the determinations of the amount of C^{14}-galactose. Experiments were performed to check this possibility. Known volumes of KRP or isotonic KCl, equivalent to the volumes contained in definite wet weights of muscle tissue,

were added to an aqueous solution containing a known amount of C^{14}-galactose. It was found that both salt solutions (either KRP or isotonic KCl) "quenched" the radioactivity of the standard C^{14}-galactose, and the decrease was found to be proportional to the amount of salt added. The "quenching" effect of salts was studied as follows: volumes of KRP or isotonic KCl (equivalent to 50, 75, 100, and 150 mg. wet weight of diaphragm muscle), corresponding to the range of wet weight of tissue used in our experiments, were added to a standard amount of C^{14}-galactose. The results obtained are shown in Table 3. Therefore, in all of the radioactive experiments a correction factor was applied to each reading, which was determined by the weight of the tissue incubated in C^{14}-galactose and subsequently extracted, planchetted and counted.

Table 3

Effect of Salt Contained in Water-Extract of Muscle on C^{14}-Galactose (4 Experiments).

Salt Content Equivalent to Wet Wt. of Tissue.	% Depression of Counts
50 mg.	10%
75 mg.	16%
100 mg.	18%
150 mg.	22%

As stated above in the description of the methods employed, a water extract of the diaphragm is made. It was assumed that during these 10

minutes of boiling, the C^{14}-galactose in the muscle is completely extracted by the water. To test this, the following experiments were carried out. Isolated rat diaphragms were incubated in KRP containing C^{14}-galactose (100 mg. %) for 60" at 38°C. Following incubation the tissues were blotted, weighed and boiled for 10 minutes in 1.0 ml. of water. Then, 0.05 ml. of the water extract was removed and planchetted. The remaining water extract plus the boiled tissue was then homogenized with alumina, centrifuged and 0.05 ml. of the clear supernatant planchetted. It will be seen from Table 4 that no further radioactivity is obtained by homogenization of the boiled tissue, thus proving the validity of our assumption.

Table 4

Muscle Aqueous Extract vs. Homogenate

I Reading of 0.05 ml. H_2O Extract (Duplicate Planchets) I & II	II Reading of 0.05 ml. H_2O Extract Homogenized Tissue (Duplicate Planchets)	III Corrected Reading for II. i.e., II+10% (since 0.1 ml. was removed for planchet I & II)
Expt. #I 383 counts/min	365	365 + 37 = 402
Expt. #II 169	152	152 + 15 = 167
Expt. #III 189	161	161 + 16 = 177

Since the results of the experiments are expressed as mg. of C^{14}-galactose taken up / gm. wet wt. of diaphragm / length of incubation, it was necessary to ascertain whether the method of determining wet wts. was accurate. The wet wts. of 86 hemidiaphragms were determined in the manner described above. The hemidiaphragms were than placed in an oven

at 100°C. and allowed to dry to constant weight. The dry wts. ranged from 19-28% of the wet wts. and averaged 24%. Although the range was from 19-28%, the great majority grouped about the average value of 24%. Therefore, the method for determining wet wts. was considered valid and reproducible.

Experiments were then performed which indicated that no demonstrable alteration of galactose occurs in the test system employed in the experiments to be reported. This was accomplished by incubating rat diaphragm in KRP plus C^{14}-galactose for 30 minutes, and by identifying the radioactive material extracted from the muscle as a single substance. This was shown to be galactose by means of paper chromatography. The chromatographic procedure of McFarren et al (113) was employed. This method consists of separating reducing sugars in an ethyl-acetate, pyridine, water system containing silver nitrate, air drying, exposing the chromatograms to NH_3 vapors, and developing the sugar spots by heating in an oven. The spot is developed as silver is deposited, as a result of the action of the reducing sugars on the ammoniacal silver nitrate. The solvent system was prepared by placing 2.5 parts of ethyl acetate, 1.0 part of pyridine, and 3.5 parts of distilled water in a separatory funnel and shaken. The mixture was allowed to separate, the water-rich layer was thrown away, and part of the solvent-rich layer was placed in the bottom of a cylindrical glass chromatography chamber. The chamber was lined with Whatman #1 filter paper. This allowed for saturation of the atmosphere of the chamber with the solvent system placed in the bottom. The remainder of the solvent-rich layer was made 0.15 N with $AgNO_3$ and poured into a solvent trough, held from the bottom of the chamber by a metal stand. The chamber is then covered with a square of plate glass ground on one side, and the union made air-tight by means of a paste.

The aqueous extract of muscle incubated with C^{14}-galactose and standard solutions of glucose and galactose were applied to Whatman #1 filter paper strips. The chromatograms were then placed in the chamber and allowed to run from 16 to 20 hours. During this time, the solvent front had progressed beyond the end of the paper and a good separation of the sugars was later demonstrated. Upon removal from the solvent chamber, the chromatograms were air-dried for 1 hour and then placed in an NH_3 chamber for 1 hour. The NH_3 chamber was prepared by pouring NH_3 into a lined chamber, and allowing the atmosphere to become saturated with NH_3 by means of an air tight ground plate glass cover. After 1 hour, the chromatograms were removed from the NH_3 chamber and placed in an oven at a temperature of $80^{o}C.$ for 20 minutes, during which time the sugar spots developed. This method was found to separate galactose from glucose; the sensitivity is about 2 gamma.

Examination of the radioactivity present in the chromatograms of the aqueous extract of muscle incubated in KRP with C^{14}-galactose revealed that only a single zone of radioactivity was present, which corresponded exactly to the spot for galactose. Hence, all of the galactose which entered the muscle tissue during the 30 minutes of incubation remained unchanged. The results also indicated that the radioactivity which left the medium could be quantitatively recovered from the tissue as galactose.

It has already been noted that muscle tissue does not contain any galactokinase (154). This means that the galactose taken up by the isolated rat diaphragm muscle cannot be transformed into glucose, and hence cannot enter into the well-known scheme of intermediary carbohydrate metabolism. What then is the metabolic fate of galactose after it is

transferred into the muscle? Wick and Drury (192) injected radioactive galactose into eviscerated-nephrectomized rabbits and measured both the blood galactose levels and the radioactive respiratory CO_2. From the specific activity of the respiratory CO_2, it was ascertained that galactose oxidation was very slight as compared to that of glucose under the same experimental conditions. How galactose is oxidized to CO_2 by tissues other than liver, kidney, or intestines which possess a galactokinase (178, 17, 18) is as yet unknown.

For all practical purposes, then, the assumption is made that the galactose taken up from the incubation medium by the isolated rat diaphragm, during an incubation period of 30 minutes or 60 minutes, remains in the muscle tissue unchanged and uncomplicated by its metabolic fate. Therefore, this procedure may be used as a test system for studying sugar transfer systems, as well as the effects of various conditions and substances on such systems. It is for this reason that the galactose uptake by isolated rat diaphragm *in vitro* has been chosen as the test system for this investigation.

In investigations on metabolism, such as were performed in this laboratory, all variables must be controlled as much as possible. For instance, rats of the same strain and weight range were used throughout the experiments. It is also known that the nutritional history of the animal at the time of removal of the tissues could have an appreciable effect on the subsequent metabolic studies of such isolated tissues. As a result, the rats were fed a standard prepared diet *ad libitum* and were fasted 24 hours before an experiment.

The insulin used in the majority of these experiments was crystalline

zinc insulin (27 units/mg.) obtained from Eli Lilly and Co. The amount of zinc insulin added was 0.27 units per ml. of incubation medium or a total of 0.54 units per Warburg flask. Several experiments were also performed using hyperglycemic-factor-free insulin, which was kindly furnished by Eli Lilly and Co. When hyperglycemic-free insulin was added to the incubation medium, the effects were identical with that resulting from the presence of zinc insulin in the medium.

Before embarking on a study of the in vitro uptake of galactose by the isolated rat diaphragm, it was necessary to ascertain whether an insulin effect could be demonstrated on glucose utilization by the isolated rat diaphragm, under essentially similar in vitro conditions, with the insulin preparations at hand. It can be seen from Table 5 that the presence of insulin in the incubation medium markedly increased the glucose utilization by isolated rat diaphragm muscle under in vitro conditions. The utilization of glucose represents the uptake of glucose from the medium plus the channeling of such glucose into the various pathways of glucose intermediary metabolism. Since glucose is constantly being metabolized by the tissue, the uptake of glucose from the medium is quite high, when compared with the uptake of a non-utilizable sugar, such as galactose. This will become apparent when galactose uptakes will be reported. It was thus concluded that the preparations of insulin at hand and the in vitro test system could now be used for studies on galactose uptake.

The d-galactose-1-C^{14} was obtained from H. S. Isbell, National Bureau of Standards, and has a specific activity of 2.94 uc./mg. (according to label). A Curie equals the number of particles emitted/sec./gm. pure

Table 5

Effect of Insulin on Utilization of Glucose by Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubated 60" in KRP plus Glucose (200 mg.%) at 38°C. with O_2 as the Gas Phase, pH 7.4.

Rat #	Glucose uptake, mg. per gm. wet wt. tissue per hour		
	In KRP-Glucose	In KRP-Glucose + Insulin	% Insulin Effect
1	2.21	2.50	13
2	2.19	3.65	67
3	3.90	5.07	30
4	2.43	3.06	26
5	2.30	3.68	60
6	2.50	4.10	64
	Avg. 2.59 mg./gm.	3.68 mg./gm.	42%

The uptake of glucose was determined by analyzing an aliquot of the medium obtained before and after incubation, by means of the Somogyi-modification of the Nelson method. The uptake in mg. per gram diaphragm was calculated from the disappearance of glucose from the medium.

S.E.M. = $\pm$ 0.21; p = $< .01$

radium element, which was found to be 3.7×10^{10} particles/sec. or 2.22×10^{12} cts./min. Therefore, 1 uc., which equals 1×10^{-6} Curies, emits 2.2×10^{6} cts./min. Since 1 mg. of d-galactose-1-C^{14} is equal to 2.94 uc., then it emits approximately 6.5×10^{6} cts./min. This C^{14}-labelled galactose was then diluted 1 to 40 with non-radioactive galactose. For example, 25 mg. of d-galactose-1-C^{14} were added to 975 mg. of non-radioactive galactose to make 1000 mg. This was mixed thoroughly and dissolved in 100 ml. of KRP. This stock solution, therefore, has a specific activity of approximately 1.63×10^{6} cts./min./ml. This stock solution was then dispensed in small pyrex test tubes, frozen and stored in the deep freeze until ready for use. For each experiment, 0.2 ml. of this stock solution was placed in the side-arm of each Warburg flask and, at an appropriate time, added to the 1.8 ml. of KRP in the main compartment of the flask. This results in 2.0 ml. of KRP containing 100 mg. % of carrier galactose plus d-galactose-1-C^{14} (40 to 1). The specific activity of the incubation medium is, then, 1.63×10^{5} cts./min./ml.

A standard solution is made up for each set of determinations of a particular experiment and is used as a standard of reference for the calculations of the galactose uptakes by the incubating tissues. 0.2 ml. of the stock solution is added to 19.8 ml. of distilled water to form 20.0 ml. of standard solution. Such a standard solution has a theoretical specific activity of 1.63×10^{4} cts./min./ml. A known volume (0.05 ml.) is then planchetted to infinite thickness on stainless steel planchets and the radioactivity determined by means of a flow-gas Geiger-Muller counter. The theoretical count for such planchets should be approximately 815 cts./min. In practice, the standard planchets show a count ranging

from 900-975 cts./min. All counts are corrected for background.

The following is a sample calculation taken from one of the experiments performed in this study. Duplicate planchets of the standard solution averaged, for this experiment, 930 cts./min. As mentioned above, the standard solution has the following composition: 0.2 ml. KRP containing 2 mg. of carrier galactose plus C^{14}-galactose + 19.8 ml. H_2O to form 20.0 ml. standard solution. This standard solution now contains 100 ug. of the galactose/ml. When 0.05 ml. of this solution was planchetted, a count of 930 cts./min. was obtained. This count multiplied by 20 gives the cts. per min./ml. of standard solution. Since 1 ml. of standard solution contains 100 gamma of galactose, then this count multiplied by 10 gives the cts. per min./mg. of galactose contained in the standard solution. E.g., 930 x 20 = 18,600 cts. per min./1 ml. standard solution/ 100 ug. galactose. This is equivalent to 186,000 cts. per min./mg. of galactose. An alternative method would be as follows: 20.0 ml. standard solution contain 2.0 mg. galactose. 0.05 ml., or 1/400 the part of the standard solution, gives a count of 930 cts./min. Therefore, 930 x 400 is equal to 372,000 cts. per minute/2 mg. of galactose. This then is equivalent to 186,000 cts. per minute/1 mg. galactose.

After incubation in KRP plus C^{14}-galactose for 30" at 38°C., HDI was removed, blotted, weighed and extracted with 1.0 ml. of boiling water. This aqueous extract was filtered through washed glass wool and 0.05 ml. aliquots planchetted to infinite thickness. The average count obtained was 300 cts./min. This tissue weighed 68 mg., and therefore a correction factor of 12% was applied to the counts/min. to correct for the absorption of radioactive emissions by the salts contained in 68 mg. of muscle tissue.

The corrected counts thus averaged 336 cts./min. This multiplied by 20 gives the counts per min./ml. aqueous extract of 68 mg. of diaphragm muscle. Thus, 336 x 20 is equal to 6720 cts./min. It is then desired to convert this figure into cts. per min./gm. of diaphragm muscle, since in the literature the uptakes of sugars by isolated tissues are expressed per gm. wet wt. of diaphragm muscle. Thus, the specific activity now reads 98, 824 cts. per min. if 1 gram of muscle tissue were contained in the aqueous extract. Since it has been established by means of the standard that 1 mg. of the galactose used for this experiment has a specific activity of 186,000 cts./min., then 98, 824 cts./min. divided by 186,000 cts./min. gives the mg. galactose contained in an extract of 1 gm. of diaphragm muscle. This, then, equals 0.53 mg. Therefore, one can say that, under the in vitro conditions employed, the galactose uptake by isolated rat diaphragm muscle was found to be 0.53 mg./gm. wet wt.

CHAPTER III

STUDIES

A. The Demonstration of an Action of Insulin on the Uptake of Galactose by Isolated Rat Diaphragm Muscle In Vitro

It was stated in Chapter II that, for all practical purposes, the assumption is made that the galactose taken up from the incubation medium by the isolated rat diaphragm, during a period of 30 or 60 minutes remains in the muscle tissue unchanged and uncomplicated by its metabolic fate. Evidence from this laboratory and from recent literature was cited to support the validity of such an assumption. Therefore, the uptake of galactose by isolated rat diaphragm muscle incubated for a period of 30 minutes (occasionally 60 minutes) in KRP plus C^{14}-galactose was chosen as the test system for studying sugar transfer under in vitro conditions.

Table 6 shows the effect of insulin on galactose uptake by isolated diaphragm muscle obtained from normal, fasted rats. The diaphragm muscle was incubated for 30 minutes in KRP plus C^{14}-galactose (100 mg. %) at 38°C., with O_2 as the gas phase. This procedure has been described in detail in Chapter II, which is devoted to methods employed in this investigation. Again, it must be emphasized that each isolated diaphragm is sectioned into two hemidiaphragms. One hemidiaphragm acts as the control tissue, the other as the experimental tissue. As a result, each animal serves as its own control and individual variations in metabolism are avoided.

Because the state of metabolism of an individual animal at a particular moment is dependent upon a host of factors such as age, sex, weight, activity, nutritional history, glandular balances and counterbalances, etc., it is a definite advantage to be able to consider each animal as an experimental unit complete unto itself. Therefore, in all of the tables

Table 6

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 38°C. O_2 - Gas Phase, pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose	In KRP-Galactose + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.54	0.65	20
2	0.51	0.73	43
3	0.60	0.71	18
4	0.62	0.82	32
5	0.58	0.78	34
6	0.64	0.88	38
7	0.58	0.68	17
8	0.56	0.71	27
9	0.59	0.63	7
10	0.56	0.65	16
11	0.61	0.64	5
12	0.57	0.71	25
13	0.52	0.68	31
14	0.49	0.67	37
15	0.68	0.68	0
	Avg. 0.58 mg./gm.	0.71 mg./gm.	23%

S.E.M. = $\pm$ 0.02; p = $< .001$.

concerning experimental work carried out in this laboratory, the values for the control tissue are presented first, followed by the values for the experimental tissue and the percentage effect between the two values. An attempt was made to keep the individual variations at a minimum. Male, white rats of a single strain, fed a standard prepared laboratory diet and fasted 24 hours were used in all of the experiments, unless otherwise indicated. The animals were killed by decapitation, since anesthetics may produce an effect on the metabolic state of the organism.

It can be seen from Table 6 that, under the in vitro conditions employed, insulin increases the uptake of C^{14}-galactose by the isolated rat diaphragm muscle. In 15 animals, the uptake of galactose in the absence of added insulin averaged 0.58 mg./gm., with a range of from 0.49 to 0.68 mg./gm. When insulin was added to the incubation medium, the uptake of galactose averaged 0.71 mg./gm., with a range of from 0.63 to 0.88 mg./gm. The percent insulin effect, that is, the increase in galactose uptake under the influence of insulin averaged 23%, with a range of from 0 to 43%. One may conclude that the effect of insulin on galactose uptake by the isolated rat diaphragm in vitro may be considered as quite consistent and reproducible. Such a conclusion may be arrived at whether one considers each animal separately as an experimental unit or whether the average of a series of animals incubated under the same conditions is considered.

B. Time-Course of Galactose Uptake, with and without Added Insulin, by the Isolated Rat Diaphragm

Since galactose is not appreciably metabolized by muscle, under these conditions, the total tissue volume available for galactose represents the difference between the wet and dry weight of the diaphragm preparation.

Since the average dry weight of the rat diaphragm was found to be 24 percent of the wet weight (86 determinations, range 19-28 percent), 76 percent of the wet weight, or 0.76 ml. per gm. diaphragm, is available for equilibration with the galactose in the medium. Since the medium contains 1.0 mg. galactose per ml., it is apparent that maximal distribution of galactose in diaphragm would be equivalent to a galactose content of 0.76 mg. per gm. tissue (range .72-.81 mg./gm.). Fig. 2 shows the galactose uptake, at time intervals ranging from 15 to 60 minutes, both in the absence and presence of added insulin. Galactose uptake at zero time is assumed to be 0.2 mg./gm. on the basis that this is the volume of the extracellular phase of diaphragm muscle as determined with inulin. This will be discussed in a later section. It will be seen that, in the absence of insulin, maximal galactose uptake has not yet occurred at 60 minutes. In the presence of insulin, galactose penetration has leveled off at 30 minutes of incubation and is close to the theoretical maximum. Since galactose penetration proceeds in the absence of added insulin, it is clear that the action of insulin is best evidenced early in the incubation period (i.e., at 15 or 30 minutes) rather than at 1 hour. Thus the insulin effect at 15 and 30 minutes is 26 and 23 percent, respectively, while at 60 minutes the insulin effect is only 6 percent. The data for Fig. 2 were obtained from Tables 7 and 7A.

C. Effect of Temperature Upon Galactose Entry into the Diaphragm Muscle Cells, with and without Added Insulin

Having demonstrated that insulin action upon galactose transfer can be evidenced at 30 minutes of incubation, this period of incubation was chosen to test a variety of factors. Accordingly, to study the influence

Fig. 2. Galactose Uptakes by Diaphragm at Varying Time Intervals of Incubation at 38°C.

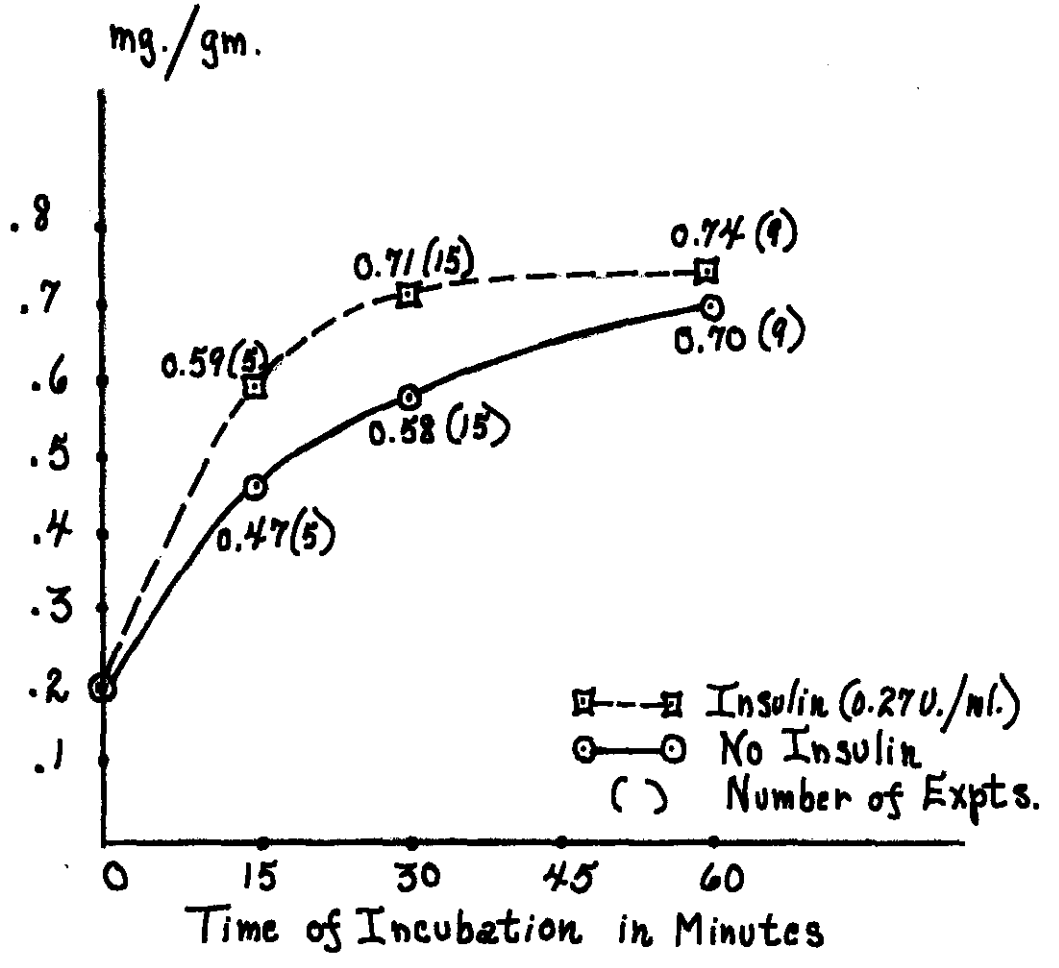


Table 7

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 60" in KRP plus C^{14}-Galactose (100 mg. %) at 38°C. O_2 - Gas Phase. pH 7.4.

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 60" of incubation		
	In KRP-Galactose	In KRP-Galactose + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.71	0.75	6
2	0.76	0.82	8
3	0.70	0.79	13
4	0.75	0.75	0
5	0.66	0.70	6
6	0.71	0.77	8
7	0.65	0.75	15
8	0.68	0.68	0
9	0.69	0.69	0
	Avg. 0.70 mg./gm.	0.74 mg./gm.	6%

S. E. M. = $\pm$ 0.013; p = .01

Table 7A

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 15" in KRP plus C^{14}-Galactose (100 mg. %) at 38°C. O_2 - Gas Phase. pH 7.4.

Rat #	Galactose uptake, mg./gm. wet wt. tissue per 15" of incubation		
	In KRP-Galactose	In KRP-Galactose + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.43	0.49	14
2	0.44	0.64	45
3	0.51	0.59	16
4	0.49	0.51	4
5	0.46	0.74	61
Avg.	0.47 mg./gm.	0.59 mg./gm.	26%

S. E. M. = $\pm$ 0.048; p = .05

of temperature, isolated hemidiaphragms were incubated in KRP for 30 minutes, with and without added insulin, at temperatures ranging from 0 to 38°C.

It can be seen from Tables 6, 8 and 9 that an action of insulin on galactose uptake by isolated rat diaphragm muscle can be demonstrated at incubation temperatures ranging from 20 to 38°C. The absolute uptake of galactose, in the absence and presence of insulin, is proportional to the incubation temperature. Nevertheless, the presence of insulin results in an increased uptake of galactose at this temperature range. The percent insulin effect at 38°C. was 23%, with an increment of 0.13 mg./gm.; at 27°C., 27%, 0.13 mg./gm.; at 20°C., 23%, 0.10 mg./gm.

When incubation is carried out at 0 or 10°C., no apparent effect of insulin on the uptake of galactose can be observed. This is shown in Tables 10 and 11. If one examines Table 10 carefully, it can be seen that in 2 out of the 8 animals a small but definite insulin effect occurred. Therefore, it is possible to consider 10°C. as approaching a critical temperature, where an action of insulin can no longer be observed on the galactose uptake. These findings thus demonstrate that the insulin effect on galactose uptake by isolated diaphragm muscle is remarkably temperature sensitive in that no effect is obtainable between 0 and 10°C., and that at 20°C. the effect already appears to be maximal.

In reviewing this data on the effect of temperature on insulin action in the test system employed, a possible explanation was considered. Stadie and his coworkers (167, 165, 166, 65, 69, 162, 66, 67, 158, 160, 163) demonstrated that insulin is bound by cells of various tissues. They dipped isolated rat diaphragm muscle into a solution containing a variety

Table 8

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 27°C. O_2 - Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose	In KRP-Galactose + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.46	0.65	41
2	0.46	0.70	52
3	0.52	0.56	8
4	0.44	0.58	32
5	0.49	0.62	27
6	0.50	0.64	28
7	0.50	0.56	12
8	0.49	0.60	23
9	0.49	0.58	18
10	0.53	0.63	19
11	0.48	0.65	35
	Avg. 0.49 mg./gm.	0.62 mg./gm.	27%

S.E.M. = $\pm$ 0.017; p = $<$ .001.

Table 9

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg.%) at 20°C. O_2 - Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wt. tissue per 30" of incubation		
	In KRP-Galactose	In KRP-Galactose + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.43	0.57	33
2	0.47	0.56	19
3	0.47	0.56	19
4	0.38	0.50	32
5	0.47	0.61	30
6	0.42	0.52	24
7	0.48	0.50	4
8	0.41	0.49	20
	Avg. 0.44 mg./gm.	0.54 mg./gm.	23%

S.E.M. = $\pm$ 0.014; p = $< .001$

Table 10

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 10°C. O_2 - Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose	In KRP-Galactose + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.42	0.42	0
2	0.38	0.42	11
3	0.44	0.40	-10
4	0.42	0.40	- 5
5	0.36	0.35	- 3
6	0.36	0.31	-16
7	0.43	0.50	16
8	0.42	0.36	-17
	Avg. 0.40 mg./gm.	0.40 mg./gm.	0%

Table 11

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 0°C. O_2-Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose	In KRP-Galactose + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.27	0.25	-8
2	0.25	0.26	+4
3	0.24	0.24	0
	Avg. 0.25 mg./gm.	0.25 mg./gm.	0%

of insulin preparations, such as, amorphous insulin, zinc-free insulin, crystallized insulin, and protamin zinc insulin. The diaphragm muscle was then thoroughly washed and incubated in a physiological salt solution containing glucose for one hour. They observed that the hemidiaphragm which had been dipped into the insulin solution synthesized more glycogen than the control hemidiaphragm, which had not been exposed to insulin. The authors interpreted this to mean that a chemical combination occurred between insulin and receptor groups of the diaphragm muscle, which withstood prolonged saline washes (even for as long as one hour). This insulin binding was demonstrated when the diaphragm was dipped into a solution containing 0.1 unit insulin per ml. for as short a period as 10 seconds. It was also shown that the binding occurs over a pH range of 6-8, and that the binding apparently increases with increasing temperature.

These authors also found that muscle, mammary and adipose tissues can also combine with insulin. More recently, these authors (67) injected radioactive insulin into rats and found that the liver and kidney appear to concentrate the injected insulin, whereas little or no insulin enters the brain. This is of interest since, as was mentioned previously, the presence of insulin seems to have no influence on the carbohydrate metabolism of brain tissue. Again, it was shown (66) that rabbit leucocytes combined with large amounts of insulin, whereas the red blood cells combined with about 1/1000th of the amount combined with the leucocytes. It has also been shown (154) that insulin seems to have no influence on the carbohydrate metabolism of the red blood cell. Finally, recent evidence (128, 19) seems to indicate that insulin combines with the reticulin of the spleen, adipose tissue and diaphragm muscle of the rat.

Therefore, since Stadie et al have shown indirectly, that insulin combines with rat diaphragm muscle and that such binding apparently increases with increasing temperature, is it possible to explain the lack of insulin action on galactose uptake by isolated diaphragm muscle incubated for 30" in KRP-C^{14}-galactose at 0°C. and at 10°C. as due to an inability of the muscle tissue to combine with insulin at such low temperatures?

An attempt was made to ascertain whether or not the above explanation is valid. Table 12 shows the effect of insulin on galactose uptake by rat diaphragm muscle. The control hemidiaphragm was incubated for 15" in KRP at 38°C., the experimental hemidiaphragm was incubated for 15" in KRP plus zinc insulin, 0.27 U./ml., at 38°C. Both tissues were then removed and blotted. The control hemidiaphragm was incubated in KRP plus C^{14}-galactose (100 mg. %) for 30" at 10°C. The hemidiaphragm exposed to insulin was also incubated in KRP plus C^{14}-galactose (100 mg. %) for 30" at 10°C. It can be seen, that although the muscle tissue was allowed to combine with insulin at 38°C., subsequent incubation in KRP plus C^{14}-galactose showed no difference in the uptake between the control tissue and the tissue exposed to insulin. In other words, there was no effect of combined insulin on the galactose uptake by the isolated diaphragm muscle.

Table 13 shows the effect of insulin on galactose uptake by rat diaphragm muscle. The control hemidiaphragm was incubated for 15" in KRP at 24°C., the experimental hemidiaphragm was incubated for 15" in KRP containing zinc insulin, 0.27 units/ml., at 24°C. Both tissues were then removed and blotted. Both tissues were then incubated in KRP plus C^{14}-

Table 12

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 10°C. O_2-Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	HDI incubated in KRP-C^{14}-galactose at 10°C. for 30"	HDII incubated in KRP plus insulin (0.27 U./ml.) for 15" at 38°C. Removed, blotted and incubated with KRP-C^{14}-galactose at 10°C. for 30"	% Insulin Effect
1	0.43	0.44	2
2	0.47	0.46	-2
3	0.45	0.45	0
4	0.41	0.38	-8
	Avg. 0.44 mg./gm.	0.43 mg./gm.	-2

Table 13

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 0°C. O_2-Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	HDI incubated in KRP-C^{14}-Galactose at 0°C. for 30"	HDII incubated in KRP plus insulin (0.27 U./ml.) for 15" at 24°C. Removed, blotted and incubated in KRP-C^{14}-Galactose at 0°C. for 30"	% Insulin Effect
1	0.34	0.35	3
2	0.35	0.34	-3
3	0.30	0.30	0
4	0.35	0.31	-13
	Avg. 0.34 mg./gm.	0.33 mg./gm.	-3

galactose (100 mg. %) for 30" at 0°C. It can be seen, that although the muscle tissue was allowed to combine with insulin at 24°C., subsequent incubation in KRP plus C^{14}-galactose showed no difference in the uptake between the control tissue and the tissue exposed to insulin. There was no effect of combined insulin on the galactose uptake by the isolated diaphragm muscle.

It must be concluded, therefore, that the lack of insulin action on galactose uptake at 0° and 10°C. cannot be due, primarily, to an inability of muscle tissue to combine with insulin at such low temperatures. This was indicated by the observation that tissues, combined with insulin at 38° or 24°C., and subsequently incubated in KRP-C^{14}-galactose at 10° or at 0°C. showed no greater galactose uptake than the untreated controls. More will be said about these observations in the general discussion at the close of this chapter.

The uptake of galactose at 0°C. and at 10°C., as seen in Tables 12 and 13, is slightly greater than that seen in Tables 10 and 11, where incubation was also carried out at 0°C. and 10°C. The tissues, tabulated in Tables 12 and 13, were incubated for 15" in KRP (with and without insulin) before being incubated in KRP containing C^{14}-galactose for an additional 30". Brown et al (15) observed that potassium is lost by isolated rat diaphragm muscle as a result of soaking in a Krebs-Henseleit solution. As a result of this, they found that such tissues could take up more glucose from the medium when incubated, subsequent to a preliminary incubation in medium without glucose. Thus, they consider that changes in cell permeability occur as a result of preliminary soaking in a physiological salt solution. Early experiments in this laboratory also

indicated that preliminary incubation in the medium before incubation in medium plus galactose resulted in a higher uptake of galactose by the isolated rat diaphragm muscle. Thus, the quantitative differences in uptakes between Tables 10 and 11 and Tables 12 and 13 might be explained on the basis of the above.

Brown et al (15) found that the effectiveness of various soaking conditions in causing a subsequent rise in glucose uptake is as follows: 0°C. in medium > 37°C. in medium > 0°C. in no medium. From Tables 10-13 it can be seen that the effect of preliminary incubation in causing a subsequent rise in galactose uptake is greater at 24°C. in medium than at 38°C. in medium.

D. Effect of Varying the Ionic Composition of the Medium on Galactose Uptake by Diaphragm Muscle

To determine whether the ionic composition of the medium had a marked influence on galactose uptake by muscle diaphragm, either in the presence or absence of insulin, the experiments shown in Tables 14-17 were performed. It will be seen from these preliminary data, that removal of K, Ca, Mg, or Mg plus Ca, were without influence upon galactose uptake, with or without added insulin. These data suggest that either these ions are not involved in galactose transfer or that sufficient amounts of such ions are associated with the tissue cells. It is interesting to note, in this connection, that the hexokinase system requires magnesium for its action (173).

E. A Demonstration of Galactose Entry Into the Cells of the Isolated Rat Diaphragm Muscle, in the Absence and Presence of Insulin

Levine, Goldstein, and coworkers (98, 57, 97, 59, 60) demonstrated

Table 14

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 27.5°C. O_2-Gas Phase. pH 7.4. The KRP does not Contain Isotonic $MgSO_4$

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose without Mg^{++}	In KRP-Galactose without Mg^{++} + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.49	0.60	23
2	0.49	0.58	18
	Avg. 0.49 mg./gm.	0.59 mg./gm.	20%

Table 15

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 27.5°C. O_2-Gas Phase. pH 7.4. The KRP Does Not Contain Isotonic $CaCl_2$.

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose without Ca^{++}	In KRP-Galactose without Ca^{++} + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.53	0.63	19
2	0.48	0.65	35
	Avg. 0.51 mg./gm.	0.64 mg./gm.	26%

Table 16

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 25°C. O_2-Gas Phase. pH 7.4. The KRP Does Not Contain Isotonic KCl

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose without K^+	In KRP-Galactose without K^+ + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.46 mg./gm.	0.65 mg./gm.	41%

Table 17

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 25°C. O_2-Gas Phase. pH 7.4. The KRP Does Not Contain Isotonic $MgSO_4$ or $CaCl_2$.

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose without Mg^{++} and Ca^{++}	In KRP-Galactose without Mg^{++} and Ca^{++} + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.46 mg./gm.	0.70 mg./gm.	52%

that when galactose was injected into an eviscerated-nephrectomized animal, such as a dog, a constant blood galactose level was attained in about 1 1/2 hours after injection and maintained for around 4 hours. Calculation based on the initial and final blood galactose concentrations and the weight of the preparation indicated that the volume of distribution of galactose in the eviscerated-nephrectomized animal was approximately 45% of the body weight. When insulin was injected together with the galactose, a constant blood level was again reached. However, the volume of galactose distribution was now approximately 70% of the body weight, which was interpreted by the authors to indicate distribution in total body water. The blood galactose was determined by the photometric method of Nelson (123) and the galactose used was non-radioactive.

These authors then administered sucrose, creatinine and urea to similar eviscerated-nephrectomized preparations. Constant blood levels were also attained within 1 1/2 hours following injection. Sucrose and creatinine attained a volume of distribution which was approximately 40-45% of the body weight. Urea attained a volume of distribution which was approximately 70% of the body weight. Insulin had no effect on the volume of distribution of these substances.

Since the volume of distribution of galactose, in the absence of insulin, was approximately 40-45% of body weight and since this was approximately the same volume of distribution as that attained by sucrose and creatinine, the authors concluded that this represents a distribution in extracellular space. Since the volume of distribution of galactose, in the presence of insulin, was approximately 70% of the body weight and since this was the same volume of distribution as that attained by such

a freely-diffusible substance as urea, the authors concluded that this represents a distribution of galactose through-out total body water (extracellular plus intracellular space). Levine et al thus postulate that the action of insulin on the uptake of galactose from the blood by the eviscerated-nephrectomized preparation is to allow for the transfer of galactose from the extracellular space into the intracellular space of certain organs and tissues. Such an action of insulin was also demonstrated on other insulin-responsive sugars, while insulin was observed not to affect the distribution of certain non-responsive sugars. A complete review of the work of this group can be found in Chapter I.

Wick and Drury (192) injected C^{14}-labelled galactose intravenously into eviscerated-nephrectomized rabbits. The radioactivity per ml. plasma, after allowing time for mixing in the body, was determined and insulin injected. The radioactivity of the plasma was then determined for several hours thereafter. These authors observed that galactose rapidly attains a volume of distribution of approximately 20-25% of body weight, a distribution equivalent to extracellular space. It can be seen from Table 18, that after the initial rapid distribution of galactose in extracellular space (20-25% body weight), galactose very slowly leaves the extracellular space even in the absence of insulin. However, this transfer from extra- to intracellular space is accelerated in the presence of insulin.

It can be seen from the work of Levine et al (98, 57, 97, 59, 60), with eviscerated-nephrectomized dogs, that 1 1/2 to 2 hours following an injection of non-radioactive galactose a volume of distribution of approximately 40-45% of body weight is attained. In the presence of

Table 18

Volume of Distribution of D-Galactose-1-C^{14} Expressed as Percentage of Body Weight (No Carrier Galactose Given). (After Wick, et al. 26)

Hour after injection of galactose	No Insulin		Insulin*	
	Expt. #226	Expt. #227	Expt. #140	Expt. #238
0.5			23	27
1.0	23	22		
1.5			72	70
2.0	43	31		
2.5			157	116
3.0		33		
3.5			146	
4.0		43		

* Insulin given immediately after taking the first plasma sample.

insulin, a volume of distribution of 70% is attained. It can also be seen from the work of Wick et al (192), with eviscerated-nephrectomized rabbits, that 2 hours following an injection of C^{14}-labelled galactose a volume of distribution of 31-43% of body weight is attained. In the presence of insulin, a volume of distribution of 70-72% is attained 1 1/2 hours following galactose administration.

In conclusion, one may say that galactose injected into eviscerated-nephrectomized animals is rapidly distributed in a volume equivalent to extracellular space. This was attained in 1 hour in eviscerated-nephrectomized rabbits (Table 18) in the absence of insulin. After approximately 2 hours, in the absence of insulin, the galactose is distributed in a volume equivalent to 31-43% in the rabbits (Wick et al) and 40-45% in the dogs (Levine et al). This means that galactose is being transferred from the extracellular to the intracellular space even in the absence of insulin. However, the rate of transfer is very slow. The presence of insulin accelerates this transfer so that the volume of distribution of galactose, 1 1/2 hours after administration, is approximately 70%. How may the observations obtained in this laboratory on the uptake of C^{14}-labelled galactose by isolated rat diaphragm from a physiological salt solution be related to in vivo work of Levine et al and Wick et al?

It, therefore, was deemed necessary to determine the extracellular space of the isolated rat diaphragm muscle incubated under the same in vitro conditions, as employed in the test system for galactose uptake. The extracellular space has been determined by many workers, using thiocyanate, bromide, chloride and sodium spaces for such a determination.

Manery and Hastings (107) postulate that tissues possess 3 phases of both chemical and morphological significance. The 3 phases are: 1. an extracellular phase, consisting primarily of a plasma ultrafiltrate and connective tissue proteins, which is in ionic equilibrium with the blood plasma; 2. an intracellular phase containing practically no sodium or chloride; 3. an intracellular phase containing chloride, with or without equivalent amounts of sodium. It was found that certain tissues, for instance, muscle fibers possess an intracellular phase which contains practically no sodium or chloride. On the other hand, blood corpuscles and connective tissue cells possess an intracellular phase which contains chloride.

It might be interesting to mention again here that it has been found that insulin promotes the transfer of certain responsive sugars from the extra- to the intracellular space by muscle tissue (98, 57, 97, 59, 60), whereas insulin has no effect on the glucose uptake of the red blood corpuscles (154). This has also been shown to be the case for brain and intestines. Might the difference in response to insulin on sugar uptake, by such tissues as muscle and erythrocytes, be explained on the basis of an existence of two kinds of intracellular spaces possessing different permeability characteristics? No definitive evidence exists as yet which could answer this question one way or the other.

Manery and Haege (109) injected a lithium chloride solution, labelled with Cl^{38}, into rabbits and determined the chloride space of various organs by comparing the activities of a tissue sample and of a plasma sample of known weight. The chloride space varied greatly from organ to organ. The chloride space for gastrocnemius muscle was found to be 9.4 gm./100 grams

tissue.

The bromide spaces of the organs of the guinea pig were determined, following injection of KBr labelled with Br^{82}, by Perlman et al (132). The apparent extracellular phase of muscle was 19.1% (grams per 100 grams fresh tissue).

The sodium spaces of the organs of a rabbit injected with NaCl labelled with Na^{24} were also determined (108). The sodium space varied from organ to organ. For example, the sodium space for gastrocnemius muscle was found to be 10%; for abdominal muscle, 19%; for heart, 30%; for liver, 22%, etc.

The extracellular space of the whole animal may also be determined by means of NaCl labelled with Na^{24}. From the ratio of the amount of $Na^{24}Cl$ injected into the blood and the amount of $Na^{24}Cl$ present after a period of time, one can calculate the total volume of extracellular fluid diluting the $Na^{24}Cl$. In rats, the volume into which the Na^{24} is distributed ranges from 23-29% of body weight (61, 108). The equilibrium is reached in 2 or 3 hours.

It has been shown by Gaudino et al (51) that the extracellular space determined by means of inulin was smaller when compared with the spaces obtained with thiocyanate, bromide and sodium. For example, these authors found that the inulin space in man is approximately 15-16% of body weight. In dogs, the inulin space averaged 21.6% of body weight.

The following idea then presented itself. Would it not be more meaningful to determine the uptake of inulin by isolated rat diaphragm muscle incubated for 30' in KRP under the in vitro conditions routinely employed? In this way, one could compare the uptake of a substance such

as inulin, which is known to diffuse only into extracellular space, and galactose by the test system. Therefore, isolated rat diaphragm muscle was prepared in the routine manner and was obtained from normal, fasted, male rats following decapitation. The muscles were then incubated in KRP for 30", with O_2 as the gas phase and at various temperatures.

Inulin is a polysaccharide composed of fructose units (some believe that the terminal end of the molecule consists of a glucose unit) and is found in the tubers and rhizomes of the Compositae plants. It is very easily hydrolyzed by acids and is composed of gamma fructose units. It has a molecular weight of approximately 5000 (3). The inulin forms a colloidal solution when dissolved in the KRP incubation medium. To make sure that the size of the inulin particles in solution in the KRP would not be a complicating factor in the inulin uptake by the incubating diaphragm muscle, KRP-inulin solutions ranging from 200 mg. % to 1000 mg. % were used as the incubation media.

After incubation in a KRP-inulin medium, the diaphragm muscle was removed, blotted firmly on filter paper and weighed on a torsion balance. This represents the wet weight of the tissue, as discussed in Chapter II. The diaphragm muscle is then placed in a test tube containing a known volume of water and placed in a boiling water bath for 10 minutes. The aqueous muscle extract was then analyzed for its inulin content by the method of Schreiner (145), which was adopted for use with the Klett-Summerson colorimeter. This determination is based upon the fact that when hexoses are heated in the presence of strong acids an hydroxymethyl-furfural is formed, which then can react with resorcinol or diphenylamine to form a colored condensation product (143). Since inulin is very easily

hydrolyzed by acids into gamma fructose units, and since the ketohexoses react very readily (more readily than aldohexoses, such as glucose), inulin may be readily and precisely determined colorimetrically.

Since the estimation of inulin by the reaction with resorcinol is dependent upon the hydrolysis of inulin to fructose, the strength of the acid used for the hydrolysis and the length and temperature of heating during which the colored condensation product is formed are very critical. The optimal conditions were found by Schreiner (145) to consist of 17% HCl in the reaction mixture and heating in a water bath at 80°C for 25 minutes. These conditions were strictly adhered to in this laboratory. In addition, a tissue blank and a standard curve were used for each set of determinations.

The aqueous extract of the diaphragm muscle was then deproteinized by means of Cd SO_4 (34.67 gms. of 3 Cd $SO_4.8H_2O$ plus 169 ml. of 1N H_2SO_4, made up to 1 liter) and 1.1 N NaOH. The aqueous extracts are placed in flasks, the deproteinizing agents are added, the flasks are stoppered, shaken and allowed to stand. The precipitate is then separated by means of centrifugation, the supernatant fluid is filtered through washed glass wool and an aliquot taken for inulin determination. In these experiments, the diaphragm tissue was extracted with 5 ml. of H_2O. After deproteinization and centrifugation, 2 ml. of the clear supernatant were placed in a pyrex test tube to which were added 2 ml. of a 0.1% solution of resorcinol in 95% ethyl alcohol and 5 ml. of 30% HCl. The test tube is then shaken, covered with a glass marble and placed in a water bath, thermostatically controlled at 80°C., for 25 minutes. 2 ml. of standard solutions of inulin (1, 2, and 3 mg. %) were similarly treated. In addition, an aqueous extract of rat diaphragm muscle not incubated in a KRP-inulin

solution was used as a tissue blank. The test tubes were then removed from the water bath, cooled, and placed in a Klett-Summerson colorimeter containing an appropriate filter and the transmission recorded. The red color developed is fairly stable for a period of several hours.

Table 19 shows the uptake of inulin by isolated diaphragm muscle obtained from normal, fasted rats. The diaphragm muscle was incubated for 30' in KRP plus inulin (200 mg. %, 400 mg.%, and 1000 mg. %) at 0°C., 10°C., 25°C., 29°C., and 38°C., with O_2 as the gas phase. Since all of the galactose uptake experiments were performed using a KRP medium containing 100 mg. % galactose, the inulin uptakes were calculated on the basis of incubation in a KRP medium containing 100 mg. % inulin. This allows for comparison of the inulin uptakes with the galactose uptakes. Since inulin is freely diffusible into the extracellular space, the uptake of inulin is proportional to the concentration of inulin in the medium. For example, the uptake of inulin from a medium containing 400 mg. % inulin was double the uptake from a medium containing 200 mg. % inulin. Also, it was found that the uptake of inulin from a medium containing 1000 mg. % was 5 times that from a medium containing 200 mg. % inulin and 2 1/2 times that from a medium containing 400 mg.%.

It can be seen from the description of the methods that a water extract of the diaphragm muscle is made. It was assumed that during 10 minutes of boiling the inulin in the muscle is completely extracted by the water. To test this, the following procedure was employed. Muscle tissue was incubated in KRP plus inulin, an aqueous extract made by boiling in water for 10 minutes and an aliquot estimated for its inulin content, as described above. The boiled tissue was then removed, blotted

Table 19

Determination of Inulin Uptake by Isolated Diaphragms Obtained from Normal Fasted Rats. Incubated 30" in KRP plus Inulin. O_2-Gas Phase. Uptake Calculated on the Basis of KRP plus Inulin (100 mg. %).

Temperature At Which Incubation Was Performed	Inulin Uptake, mg. per gm. wet wt. Tissue per 30" of Incubation
0°C.	0.10 0.10 0.12 0.11 Avg. 0.11 mg./gm.
10°C.	0.14 0.14 0.14 0.13 0.15 0.15 Avg. 0.14 mg./gm.
25°C.	0.16 0.15 0.15 0.19 Avg. 0.16 mg./gm.
29°C.	0.21 0.19 0.19 Avg. 0.20 mg./gm.
38°C.	0.23 0.20 0.18 0.17 0.17 0.18 0.20 Avg. 0.19 mg./gm.

lightly with filter paper, placed in a tube containing a known volume of water and homogenized with alumina. The homogenate is then centrifuged, the supernatant is then deproteinized by Cd SO_4 and NaOH, as described above; centrifuged, and an aliquot of the clear supernatant analyzed for its inulin content. It was found, using 4 animals, that no color developed. Therefore, all of the inulin was extracted by 10 minutes of boiling in water and hence the procedure is considered valid.

Table 20 shows a comparison between the inulin uptakes and galactose uptakes at 30" of incubation at various temperatures. All of the incubations carried out in this entire study were in Warburg flasks, placed in a constant temperature Warburg water bath and shaken for the entire period of incubation. Such shaking allows for continuous movement of the medium bathing the tissue, thus promoting exchanges and preventing stagnation.

It can be seen that, at each temperature of incubation, the inulin uptake is significantly less than the galactose uptake by the diaphragm muscle. It can also be observed that 30" of incubation in KRP plus inulin at 38°C. apparently results in a maximum uptake of inulin by the isolated diaphragm muscle. This argument is strengthened by the observation that the inulin uptake was already maximal after 30" incubation of the tissue at 29°C. Therefore, an inulin uptake of approximately 0.20 mg./gm. will be considered as maximal diffusion of inulin into the extracellular space of the diaphragm muscle, with the establishment of a state of equilibrium with the surrounding medium. This assumption is strengthened by the observation, already mentioned, that the uptake of inulin is proportional to the concentration of inulin in the surrounding medium. Therefore, the

inulin can readily diffuse into and out of the extracellular space until an equilibrium state is attained. This was shown to be the case for the various incubation temperatures employed. In conclusion, one may state that inulin is a freely-diffusible substance, its diffusion being proportional to the concentration of inulin in the medium. In addition, since the uptake of inulin under these conditions apparently is an expression of a free diffusion process, then one would expect the inulin uptake to be less at lower temperatures and greater at higher temperatures. Table 20 indicates that such is the case.

It can also be seen from Table 20 that, at each temperature of incubation, the uptake of galactose is considerably greater than the inulin uptake by the diaphragm muscle. The effect of temperature on insulin action on galactose uptake by diaphragm muscle has already been discussed in this chapter. One may, therefore, postulate that during the 30" of incubation of diaphragm muscle in KRP-plus C^{14}-galactose, at temperatures ranging from 0° to $38^{\circ}C.$, the galactose not only enters the extracellular space but also the intracellular space of the muscle tissue. This occurs even in the absence of insulin. It has already been mentioned that Levine et al (98, 57, 97, 59, 60) and Wick et al (192) demonstrated in eviscerated-nephrectomized dogs and rabbits, respectively, that such a transfer, in the absence of insulin, occurs in vivo. However, this transfer of galactose from extra- to intracellular space in vivo was found to occur at a very slow rate (1-4 hours following galactose administration). In both the in vivo experiments of Levine et al and Wick et al and in the in vitro experiments carried out in this laboratory, the presence of insulin accelerated the transfer of galactose from the

Table 20

A Comparison Between the Inulin Uptake and the Galactose Uptake by Isolated Rat Diaphragms Obtained from Normal, Fasted Animals. Incubated 30" in KRP plus Inulin or KRP plus C^{14}-Galactose (100 mg. %). O_2-Gas Phase. pH 7.4.

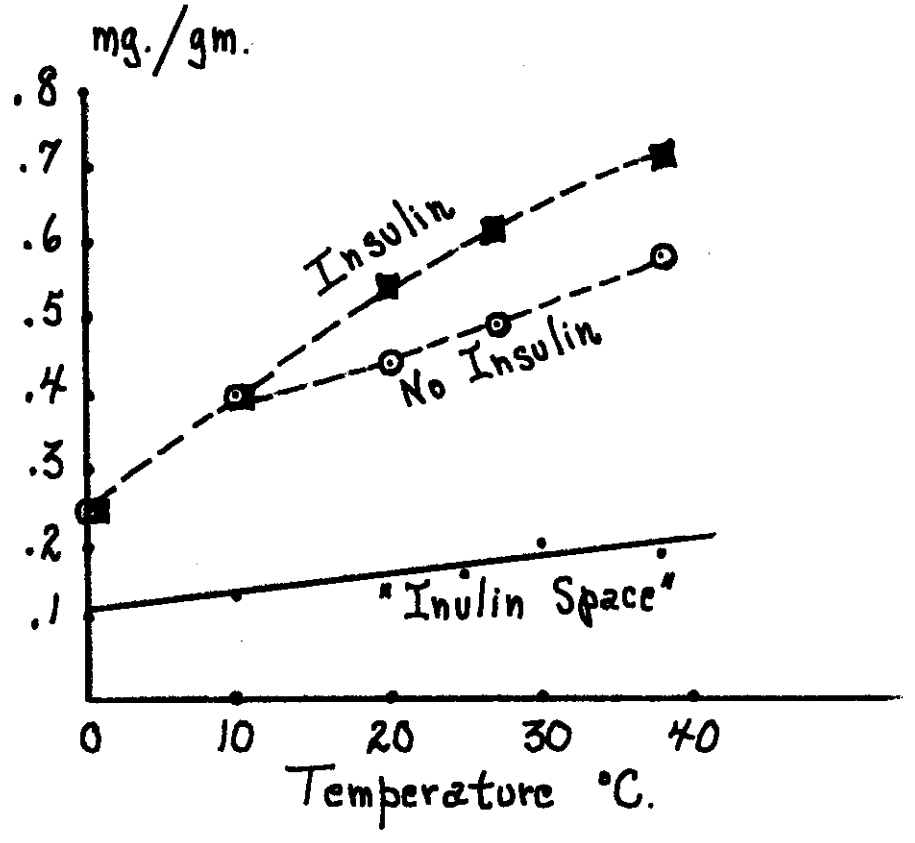
Temp. of Incubation	In KRP + Inulin mg./gm./30"	In KRP + C^{14}-Galactose mg./gm./30"	In KRP + C^{14}-Galactose + Insulin (0.27 U./ml.) mg./gm./30"
0°C.	0.11 (4 rats)	0.25 (3 rats)	0.25 (3 rats)
10°C.	0.14 (6 rats)	0.40 (8 rats)	0.40 (8 rats)
20°C.	--	0.44 (8 rats)	0.54 (8 rats)
25°C.	0.16 (4 rats)	--	--
27°C.	--	0.49 (11 rats)	0.62 (11 rats)
29°C.	0.20 (3 rats)	--	--
38°C.	0.19 (7 rats)	0.58 (15 rats)	0.71 (15 rats)

Table 21

Entry of Galactose into the Muscle Cells of Isolated Rat Diaphragms Incubated 30" in KRP + C^{14}-Galactose (100 mg. %) at Varying Temperatures. O_2-Gas Phase pH 7.4

Temp.	No. of Rats	Galactose uptake, less inulin uptake, mg./gm. wet wt.		
		In KRP + C^{14}-Galactose	In KRP + C^{14}-Galactose + Insulin (0.27 U./ml.)	Insulin Effect
0°C.	4	0.14 mg./gm.	0.14 mg./gm.	0
10°C.	8	0.26 mg./gm.	0.26 mg./gm.	0
20°C.	8	0.29 mg./gm.	0.39 mg./gm	0.10 mg./gm.
27°C.	11	0.33 mg./gm.	0.46 mg./gm.	0.13 mg./gm.
38°C.	15	0.38 mg./gm.	0.51 mg./gm.	0.13 mg./gm.

Fig. 3. Galactose Uptake by Diaphragm at Varying Temperatures.



extracellular to the intracellular tissue spaces.

F. The Ability of Galactose to Diffuse Out of Muscle Tissue

Since it had been established, both *in vivo* by other workers and *in vitro* in this laboratory, that galactose readily enters the extracellular phase of muscle and then slowly is transferred intracellularly, it was thought desirable to ascertain if galactose can readily diffuse out of muscle tissue.

Therefore, the following experiments were carried out. Diaphragms obtained from normal, fasted rats were sectioned into right and left hemidiaphragms. Each hemidiaphragm was in turn sectioned into two slices. Thus each diaphragm yielded 4 slices of muscle tissue. All 4 slices were then incubated in KRP plus C^{14}-galactose (100 mg. %) containing insulin for various lengths of time and at various temperatures. After incubation, all of the diaphragm slices were removed from the KRP-C^{14} galactose medium and blotted lightly between filter paper. One diaphragm slice was immediately weighed and analyzed for galactose in the usual manner. The remaining three slices were placed in flasks containing KRP and incubated with shaking. After incubation in the KRP medium for a definite period of time, a diaphragm slice would be removed, blotted, weighed and analyzed for its galactose content. In this way, one could determine whether or not galactose which entered the diaphragm during incubation in KRP-galactose can diffuse out of the tissue under the proper conditions.

It can be seen from Table 22 that the galactose which entered the diaphragm during incubation can very readily diffuse out of the tissue, when incubated in a medium containing no galactose. The galactose, however, is not removed completely by such a procedure. A very slight amount

remained in the tissue even after 60" of incubation in KRP. In order to explain this, the following was performed. Two slices of isolated rat diaphragm muscle were dipped for a fraction of a second into a flask containing KRP-C^{14}-galactose (100 mg.%), removed immediately, blotted vigorously and analyzed for galactose. The two slices were found to contain 0.03 mg./gm. and 0.04 mg./gm. galactose, respectively. This was taken to mean that a very small quantity of galactose adheres to the surface of the muscle slice.

Therefore, the effect of insulin to accelerate the entry of galactose into the muscle cells is not the result of a reduced rate of galactose exit from the cells.

G. The Effect of Known Constituents of Muscle on Galactose Uptake by the Isolated Rat Diaphragm

The clinical fact that intense muscular work or exercise can lead to a state of insulin hypersensitivity in a diabetic patient (77, 135) has been mentioned in Chapter I. Also, Goldstein et al (60) studied the effect of muscular work on the transfer of sugars by eviscerated-nephrectomized preparations, with or without section of the spinal cord. They found that those sugars which were insulin-responsive responded to muscular work in the same manner. Those sugars which were non-responsive to insulin were unaffected by muscular work.

Since, throughout this study, an attempt has been made to characterize and elucidate the mechanism of insulin action on the galactose uptake by isolated muscle, several of the many substances known to have important physiological and biochemical roles in muscle tissue were added directly to the incubation medium. These are preliminary experiments and further work is contemplated. Tables 23 and 24 show that essentially normal

Table 22

Effect of Incubation of Isolated Diaphragm Slices, Obtained from Normal, Fasted Rats, in KRP Medium on the Galactose Taken Up By Such Slices Following Incubation for 60" in KRP-C^{14}-Galactose (100 mg. %) plus Insulin. O_2-Gas Phase. pH 7.4

No Incubation in KRP	5" Incubation in KRP	15" Incubation in KRP	30" Incubation in KRP	60" Incubation in KRP
Galactose Content mg./gm. wet wt.	Galactose Content mg./gm. wet wt.	Galactose Content mg./gm. wet wt.	Galactose Content mg./gm. wet wt.	Galactose Content mg./gm. wet wt.
0.65 (Inc. 38°C.)	—	0.15	0.09	0.03
0.69 (Inc. 24°C.)	0.37	0.16	0.10	--
0.78 (Inc. 24°C.)	0.37	0.18	0.11	--
0.37 (Inc. 0°C.)	0.28	0.17	0.12	--
— "	0.27	0.20	0.12	--

galactose uptakes, in the absence and presence of insulin, are obtained when the diaphragm muscle is incubated in the presence of ATP at a concentration of 1×10^{-5}M or 1×10^{-2}M. It can be seen that in general the insulin effect, though consistent, is at the low normal range. This is especially apparent when the higher concentration of ATP is used. This might reflect a physical hindrance to diffusion of the sugar as a result of the presence of ATP. When the tissues were removed from the incubation flasks it was noted that the muscle slice has a tough rubbery consistency with its edges curled up, suggesting a state of contracture. This was most noticeable with the higher concentration of ATP.

Table 25 shows the effect of insulin on galactose uptake by isolated diaphragm muscle obtained from normal, fasted rats. The diaphragm muscle was incubated for 30" in KRP plus C^{14}-galactose (100 mg. %) containing creatine hydrate at a concentration of 1×10^{-2}M. Incubation was carried out at 38°C., with O_2 as the gas phase. In 4 animals, the uptake of galactose in the absence of added insulin averaged 0.56 mg./gm., with a range of from 0.54 to 0.58 mg./gm. When insulin was added to the incubation medium, the uptake of galactose averaged 0.63 mg./gm., with a range of from 0.57 to 0.69 mg./gm. The percent insulin effect averaged 13%, with a range of from -2 to 23%. The average increment of galactose uptake was 0.07 mg./gm.

If, however, one examines the 4 animals individually, it becomes apparent that a very prominent action of insulin on galactose uptake occurs with the diaphragms isolated from 2 animals. The diaphragm from the other 2 animals showed no response to the presence of insulin. The uptakes of galactose, in the absence of insulin, by the 2 insulin-

Table 23

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubation 30" in KRP plus C^{14}-Galactose (100 mg. %) Containing Adenosine Triphosphate (1 x 10^{-5} M). O_2-Phase. Incubation Performed at 38°C. pH 7.4.

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose + ATP	In KRP-Galactose + ATP + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.57	0.63	11
2	0.53	0.75	42
3	0.62	0.67	8
4	0.61	0.70	15
	Avg. 0.58 mg./gm.	0.69 mg./gm.	19%

S. E. M. = $\pm$ 0.04; p = $>$.05

Table 24

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) Containing Adenosine Triphosphate (1×10^{-2} M). O_2-Gas Phase. Incubation Performed at 38°C. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose + ATP	In KRP-Galactose + ATP + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.58	0.62	7
2	0.56	0.60	7
3	0.42	0.60	43
4	0.61	0.72	18
5	0.75	0.73	-3
	Avg. 0.54 mg./gm.	0.65 mg./gm.	20%

S. E. M. = $\pm$ 0.03; p = .1

responsive diaphragms were 0.56 and 0.54 mg./gm.; in the presence of insulin, 0.69 and 0.66 mg./gm. the percent insulin effect, 23 and 22%; the increment, 0.13 and 0.12 mg./gm. The uptakes of galactose, in the absence of insulin, by the 2 insulin-nonresponsive diaphragms were 0.57 and 0.58 mg./gm.; in the presence of insulin, 0.58 and 0.57 mg./gm.; the percent insulin effect, 2 and -2%; the increment, 0.01 and -0.01 mg./gm. Thus, one may conclude that a maximal insulin effect was demonstrated in 2 out of 4 animals. This might reflect a physical hindrance to diffusion of the sugar as a result of the presence of creatine hydrate.

The same picture was obtained when adenylic acid (AMP) was added to the incubation medium. Table 26 shows the effect of insulin on galactose uptake by isolated diaphragm muscle obtained from normal, fasted rats. The diaphragm muscle was incubated for 30' in KRP plus C^{14}-galactose (100 mg. %) containing adenylic acid (1×10^{-2} M) at 38°C., with O_2 as the gas phase. It can be seen, that the diaphragm of one animal responded maximally under these conditions. The diaphragm from the other animal showed no response to the presence of insulin. Thus, it may be concluded that an insulin effect may be observed, on the routine test system, in the presence of a high concentration of ATP, creatine hydrate and AMP. Such an effect, however, was shown to be inconsistent. These experiments are to be considered as very preliminary. They are included with the idea that as much information as possible should be presented so as to aid in the attempt to analyze and understand the mechanism of insulin action.

H. The Effect of Several Inhibitors on Galactose Uptake by Isolated Diaphragm Muscle

It has been shown that fermentation is inhibited by cyanide and that

Table 25

Effect of Insulin on Galactose Uptake By Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) Containing Creatine Hydrate (1 x 10^{-2} M) at 38°C. O_2-Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" incubation		
	In KRP-Galactose + Creatine Hydrate	In KRP-Galactose + Creatine Hydrate + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.56	0.69	23
2	0.57	0.58	2
3	0.54	0.66	22
4	0.58	0.57	-2
	Avg. 0.56 mg./gm.	0.63 mg./gm.	13%

S. E. M. = $\pm$ 0.04; p = .2

Table 26

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubated 30" in KRP Plus C^{14}-Galactose (100 mg. %) Containing Adenylic Acid (1×10^{-2} M) at 38°C. O_2-Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" incubation		
	In KRP-Galactose + Adenylic Acid	In KRP-Galactose + Adenylic Acid + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.54	0.53	-2
2	0.56	0.68	21
3	Avg. 0.55 mg./gm.	0.61 mg./gm.	11%

fluoride inhibits enolase, which dehydrates 2-phosphoglyceric acid to enol-phosphopyruvic acid (114). Najjar (122) has shown that phosphoglucomutase is inhibited by fluoride. Haft and Mirsky (63) observed that the action of insulin upon glucose uptake in muscle is inhibited by iodoacetate and by pentobarbital (64). In addition, a whole host of enzymes have been shown to be inhibited either by cyanide or fluoride. It will suffice here to mention only a few instances: ATP ase is inhibited by fluoride ions (83) and cytochrome C peroxidase is inhibited by cyanide ions (2).

Tables 27 and 28 show that essentially normal galactose uptakes, in the absence and presence of insulin, are obtained when the diaphragm muscle is incubated in the presence of sodium cyanide or sodium fluoride at a concentration of 1×10^{-4} M.

It was next decided to examine what effects the addition of 2,4-dinitrophenol (DNP) to the incubation medium might have on the routine test system employed.

It can be seen from Tables 29 through 32 that, under the in vitro conditions employed, insulin does not increase the uptake of C^{14}-galactose by the isolated rat diaphragm in the presence of DNP (2×10^{-4} M). This was found to be the case when incubation was carried out at 38°C., 25°C., 20°C., and 0°C.

It has been shown that 2,4-dinitrophenol considerably enhances the rate of adenosine triphosphate (ATP) breakdown by toad embryo brei under certain conditions (111). These authors employed a concentration of DNP of 1 to 2×10^{-4} M. It has also been shown that the breakdown of ATP by rat liver mitochondria is greatly enhanced by DNP, at a concentration of

Table 27

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) Containing Sodium Cyanide (1×10^{-4} M). O_2-Gas Phase. Incubation Performed at 38°C. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose + NaCN	In KRP-Galactose + NaCN + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.59	0.77	31
2	0.62	0.77	24
3	0.72	0.77	7
4	0.53	0.63	19
5	0.50	0.62	24
	Avg. 0.59 mg./gm.	0.71 mg./gm.	20%

S. E. M. = $\pm$ 0.02; p = $< .01$

Table 28

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubated 30" in KRP Plus C^{14}-Galactose (100 mg. %) Containing Sodium Fluoride (1 x 10^{-4} M). O_2-Gas Phase. Incubation Performed at 38°C. pH 7.4.

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose + NaF	In KRP-Galactose +NaF + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.51	0.78	53
2	0.66	0.82	24
3	0.67	0.74	10
4	0.46	0.76	65
5	0.51	0.72	41
	Avg. 0.56 mg./gm.	0.76 mg./gm.	36%

S. E. M. = $\pm$ 0.04; p = $<$ .01

Table 29

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) Containing Dinitrophenol (2×10^{-4} M) at 38°C. O_2-Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP + Galactose + DNP	In KRP-Galactose + DNP + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.50	0.49	-2
2	0.51	0.56	10
3	0.50	0.48	-4
4	0.65	0.63	-3
5	0.58	0.55	-5
	Avg. 0.55 mg./gm.	0.54 mg./gm.	-2%

Table 30

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) Containing Dinitrophenol (2×10^{-4} M) at 25°C. O_2-Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose + DNP	In KRP-Galactose + DNP + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.39	0.37	-5
2	0.39	0.39	0
3	0.41	0.41	0
4	0.38	0.37	-3
	Avg. 0.39	0.39 mg./gm.	0%

Table 31

Effect of Insulin on Galactose Uptake By Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) Containing Dinitrophenol (2×10^{-4} M) at 20°C. O_2-Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose + DNP	In KRP-Galactose + DNP + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.35	0.35	0
2	0.38	0.39	3
	Avg. 0.37 mg./gm.	0.37 mg./gm.	0%

Table 32

Effect of Insulin on Galactose Uptake By Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) Containing Dinitrophenol (2×10^{-4} M) at 0°C. O_2-Gas Phase. pH 7.4.

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose + DNP	In KRP-Galactose + DNP + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.22	0.22	0
2	0.24	0.25	4
3	0.29	0.25	-16
	Avg. 0.25 mg./gm.	0.24 mg./gm.	-4%

3×10^{-5} M (137). These results have been confirmed by numerous workers.

Therefore, experiments were designed to ascertain whether or not the observed inhibitory action of DNP on insulin action, on the uptake of galactose by isolated diaphragm muscle, could be reversed by the addition of a large concentration of ATP. Table 33 shows the influence of ATP on the action of insulin on galactose uptake by isolated diaphragms obtained from normal, fasted rats. Each diaphragm was sectioned into 3 slices; one slice was incubated for 30" in KRP plus C^{14}-galactose containing DNP (2×10^{-4} M) at 38°C.; the second slice was incubated for 30" in KRP plus C^{14}-galactose containing DNP (2×10^{-4} M) and insulin at 38°C.; the third slice was incubated for 30" in KRP plus C^{14}-galactose containing DNP (2×10^{-4} M), ATP (2.5×10^{-3} M) and insulin. It can be seen that, in 3 animals, the addition of a high concentration of ATP to the incubation medium had no effect on the inhibition of insulin action by DNP.

The nitro and similarly substituted phenols have been shown to possess biological activity. For example, it was shown in 1936 that the nitrophenols and the dihalo and trihalophenols influence growth, differentiation, cleavage and respiration in sea urchin embryos (24, 86). It was also shown (86) that the biological activity of these compounds is derived from the properties of the phenolic -OH group. Therefore, experiments were performed to ascertain whether or not dinitronaphthol exhibited any influence on insulin action. Dinitronaphthol has been shown by Szent-Gyorgyi to have biological activity (175) on muscular contraction. It can be seen from Table 34 that dinitronaphthol inhibits the action of insulin on the galactose uptake by diaphragm muscle, under the <u>in vitro</u> conditions employed.

Table 33

The Influence of Adenosine Triphosphate on the Action of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) Containing Dinitrophenol (2×10^{-4} M) at 38°C. O_2-Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose + DNP	In KRP-Galactose + DNP + Insulin (0.27 U./ml.)	In KRP-Galactose + DNP + ATP (2.5×10^{-3} M) + Insulin (0.27 U./ml)
1	0.67	0.65	0.60
2	0.58	0.56	0.54
3	0.59	0.57	0.57
	Avg. 0.61 mg./gm.	0.59 mg./gm.	0.57 mg./gm.

Table 34

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 30" in KRP-C^{14}-Galactose (100 mg. %) Containing Dinitronaphthol (2×10^{-4} M) at 38°C. O_2-Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. per 30" of incubation		
	In KRP-Galactose + DNN	In KRP-Galactose + DNN + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.61	0.64	5
2	0.61	0.59	-3
3	0.54	0.55	2
4	0.59	0.59	0
	Avg. 0.59 mg./gm.	0.59 mg./gm.	0%

Is the inhibitory influence of DNP on insulin action in some way related to an effect of DNP on cellular respiration? It has been shown (24, 104) that DNP may have no effect on respiration, may stimulate respiration or may inhibit respiration depending on the concentration of DNP and the test system employed.

Table 35 shows the influence of DNP at a concentration of 2×10^{-4} M on the O_2-consumption of isolated diaphragms incubated for 30" in KRP plus C^{14}-galactose at 38°C., with O_2 as the gas phase. It can be seen that the O_2-consumption by diaphragm tissue is considerably less in the presence of DNP at this concentration. Therefore, it was assumed that the inhibitory influence of DNP on insulin action occurred at a concentration of DNP inhibitory to the respiration of the isolated diaphragm. This is interesting in light of the work of Clowes et al (24) who found that the reversible block to division by Arbacia eggs resulting from the action of certain nitro compounds coincided with the down curve of respiration. In other words, the biological activity of the nitrophenols was observed at a concentration range in which the respiratory rate was decreasing from the optimum value.

Table 36 further indicates that DNP at a concentration of 2×10^{-4} M is at the inhibitory range for respiration of diaphragm muscle, under the in vitro conditions employed. It will be seen that the O_2-consumption of the diaphragm muscle obtained from fed rats, incubated in the presence of DNP is approximately one half that of the muscle incubated in the absence of DNP. The absolute values are higher. This may indicate that the endogenous respiration, which is here being measured, is greater in diaphragm muscle isolated from fed animals.

Table 35

The Influence of DNP (2×10^{-4} M) on the O_2-Consumption of Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 38°C. O_2-Gas Phase. pH 7.4.

Rat #	O_2-Consumption, ul.O_2/100 mg. wet wt./hr. per 30" of incubation			
	In KRP-Galactose	In KRP-Galactose + Insulin (0.27 U./ml.)	In KRP-Galactose + DNP	In KRP-Galactose + DNP + Insulin (0.27 U./ml.)
1		197	54	61
2	190	155	85	119
3	137		138	
4	173		103	
	Avg. 167	176	95	90

Table 36

The Influence of DNP (2×10^{-4} M) on the O_2-Consumption of Isolated Diaphragms Obtained from Normal, Fed Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 38°C. O_2-Gas Phase. pH. 7.4.

Rat #	O_2-Consumption, ul. O_2/100 mg. wet wt./hr. per 30" of incubation			
	In KRP-Galactose	In KRP-Galactose + Insulin (0.27 U./ml.)	In KRP-Galactose + DNP	In KRP-Galactose + DNP + Insulin (0.27 U./ml.)
1			106	116
2			137	144
3			113	151
4	231	298		
	Avg. 231	298	119	137

Tables 36A and 37 show that essentially normal galactose uptakes, in the absence and presence of insulin, are obtained when the diaphragm muscle is incubated in the presence of DNP at a concentration of 2×10^{-5} M or 2×10^{-6} M. It can be seen that, on the average, DNP at a concentration of 2×10^{-6} M does not seem to influence respiration to any significant extent; at a concentration of 2×10^{-5} M, respiration seems to be stimulated. It is possible that an effect on O_2-consumption might have been more definitive if incubation had been carried out for a longer period of time.

I. The Effect of Anaerobiosis on Galactose Uptake by the Isolated Rat Diaphragm

Since it had been shown that DNP inhibits insulin action on galactose uptake, it was considered of interest to ascertain whether anaerobiosis would have any effect on insulin action in the test system employed. It can be seen from Table 38 that 4 out of 5 animals showed an insulin effect on galactose uptake. However, the percent insulin effects were significantly lower than those obtained when diaphragms were incubated under the same *in vitro* conditions, but with O_2 as the gas phase. The average percent insulin effect, with O_2 as gas phase, was found to be 23% (See Table 6).

It has been shown (64) that the uptake of glucose by isolated rat diaphragm was identical under both aerobic and anaerobic conditions. The diaphragms were incubated for 90' in phosphate saline buffer containing glucose (200 mg. %) at 37°C. When anaerobiosis was employed, incubation was carried out in its entirety under an atmosphere of 95% N_2 - 5% CO_2. These authors found that, under such anaerobic *in vitro* conditions, an

Table 36A

The Influence of DNP (2×10^{-5} M) on the O_2-Consumption and Galactose Uptake by Isolated Diaphragms Obtained from Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 38°C. O_2-Gas Phase. pH 7.4

Rat #	Galactose uptake, mg./gm. wet wt. per 30" of incubation ul. O_2/100 mg. wet wt./hr. per 30" of incubation		
	In KRP + Galactose + DNP	In KRP + Galactose + DNP + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.64 mg./gm.	0.63 mg./gm.	-2
2	0.59 mg./gm.	0.60 mg./gm.	2
3	0.52 mg./gm.	0.64 mg./gm.	23
4	0.54 mg./gm.	0.59 mg./gm.	9
5	0.50 mg./gm. 358 ul. O_2	0.67 mg./gm. 445 ul. O_2	34
6	0.43 mg./gm. 159 ul. O_2	0.58 mg./gm. 328 ul. O_2	35
7	0.59 mg./gm. 374 ul. O_2	0.78 mg./gm. 300 ul. O_2	32
8	0.63 mg./gm. 268 ul. O_2	0.69 mg./gm. 450 ul. O_2	10
	Avg. 0.56 mg./gm.	0.65 mg./gm.	16%

S. E. M. = $\pm$ 0.03; p = $< .02$

Table 37

The Influence of DNP (2×10^{-6} M) on the O_2-Consumption and Galactose Uptake by Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 38°C. O_2-Gas Phase. pH 7.4

Rat #	Galactose uptake, mg./gm. wet wt. per 30" incubations; O_2-consumption, ul. O_2/100 mg. wet wt./hr. per 30" of incubation					
	In KRP-Galactose	In KRP-Galactose + Insulin	% Insulin Effect	In KRP-Galactose + DNP	In KRP-Galactose + DNP + Insulin	% Insulin Effect
1	0.69 mg./gm. 180 ul. O_2	0.78 mg./gm. 198 ul. O_2	13%	0.64 mg./gm. 156 ul. O_2	0.70 mg./gm. 193 ul. O_2	9%
2	0.54 mg./gm. 183 ul. O_2	0.65 mg./gm. 236 ul. O_2	20%	0.62 mg./gm. 300 ul. O_2	0.66 mg./gm. 137 ul. O_2	6%
3				0.55 mg./gm.	0.76 mg./gm.	38%
4				0.67 mg./gm.	0.80 mg./gm.	19%
5				0.63 mg./gm.	0.62 mg./gm.	-2%
6				0.58 mg./gm.	0.65 mg./gm.	12%
Avg.	0.62 mg./gm.	0.72 mg./gm.	16%	0.62 mg./gm.	0.70 mg./gm.	13%

S.E.M. = $\pm$ 0.03; p = $< .05$

Table 38

Effect of Insulin on Galactose Uptake by Isolated Diaphragms Obtained From Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) at 38°C. Gassed with N_2 for 5 minutes. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP-Galactose	In KRP-Galactose + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.62	0.68	10
2	0.63	0.75	19
3	0.64	0.70	9
4	0.67	0.66	0
5	0.71	0.79	11
	Avg. 0.65 mg./gm.	0.72 mg./gm.	10%

S. E. M. = $\pm$ 0.02; p = $< .05$

action of insulin on the uptake of glucose from the medium could not be demonstrated.

Walaas and Walaas (187) also found that the uptake of glucose by isolated rat diaphragm was identical under aerobic and under anaerobic conditions. They also found that, under anaerobic *in vitro* conditions, an action of insulin on the uptake of glucose from the medium could be demonstrated. This insulin effect, however, was much less than that observed under aerobic conditions.

Demis and Rothstein (39) recently have found that there is no significant effect of anaerobiosis on the uptake of glucose, fructose and galactose by isolated diaphragms incubated in KRP plus non-radioactive sugars at 38°C. When insulin was added to the incubation medium, an increased uptake of these 3 sugars occurred under aerobic conditions. When the medium was gassed with nitrogen for 10 to 20 minutes, an action of insulin on the uptake of these 3 sugars from the medium could not be demonstrated.

It can be seen from Table 38 that gassing with N_2 for 5" followed by incubation for 30" still allows for an insulin effect on galactose uptake. However, the percent insulin effect averages less than half that observed under aerobic conditions. How may these results be reconciled in the light of the results of Demis and Rothstein (39)? First, in the experiments carried out in this laboratory the incubation medium was gassed with N_2 for only 5", as against 10-20" by Demis *et al* (39). Secondly, in these experiments the insulin was already in the medium before the gassing with N_2, and the uptake of galactose was determined during the 30" immediately following such gassing. In the experiments of

Demis et al (39) the tissues and medium were gassed for 10-20" with N_2, the insulin then added and the uptake of galactose determined 60" after the addition of insulin. Since it has already been shown by Walaas and Walaas (187) that when the isolated diaphragm muscle is made anaerobic, the insulin effect lasts for about 15" and then disappears, one may interpret the results tabulated in Table 38 as due to a persistence of insulin action during the first 15" of incubation followed by a disappearance of insulin action during the second 15" of incubation. This resulted in an apparent partial insulin effect. On the other hand, in the experiments of Demis et al (39) the diaphragms and the medium were gassed for 10-20" with N_2 before the addition of insulin and the onset of incubation. Hence, no insulin effect was apparent under those conditions, since at least 15-20" elapsed before the addition of insulin.

The same reasoning may be used to explain the slight insulin effect on glucose uptake under anaerobic conditions, as observed by Walaas and Walaas (187), and the lack of an insulin effect on glucose uptake by isolated diaphragm muscle incubated under anaerobic conditions as observed by Haft and Mirsky (64).

Demis et al (39) found that diaphragm muscle made unresponsive to insulin action, by means of anaerobiosis, is still able to respond to insulin when returned to aerobic conditions. They state that "when muscle is re-exposed to air after an anaerobic period, the insulin effect reappears only after a delay of some 15-30 minutes. The delay in the disappearance and reappearance of the insulin effect suggests that the action of insulin on sugar uptake is dependent on some cellular constituent which is maintained in adequate amount only under aerobic conditions. When

the muscle is made anaerobic there is a sufficient reservoir of this constituent to maintain the insulin effect for a time. When the muscle is returned to aerobic conditions, it takes some 15 minutes for the constituent to be resynthesized, accounting for the delay in the reappearance of the insulin effect."

The significance of anaerobiosis on insulin action will be discussed later. Suffice it here to point out that Walaas and Walaas (187) found that, following 15" of anaerobic incubation, the phosphocreatine of the isolated diaphragm muscle disappeared completely and the level of ATP fell to 2-3 mg. labile P per 100 gm. muscle. Nevertheless, it has been pointed out by Walaas and Walaas (187), by Haft and Mirsky (64), by Demis and Rothstein (39) that the uptake of glucose, fructose and galactose under such anaerobic conditions is essentially the same as under aerobic conditions. This has also been confirmed in this laboratory. Yet, it is possible to demonstrate an inhibition of insulin action under such anaerobic conditions (64, 187, 39). Therefore, one may consider that the action of insulin on the uptake of certain sugars is dependent in some way upon aerobic processes of the tissues. The normal uptake of sugars by muscle tissue, apparently does not seem to be dependent upon such aerobic processes.

CHAPTER IV

DISCUSSION
and
SUMMARY

A. Discussion

Evidence has been presented which indicates that, under certain *in vitro* conditions, an action of insulin on galactose uptake by isolated rat diaphragm muscle can be observed. Such an action can be considered as being quite consistent. The optimal conditions for the test system employed for such a demonstration of insulin action have also been presented. These experiments were performed with non-radioactive carrier galactose containing tracer amounts of D-galactose-1-C^{14}.

Demis and Rothstein (39), working independently, were also recently able to demonstrate an insulin effect on the uptake of non-radioactive galactose by isolated rat diaphragm, incubated for 1 hour in KRP at 38°C. The uptake of galactose by the diaphragms was determined by the rate of the disappearance of galactose from the medium, aliquots of which were analyzed by a modification of the Nelson photometric method (123). The same concentration of hexose in the medium was used as in the experiments reported here. However, these authors placed the isolated diaphragms in chilled KRP for 15" before using in the test system. This might possibly explain why these authors were able to demonstrate an insulin effect at 60" of incubation, whereas in this laboratory the optimal insulin effect was observed at 30" of incubation. It is interesting to note that the uptake of galactose by diaphragms incubated at 38°C. for 1 hr. in KRP was found by Demis *et al* to be 0.62 mg./gm. in one series and 0.56 mg./gm. in another series. In the presence of insulin the uptake was 0.86 mg./gm., in the latter series. If one consults Tables 6 and 7 it will be seen that the uptake of C^{14}-galactose by diaphragms incubated at 38°C. for 30" averaged 0.58 mg./gm.; for 60" incubation, 0.72 mg./gm. In the presence

of insulin, the uptake for 30" incubation averaged 0.71 mg./gm.; for 60" incubation, 0.76 mg./gm.

As already mentioned, Demis et al used non-radioactive galactose and determined the galactose uptake by analyzing an aliquot of the medium before and after incubation. The experiments performed in this laboratory were with C^{14}-galactose plus carrier galactose. The galactose uptake was determined by extracting the tissue and determining its radioactivity. The fact that an approximate quantitative relationship exists between the data obtained via highly divergent methods is indeed heartening.

Haft et al (62) were also recently able to demonstrate an insulin effect on the uptake of non-radioactive galactose by isolated rat diaphragm, incubated in KRP at 37°C. The uptake of galactose by the diaphragm was determined by the rate of the disappearance of galactose from the medium, aliquots of which were analyzed before and after incubation by a modification of the Nelson photometric method (123). The incubation medium used was KRP plus galactose (200 mg. %). They were able to observe a sizeable insulin effect after 30" and 60" of incubation. After 30" incubation, the galactose uptake in the absence of insulin was 0.67 mg./gm.; in the presence of insulin, 1.00 mg./gm. After 60" incubation, the galactose uptake in the absence of insulin was 0.74 mg./gm.; in the presence of insulin, 1.18 mg./gm. After 120" incubation, the galactose uptake in the absence of insulin was 1.26 mg./gm.; in the presence of insulin, 1.37 mg./gm. (the increase with insulin was less than the experimental error). After 240" incubation, the galactose uptake in the absence of insulin was 1.30 mg./gm.; in the presence of insulin, 1.53 mg./gm. (the increase with insulin equalled approximately the experimental error).

How may the galactose uptakes obtained by these workers be reconciled with the values obtained by Demis et al using non-radioactive galactose and by the writer using C^{14}-labelled galactose? During the course of the various experiments it was found in this laboratory that the uptake of galactose by the isolated rat diaphragm muscle is proportional to the concentration of galactose in the medium. Since these authors used KRP containing 200 mg. % galactose and Demis et al and the writer used KRP containing 100 mg. % galactose, dividing the results obtained by Haft et al by 2 would express the galactose uptakes based upon an incubation medium containing 100 mg. %. This procedure seems justifiable in the light of the experiences of the writer. By such recalculation, the results of Haft et al may be considered to be as follows:

	KRP + Galactose	KRP + Galactose + Insulin
30"	0.67 0.34 (100 mg. %)	1.00 0.50 (100 mg. %)
60"	0.74 0.37 (100 mg. %)	1.18 0.59 (100 mg. %)
120"	1.26 0.63 (100 mg. %)	1.37 0.69 (100 mg. %)
240"	1.30 0.65 (100 mg. %)	1.53 0.77 (100 mg. %)

The recalculated values are now seen to be lower than the values obtained by Demis et al and the writer, especially for the 30" and 60"

incubation periods. If one looks at the methods employed by Haft et al it will be seen that the isolated diaphragms are first placed in chilled KRP containing galactose (200 mg. %) and shaken in this chilled medium for 5 to 7 minutes. Then the diaphragms are removed, blotted lightly, and placed in Warburg flasks containing KRP + galactose (200 mg. %) and incubation is carried out at 37°C. The initial aliquot of medium is analyzed at that time. Now, it was demonstrated in section C of Chapter III that isolated diaphragms incubated in KRP containing galactose can take up galactose from the medium at 0°C. or at 10°C. This uptake was quite sizeable. These authors did not take into account any galactose uptake which must have occurred in the chilled medium plus galactose (200 mg. %). If this were done, then their values for galactose uptakes would surely be higher.

In reviewing the in vitro work on galactose uptake by isolated rat diaphragm, it became apparent that the concepts previously obtained from the literature concerning the entry of certain sugars into muscle tissue and the influence of insulin thereon must be modified. An historical review and evaluation of the various concepts of insulin action are given in Chapter I in great detail. To review briefly, Cori and Cori (29) in 1929 found that insulin stimulated the utilization of glucose, but not that of fructose or mannose by eviscerated rats. Since muscle possesses 2 phosphorylating enzymes, glucokinase and fructokinase (150), the authors interpreted this to mean that the prime action of insulin is to influence favorably the phosphorylation of glucose by ATP in the presence of hexokinase. Further work in Cori's laboratory demonstrated that the concentration of ATP in muscle from normal and diabetic animals is not a limiting factor for glucose phosphorylation in the presence of hexokinase (150, 187, 85). Thus, for a long period of time

such a concept of insulin action was widely held and confirming work soon appeared (50, 21, 22).

From 1949 to the present, evidence has accumulated in the literature showing that it is possible to demonstrate an increased uptake of various sugars, in addition to glucose by the action of insulin. This has been demonstrated both *in vivo* and *in vitro*. Such an action of insulin has been demonstrated on galactose (98, 57, 97, 59, 60, 192, 39, 62), fructose (39, 62, 106), mannose (41), l-arabinose (59) and d-xylose (59). The reasons why Cori and Cori (29) and others were unable to demonstrate an insulin effect on fructose and mannose were put forth by Mackler and Guest (106) for fructose and by Drury and Wick (41) for mannose.

The fact that insulin can increase the uptake of galactose by the extrahepatic tissues of eviscerated-nephrectomized animals led Levine, Goldstein and coworkers (98, 57, 97, 59, 60) to postulate that insulin facilitates the transfer of certain sugars into the cells of certain tissues and organs. They argue that since only the liver, kidneys and intestines contain a galactokinase (178, 17, 18, 154) and since these organs were removed, then the extrahepatic tissues cannot transform galactose into glucose with subsequent phosphorylation. Therefore, hexokinase (glucokinase) is not involved in the action of insulin on the uptake of galactose by the eviscerated-nephrectomized preparation.

Since then, Demis and Rothstein (39), Haft *et al* (62) and the writer have demonstrated an influence of insulin on galactose uptake by isolated rat diaphragm muscle. In addition the writer has demonstrated, by means of paper chromatography, that the galactose taken up from the medium by the diaphragm and subsequently extracted by boiling water is galactose. This

would seem to indicate that the galactose which enters into the muscle cell is not phosphorylated. An alternative explanation would be to assume that the galactose taken up by the muscle is phosphorylated at the cell surface and that the compound is broken down after transfer into the muscle cell.

Levine, Goldstein, and coworkers postulate that the action of insulin on the uptake of galactose and certain other insulin-responsive sugars is to allow for the transfer of these sugars from the extracellular space into the intracellular space of certain tissues. In section E of Chapter III, the view was put forth that galactose (and presumably the other insulin-responsive sugars) enters the intracellular space of certain tissues even in the absence of insulin. However, the rate of such transfer is slow. The presence of insulin, then, would accelerate such transfer. Stated differently, one could say that the influence of insulin on the transfer of certain sugars into certain tissues is superimposed upon a process which is going on at an intrinsic rate. This concept was based on the in vitro work performed in this laboratory. It will be recalled that, at temperatures ranging from 0-38°C., the inulin uptake by isolated diaphragm muscle was found to be significantly less than the galactose uptake in the absence of insulin. One may, therefore, postulate that galactose not only enters the extracellular space of muscle but also the intracellular space, even in the absence of insulin. Under the proper in vitro conditions, this rate of transfer is accelerated by the action of insulin.

Additional evidence is available which also seems to indicate that two processes are involved when one studies the uptake of galactose by isolated rat diaphragm muscle and the influence of insulin thereon. One may conceive that galactose enters into the intracellular space of muscle tissues at an

intrinsic rate and that this process may be considered as diffusion across semi-permeable membranes. Such a process may possibly be conceived for all sugar molecules which enter muscle tissues. The factors governing the amount of galactose (or any other permeable sugar) which will enter the muscle tissue, as well as the rate of such entry, would be those encountered in a study of the phenomenon of permeability: size, spatial configuration, polarity, mobility, electrical charge, lipid solubility, etc. of the diffusing molecules; porosity and physico-chemical state of the semi-permeable membrane; temperature and characteristic of the milieu.

That such an underlying process as diffusion through a semi-permeable membrane may exist for the entry of galactose (and the other permeable sugars) into muscle tissue is indicated by the following experimental evidence:

1. It was observed in this laboratory that the uptake of galactose by the isolated rat diaphragm muscle is proportional to the concentration of galactose in the medium; e.g., the uptake of galactose was doubled if the concentration of galactose in the medium was doubled, etc. Also, the maximal concentration of galactose in the muscle tissue, attained either in the presence of insulin or after long-term incubation, represented a volume of distribution approximating total tissue water. In addition, the maximal concentration of galactose in tissue water of the muscle corresponded approximately to the concentration of galactose in the incubation medium. Since galactose is not metabolized by muscle, under these <u>in vitro</u> conditions, the total tissue volume available for galactose represents the difference between the wet and dry weight of the diaphragm preparation. Since the average dry weight of the rat diaphragm was found

to be 24 percent of the wet weight (86 determinations, range 19-28 percent), 76 percent of the wet weight, or 0.76 ml. per gm. diaphragm is available for equilibrating with the galactose in the medium. Since the medium contains 1.0 mg. galactose per ml., it is apparent that maximal distribution of galactose in diaphragm would be equivalent to a galactose content of 0.76 mg. per gm. tissue (range .72-.81 mg./gm.).

2. It was shown in section B of Chapter III that if the isolated rat diaphragm muscle is allowed to incubate for 60" in KRP + C^{14}-galactose at 38°C., instead of 30", then the uptake of galactose in the absence of insulin is almost maximal (total body water distribution). Thus, if insulin is added to the medium, a very slight effect or no effect on galactose uptake is observed. It will also be recalled that Haft et al (62) were able to show an insulin effect on galactose uptake by isolated rat diaphragms after 30" or 60" of incubation at 38°C., but not after 120" or 240" of incubation. There, again, the uptake of galactose was already maximal in the absence of insulin, so that an action of insulin would not be apparent.

3. If a permeable sugar, such as galactose, can readily diffuse into the tissues, then it should be able to diffuse out with equal facility. This was shown to be the case for the galactose taken up by isolated rat diaphragms incubated in KRP plus C^{14}-galactose. These experiments were described in section F of Chapter III.

4. It was shown in section C of Chapter III that the amount of galactose taken up by isolated rat diaphragms for KRP + C^{14}-galactose was least when incubation was carried out at 0°C. The galactose uptake increased with increasing temperatures of incubation with a maximum uptake

at 38°C. This result should be expected if one is dealing with diffusion through a semi-permeable membrane.

5. It has been shown by Walaas and Walaas (187), Demis and Rothstein (39) and the writer that the uptake of glucose, galactose and fructose by isolated rat diaphragm muscle, in the absence of insulin, is unaffected by anaerobiosis. However, insulin action is inhibited in such a system after 15" of anaerobiosis. This was shown to be the case for glucose (187) and glucose, fructose and galactose (39). This inhibition of insulin action could be reversed by returning the diaphragms to aerobic conditions (39).

After 15" of anaerobic incubation, Walaas and Walaas (187) showed that the phosphocreatine of the isolated rat diaphragm disappeared completely and that the level of ATP dropped to 2-3 mg. % of labile P (normal concentration, 17-22 mg. %). Yet the in vitro uptake of glucose by diaphragm muscle incubated under such anaerobic conditions was the same as when incubation was carried out under aerobic conditions. The most striking result was the demonstration that even though no action or only a very slight action of insulin on glucose uptake by isolated rat diaphragms incubated anaerobically could be demonstrated, the uptake of glucose could be doubled by raising the concentration of glucose in the medium from 140 to 420 mg. %. This could be demonstrated both for anaerobic and aerobic incubation.

6. The uptake of galactose by isolated rat diaphragm was shown to be uninfluenced by changes in the ionic composition of the medium. (The incubation medium was always kept isotonic with respect to rat tissues.) The uptake was also uninfluenced by NaF and NaCN added to the medium.

Therefore, the evidence obtained by studying the in vitro uptake of galactose by isolated diaphragm muscle seems to indicate that: 1. galactose is capable of entering the intracellular space of muscle tissue, even in the absence of insulin; 2. if a sufficient period of incubation is allowed, the final volume of distribution would indicate a distribution in total tissue water, even in the absence of insulin; 3. when an action of insulin on galactose uptake is manifested, it is taken to mean that insulin increases the rate of galactose entry into the muscle cells.

Could this concept be applied to other permeable sugars as well? It is possible to consider that each permeable sugar has its own rate of entry, which is governed by the properties of the sugars and those of the semi-permeable membranes involved. Superimposed upon this, a control of insulin on the entry of only certain of these sugars can be demonstrated. Goldstein et al (59) showed that, in eviscerated-nephrectomized preparations, the volume of distribution in the absence of insulin of d-galactose, d-xylose, l-arabinose, d-mannose, d-fructose, l-sorbose, d-arabinose, l-rhamnose and d-sorbitol was essentially the same for all of these sugars. In the presence of insulin and a normal blood glucose, however, only d-galactose, d-xylose and l-arabinose responded by means of an increased volume of distribution. If a higher concentration of blood glucose had been maintained, d-galactose would not have responded to insulin action (192). If the animals had been rendered aglycemic, then fructose would have responded to the presence of insulin (106) as well as mannose (41). Thus, it can be seen that the superimposed mechanism of insulin action demonstrates both specificity with respect to which sugars will be influenced and a competition amongst the insulin-responsive sugars for

this favorable influence of insulin. It is interesting to note that, in this preparation, Goldstein et al (59) observed that the level of blood glucose constantly falls, indicating utilization of this sugar by the preparation in the absence of insulin. They also observed that the responsiveness of a sugar to insulin was not correlated with its utilizability by the preparation (see Chapter I for a complete discussion of this work). Essentially similar inferences may be drawn from the in vitro work of Demis and Rothstein (39), Haft et al (62) and the writer.

The fact that certain tissues, such as the brain and the red blood cells, do not require insulin, do not combine with insulin, and do not respond to the presence of insulin and yet undergo carbohydrate metabolism seems to me to indicate the presence of an independent, pristine mechanism for sugar entry into cells. This is also the case for the embryo. Other tissues, however, have evolved an hormonal complement which can influence this pristine mechanism. If one wishes to be teleological, it is possible to elaborate theories to explain why some tissues developed an hormonal complement while others did not. For instance, when an animal is called upon to fight its enemies or to flee from its enemies, its muscles must be geared for maximum activity and efficiency. It would, therefore, be advantageous to possess a mechanism which would accelerate the entry of sugar into the muscles. On the other hand, since the red blood cells are suspended in a medium laden with glucose, a mechanism to accelerate sugar transfer would not be necessary. On the other hand, in cases of emergency it would seem that the brain would need as much energy substrate as it can possibly take up. However, because of the peculiar blood-brain barrier it would be at a disadvantage if it had to rely on a

mechanism to increase uptake of sugars from the blood and tissue fluid. As a result, the brain tissue became adapted so that maximal uptake of energy substrate occurs in the absence of any superimposed hormonal influence. Whether such teleological reasoning is valid or not, it is presented merely to emphasize that certain mammalian tissues can function utilizing only the underlying process of sugar entry through semi-permeable membranes, without superimposed hormonal complements.

What is the nature of the superimposed influence of insulin on sugar entry? How does the presence of insulin result in an acceleration of transfer of certain sugars into certain tissues, while other sugars and tissues are uninfluenced by the presence of insulin? For example, the presence of insulin may result in an increased uptake of such hexoses as d-glucose, d-galactose, d-mannose, and d-fructose; but insulin will not influence the uptake of certain other hexoses, such as l-sorbose, l-rhamnose, and d-sorbitol (a sugar alcohol). In addition the presence of insulin may result in an increased uptake of such pentoses as d-xylose and l-arabinose, but will not influence the uptake of d-arabinose (See Table 2). This appears to be an unusual specifity. For example, insulin may influence d-xylose and l-arabinose, but not d-arabinose. In addition, it has already been mentioned that a competition exists amongst the insulin-responsive sugars for insulin influence. Such a competition has been shown to exist between d-glucose, d-galactose, d-fructose and d-mannose. Whether such a competition includes the other insulin-responsive pentose sugars has not been demonstrated as yet. The nature of this competition has not been completely worked out. For example, Mackler and Guest (106) demonstrated that when isolated rat diaphragm muscles were incubated in a medium containing equimolar concentrations of

glucose and fructose, insulin increased the uptake of glucose but not of fructose. Similarly, Goldstein et al (59) observed that when the eviscerated-nephrectomized animal is kept aglycemic, mannose will show a greater rate of disappearance from the blood in response to the presence of insulin. If the blood glucose level of the preparation is maintained at a normal level, mannose will not respond to the presence of insulin. Wick and Drury (192) also demonstrated that a high concentration of glucose (800 mg. %) depressed the entry of galactose into the cells of the extrahepatic tissues of eviscerated-nephrectomized animals. A normal blood glucose concentration was shown by Goldstein et al (59) not to interfere with the insulin response to galactose in eviscerated-nephrectomized preparations. Also, it has been shown in this laboratory that when isolated rat diaphragms are incubated in KRP containing both 100 mg. % glucose and 100 mg. % galactose, the uptake of galactose and the action of insulin on the galactose uptake are not interfered with (see Table 39) in 3 out of 4 animals.

In an attempt to characterize the mechanism of insulin action, the specificity of action with respect to the target sugars and the competition amongst these sugars for insulin action has been noted. What additional properties may be ascribed to insulin action? It has already been noted that the uptake of glucose, galactose and fructose by isolated rat diaphragm muscle, in the absence of insulin, is unaffected by prolonged anaerobiosis. However, insulin action on these sugars is reversibly inhibited by prolonged anaerobiosis. It has also been noted that after 15" of anaerobic incubation, the phosphocreatine of the isolated rat diaphragm disappeared completely and that the level of ATP dropped to

Table 39

Effect of Insulin on Galactose Uptake by Isolated Diaphragms From Normal, Fasted Rats. Incubated 30" in KRP plus C^{14}-Galactose (100 mg. %) and Glucose (100 mg. %) at 38°C. O_2-Gas Phase. pH 7.4

Rat #	Galactose uptake, mg. per gm. wet wt. tissue per 30" of incubation		
	In KRP + Galactose + Glucose	In KRP + Galactose + Glucose + Insulin (0.27 U./ml.)	% Insulin Effect
1	0.53	0.72	36
2	0.63	0.71	13
3	0.60	0.61	2
4	0.58	0.65	12
	Avg. 0.59 mg./gm.	0.67 mg./gm.	16%

S. E. M. = $\pm$ 0.02; p = $< .05$

2-3 mg. % of labile P (normal concentration, 17-22 mg. %). This would, then, indicate a very much reduced formation of high-energy phosphate containing compounds by the muscle tissue under anaerobic conditions. This consideration, together with the reversible nature of this insulin inhibition, suggested the possibility of an altered oxidative-phosphorylation by muscle tissue incubated under anaerobic conditions.

Therefore, the effect of 2,4-dinitrophenol on the uptake of galactose by isolated rat diaphragms was studied. Dinitrophenol is used extensively as an uncoupler of oxidation and phosphorylation (104, 36, 82, 176, 78, 184, 194, 195). The literature on this subject is extremely voluminous. It will be recalled that DNP, at a concentration of 2×10^{-4} M, was found to inhibit the action of insulin on the uptake of galactose by isolated rat diaphragm muscle incubated for 30" at temperatures ranging from 0°C. to 38°C. It was also observed that addition of ATP to the medium did not reverse the inhibition of DNP. A 2×10^{-4} M concentration of DNP was found to depress the O_2-consumption of the isolated rat diaphragm muscle. A 2×10^{-6} M concentration of DNP, for a period of 30" of incubation, either had no effect or a stimulatory effect on O_2-consumption. At the latter concentration of DNP, it was seen that insulin is capable of increasing the galactose uptake by isolated rat diaphragm muscle.

Sacks et al (144) studied the effects of DNP on isolated rat diaphragm muscle incubated for 1 to 2 hours in KRP plus glucose (100 or 200 mg. %) at 38°C., with O_2 as the gas phase. The following effects of DNP on respiration of diaphragm muscle were observed:

a. 2×10^{-5} M = marked increase in O_2-consumption;

b. 1×10^{-4} M = initial increase, followed by considerable depression

of O_2-consumption;

c. 2×10^{-4} M = slight transitory stimulation, followed by a marked and progressive depression of O_2-consumption.

Even though the incubations were carried out only for 30", essentially similar results were obtained in this laboratory, using KRP plus C^{14}-galactose (100 mg. %).

Sacks et al (144) also found that at all of the concentrations of DNP used there was a complete disappearance of phosphocreatine from the muscle and a marked reduction in the content of ATP. In addition, at a concentration of 1 to 2×10^{-4} M of DNP, there was a decreased turnover of ATP in the incubating diaphragm muscle.

These effects of DNP on the isolated, incubating rat diaphragm remarkably resemble the effects of prolonged anaerobiosis on isolated rat diaphragm (187). It will be recalled that prolonged anaerobiosis reversibly inhibits insulin action on the uptake of glucose, galactose and fructose by the isolated rat diaphragm. It is also interesting to note that it has been demonstrated that the action of DNP in uncoupling oxidative-phosphorylation in cell particulates can be reversed by washing the DNP away (104). Whether the inhibitory action of insulin on galactose uptake by isolated rat diaphragms, as observed in this laboratory, can be reversed by washing the DNP away is difficult to determine because DNP combines firmly with rat diaphragm muscle tissue (164).

What are the effects of DNP on glucose utilization by isolated rat diaphragm muscle? It is difficult to interpret effects on glucose utilization, since two sets of phenomena are involved: 1. glucose uptake by the tissue; 2. metabolic fate of such sugar by the tissue.

Stadie et al (164) tested the effect of DNP on glycogen synthesis from glucose by isolated rat diaphragm incubated in KRP plus glucose. When DNP was added to the medium (10 gamma/ml.), Stadie found an increase in the O_2-consumption and a decrease in the glycogen synthesis.

Villee et al (184) incubated isolated rat diaphragm muscle in KRP plus glucose containing various concentrations of DNP, in the presence and absence of insulin. They observed that with increasing concentrations of DNP the glucose utilization was decreased, the formation of glycogen was inhibited and the effect of insulin on glucose utilization was decreased to a negligible amount. When ATP (1×10^{-3} M) was added to the incubation medium, the effects of DNP were not changed. It is also of interest here to mention that Haft and Mirsky (64) determined the effect of pentobarbital, added to the incubation medium, on isolated rat diaphragm muscle. They found that with increasing concentrations of pentobarbital, respiration was depressed, the glucose uptake was increased, the glycogen concentration was decreased, and the action of insulin on glucose utilization was progressively inhibited. One of the hypotheses formulated by these workers is that the concentrations of pentobarbital which depress respiration might also inhibit oxidative phosphorylation. It has been shown, however, by Eiler et al that pentobarbital inhibits both respiration and phosphate by rat brain homogenates proportionately. Therefore, the P:O ratio remains fairly constant (42).

Although there is a striking resemblance between these effects of DNP on glucose utilization and the effects of DNP on galactose uptake, it would be unwise, at this time, to make any correlations. It is difficult to ascertain whether a certain effect of a substance is due to its action on glucose uptake or on glucose utilization or on both.

As previously mentioned, a tremendous amount of work on the action of DNP has been performed. However, most of this work has been done using tissue homogenates, cell-particulates and mitochondrial preparations. It is difficult, as well as dangerous, to refer results obtained with such preparations with results obtained with tissue slices or whole animals, and vice versa. When a suitable correlation seems to exist, one is tempted to make use of it. When a suitable correlation does not seem to exist, then one is comforted by the axiom that one should not equate results obtained from one type of preparation with those obtained from another type. With respect to possible mechanisms of insulin action, both situations frequently arise.

Clowes et al (24) showed that DNP inhibited growth, cleavage and differentiation in sea urchin eggs. The effects of DNP on isolated rat diaphragm muscle have already been discussed (144). It has been shown that DNP inhibits acid secretion by mouse stomachs without affecting O_2-consumption (37). It has been shown that DNP inhibits K accumulation by surviving kidney slices (120). Fuhrman has shown that DNP inhibits active sodium transport in the isolated frog skin (49). These are just a few of the countless and varied isolated experiments involving the action of DNP. A concerted and extensive attempt to ascertain the action of DNP on oxidative-phosphorylation has been carried out using tissue homogenates, cell-particulates and mitochondrial preparations. A few results of such a concerted effort will be discussed now; the purpose being to try to correlate, if and when possible, such results with the results obtained on the influence of DNP on insulin action. The concensus of opinion seems to be that DNP influences oxidative phosphorylation, which is a cellular

mechanism whereby high-energy phosphate bond regeneration is coupled with oxidative reactions. It has already been mentioned that DNP has been shown to be able to reversibly uncouple phosphorylation from oxidation (104, 36, 82, 176, 78, 184, 194, 195). In studying the action of DNP on kidney homogenates, it was demonstrated that DNP at concentrations ranging from 5×10^{-5} to 2×10^{-4} M prevented phosphorylation by the test system. Oxidation of the substrate, sodium glutamate, however, was unaffected or slightly stimulated (104). It has already been mentioned that this action of DNP can be reversed by washing out the DNP with fresh medium (104). These workers also found that the respiration of such kidney homogenates was inhibited with high concentrations of DNP. In general, working with particulate fractions of tissues, the presence of DNP usually results in a significant drop in P:O ratios when members of the tricarboxylic acid cycle (Krebs Cycle) are oxidized. The P:O ratio is the ratio of synthetic organic phosphorylation reactions to oxidative reactions. Since the rate of oxidation of the members of the Krebs Cycle is either unaffected or slightly stimulated (104, 36, 79, 5), the level of ATP in the system falls (194). This is usually explained as the result of inhibition of resynthesis of adenine nucleotides via the transphosphorylation reactions of oxidative phosphorylation (194). However, the fall of the level of ATP in the systems could overtly resemble an increased rate of ATP breakdown. In fact it has been shown that the ATPase activity of fresh rat liver mitochondria, in which case the ATPase activity is very low, can be greatly enhanced by DNP (93). This was also found to occur, using a total brei of the toad embryo (111). In washed particles, DNP also activates phosphatases (194). These authors

believe that an activation of phosphatases or of an ATPase plays a secondary role in uncoupling oxidative phosphorylation. Their reasoning is as follows: DNP reversibly inhibits fatty acid oxidation by particulate fractions of tissues; the addition of ATP to the system does not reactivate fatty acid oxidation. The inhibitory action of DNP on fatty acid oxidation in particulate system is not demonstrable in liver slices. No explanation has been given to explain this phenomenon (43, 55). The inhibition of fatty acid oxidation of DNP in particulate systems is believed to be due to a lack of energy-rich P bonds necessary to maintain the reactions, rather than an inhibition of fatty acid oxidases by DNP (36). These investigations may, in the future, shed light on the derangements of fatty acid metabolism found in the alloxan-diabetic mammal.

These actions of DNP on cell-particulates have been shown only when the mitochondria of the preparations are allowed to remain intact. Possibly, the reason for this is that most of the enzymes which catalyze the Krebs Cycle and fatty acid oxidation are localized in the mitochondria (70, 80, 81). This also seems to apply for the ATPase and phosphatases activated by DNP (194).

Finally, some authors have found no effect of insulin on oxidative phosphorylation in cell-particulates. On the other hand, Polis et al (136) found that insulin increased the amount of inorganic phosphate esterified by the "cyclophorase system" coupled with α-ketoglutarate oxidation. No stimulation of O_2-consumption in the presence of insulin could be observed. The insoluble residue of rat liver homogenates comprises the "cyclophorase system". Judah et al (78) found that DNP does not inhibit the hexokinase reaction in cell-particulate systems.

Therefore, Polis et al (136) assume that insulin increased high-energy phosphate bond formation via the aerobic oxidation of α-ketoglutarate. These authors also demonstrated that the addition of an adequate amount of insulin to the test system reversed the inhibition of O_2-uptake and inorganic phosphate esterification produced by the addition of MgATPase.

The mechanism of DNP action has not yet been elucidated. Loomis and Lipmann (104) found that DNP could replace inorganic phosphate for the oxidation of sodium glutamate. The test system contained rabbit kidney homogenate in buffer; yeast hexokinase; $MgCl_2$; NaF; AMP; phosphate buffer, pH 7.2; fructose; and sodium glutamate as substrate. Inorganic phosphate must be present in this system, if sodium glutamate is to be oxidized. The addition of DNP obviates this requirement. It was also found that the oxidation reactions of the complete system was slightly stimulated by DNP. If inorganic phosphate was removed, the oxidative reactions by this system was greatly stimulated by DNP. It was later found by Lipmann et al (101) that DNP also can replace the adenylate (AMP) required for oxidation reactions by this system. These findings have been confirmed (78).

From the discussion of the actions of DNP it is also possible to conceive that DNP could eliminate the inorganic phosphate and adenylate requirements by causing the immediate breakdown of primary phosphorylated intermediates. This would result in a continuous release of inorganic phosphate, with no energy-rich P bonds being transferred to the ATP system via AMP. Overtly, however, this would appear as if phosphorylation was uncoupled from oxidation.

Finally, in employing a test system such as that used by Loomis and

Lipmann (104), Mg^{++} ions must be added. Mg^{++} ions are necessary for the overall reactions of pyruvate oxidation coupled with phosphorylation. This may be due to certain dehydrogenases requiring Mg^{++} ions. For example, Mg^{++} ions are required by pyruvate dehydrogenase (100) and by α-ketoglutaric dehydrogenase (125). It has also been found that inorganic phosphate is necessary for complete oxidation of pyruvate (124).

The inhibition of insulin action by DNP on galactose uptake by the isolated rat diaphragm and on glucose utilization by isolated rat diaphragms (184) has been discussed. The reversible inhibition of insulin action by prolonged anaerobiosis on glucose uptake (187) and on glucose, fructose and galactose uptakes (39) by the isolated rat diaphragms has also been noted. It has also been demonstrated that both anaerobiosis (187) and DNP (144) result in a complete disappearance of phosphocreatine from rat diaphragm muscle and in a very marked reduction in the concentration of ATP. The similarity is further strengthened by the reversibility of the inhibition by anaerobiosis of insulin action by returning the diaphragm to aerobic conditions (39), and by the reversibility of the effects of DNP on cell-particulates (104). This would be difficult to establish with diaphragm muscle, since DNP firmly combines with rat diaphragm tissue (164). It has also been mentioned that DNP does not inhibit the hexokinase reaction in cell particulates (78), nor does it inhibit yeast hexokinase (101). Finally, ATP added to the medium did not reverse the DNP inhibition of insulin action on galactose uptake by isolated diaphragms. Also, ATP did not reverse the DNP inhibition of insulin action on glucose utilization (184). Finally, ATP does not reactivate the inhibition of DNP of fatty acid oxidation in particulate systems.

As a result, it might be possible to postulate that coupled oxidative-phosphorylation is necessary for an action of insulin on galactose uptake by isolated rat diaphragm muscle. Such may also be the case for glucose uptake by the isolated rat diaphragm. Further work is necessary before oxidative phosphorylation can be definitely implicated with a mechanism of insulin action. Other substances known to inhibit respiratory phosphorylation should be investigated. Such substances are, in addition to DNP, azide, arsenate, arsenite, ethyl carbylamine and sodium nitrite (78). It has also been observed that sodium azide counteracted the activation of ATPase by DNP on a total brei of the toad embryo (111) and thus acted as an inhibitor of ATPase. This worker also found that DNP stimulated the O_2-consumption of intact toad embryoes, and that this stimulation was inhibited by azide. Sodium azide was also found to inhibit the ATPase activity of extracts and homogenates of sarcoma, brain and some other organs by about 40% (115). A few preliminary experiments, testing the effects of sodium azide on DNP inhibition of insulin action on the galactose uptake by isolated rat diaphragm muscle, were performed. The results seem to indicate that sodium azide does not release the inhibition of DNP on insulin action in this test system. Interpretation of such results must be kept in abeyance until the effects of sodium azide, and the other compounds mentioned, on this test system have been worked out more fully.

It is possible for one to postulate other mechanisms whereby DNP might inhibit insulin action. Since it has been shown that both insulin (165, 166, 65, 69, 162, 66, 67) and DNP (164) combine firmly with rat diaphragm muscle tissue, is it possible that DNP competes with insulin

for such sites of combination? If DNP can combine with such tissue sites more readily than insulin, this could conceivably explain the inhibition of insulin action by DNP. It can be seen from the work of Stadie et al (167, 165, 166, 65, 69, 162, 66, 67, 158, 160) that probably insulin must combine with tissues before an action on sugar uptake can be manifested. Conversely, such tissues as brain and red blood cells, which do not respond to insulin, have been found to be unable to combine with insulin (167, 67). It may be possible to test this postulate by means of labelled insulin and DNP. This hypothesis however would seem difficult to accept, since Stadie et al (164) found that DNP resulted in an increased O_2-consumption and in a decreased glycogen formation by isolated rat diaphragm incubated in KRP plus glucose in the absence of insulin.

Another possible explanation for the inhibition of insulin action by DNP could be a decreased permeability of the muscle tissues, as a result of the complete breakdown of phosphocreatine and the almost complete disappearance of ATP. Villee et al (184) found that, even in the absence of insulin, high concentrations of DNP resulted in a decreased glucose utilization (measured as uptake from medium), an inhibition of glycogen formation and a stimulation of glucose oxidation. This stimulation of glucose oxidation might be interpreted as a consequence of the phosphocreatine and ATP breakdown, resulting in a stimulation of oxidative reactions. In the experiments carried out in this laboratory, the uptake of galactose by diaphragm muscle incubated in the presence of DNP was not decreased or was only very slightly decreased. However, when the diaphragm tissue was removed from the flask containing DNP, it had an appearance which would suggest that of contracture.

It will be recalled that an action of insulin on galactose uptake by diaphragm muscle may be demonstrated, under certain in vitro conditions, at incubation temperatures of 38°C., 27°C., and 20°C. The percent insulin effects were found to be 23%, 27% and 23%, respectively. This would indicate that the action of insulin, in this test system, is both consistent and maximal at incubation temperatures ranging from 20°C. to 38°C. When incubation was carried out at 10°C. or at 0°C., no influence of insulin on galactose uptake by the diaphragm muscle could be demonstrated. In all of the above experiments, the only condition which was changed was the temperature at which incubation was carried out. It was then shown that the lack of insulin action on galactose uptake at 0°C. and 10°C. cannot be due, primarily, to an inability of muscle tissue to combine with insulin at such low temperatures. A possible explanation may be that the diffusion of galactose into the muscle tissue, at such low temperatures, progresses at such a slow rate that the influence of insulin, thereon, cannot be made apparent. Such an explanation might be valid at 0°C. where the galactose uptake is quite low. However, the galactose uptake in the absence of insulin at 10°C. is only 10% lower than the uptake at 20°C. Yet, no action of insulin can be demonstrated at 10°C., while a maximum insulin action may be demonstrated at 20°C. A possible explanation would be to consider that, at such low temperatures as 0°C. and 10°C., the oxidative-phosphorylation reactions or other reactions necessary for insulin action are sufficiently slowed down to prevent insulin from acting on galactose uptake by the diaphragm muscle.

Finally, in all of the work done on the mechanism of insulin action, not one clue exists which would shed light on the mechanism of the

specificity of insulin action or on the competition for insulin action by the responsive sugars. The exact definition of the mechanism(s) of insulin action in mammalian processes is still elusive. That this is the case, despite the tremendous amount of work on the subject, only emphasizes the complexity of trying to analyze the mechanisms of action of a substance which has been shown to influence carbohydrate, fat, protein and mineral metabolism. With the much simplified test system employed in this study, it is hoped that the data obtained will shed some additional light on the subject.

In Chapter I, the influence of certain hormones on carbohydrate metabolism has been discussed. These hormones included those from the anterior pituitary, adrenal cortex, and adrenal medulla. The majority of the work concerning the interplay of hormones has revolved around glucose utilization. With the test system employed in this laboratory, it would be possible to determine whether these hormones also influence the uptake of a sugar which is, for all intents and purposes, not utilized by the preparation. The same applies for the many other factors, both known and unknown, which seem to influence glucose utilization. For example, Lang, Goldstein and Levine observed that the glucose utilization of the peripheral tissues of eviscerated dogs is approximately half that exhibited by the same tissues of the intact dog (92). It was shown, in this laboratory, that the galactose uptake of isolated rat diaphragm muscle obtained from eviscerated-nephrectomized rats was the same as that shown by normal diaphragm tissue. In addition, the response to insulin was the same in both cases. These results were obtained with incubation carried out at 38°C. and at 22°C. It is hoped that such an approach will

prove fruitful in an attempt to characterize insulin action.

B. Summary

The evidence presented seems to indicate that two processes are involved when one studies the uptake of galactose by isolated rat diaphragm muscle and the influence of insulin thereon. These two processes are:

a. An entry of galactose into the muscle cells, independent of insulin, which is not affected markedly by temperature, anaerobiosis or DNP.

b. An entry of galactose into the muscle cells which requires insulin and is markedly influenced by temperature, anaerobiosis and DNP.

The evidence presented demonstrates that insulin increases the rate of galactose entry into the muscle cells.

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STUDIES ON REGULATORY FACTORS IN CARBOHYDRATE METABOLISM, WITH ESPECIAL REFERENCE TO THE MECHANISM OF INSULIN ACTION

Abstract of a Dissertation

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Introduction

It is now generally recognized that insulin facilitates the transfer of certain sugars through cytostructural barriers. If the transfer of a utilizable sugar into the cell is affected by a rate-limiting reaction, then all subsequent enzymatic reactions of this sugar in the cell would be dependent upon this reaction.

This study has involved an investigation of factors which regulate the transfer of sugar into muscle cells.

Methods

Since sugar transfer can best be studied by use of a sugar which is not appreciably metabolized by muscle tissue, the transfer of galactose into isolated rat diaphragm muscle was studied. Galactose, an isomer of glucose, was selected for study because muscle tissue does not contain any galactokinase. This was demonstrated by an inability to detect any disappearance of galactose from a test system during the course of a balance study.

Hemidiaphragms obtained from fasted, male, white rats were incubated with 2.0 ml. Krebs-Ringer phosphate (KRP) medium containing 100 mg. per cent C^{14}-galactose, at pH 7.4. The extent of galactose uptake was determined after incubation by measuring the radioactivity present in the muscle tissue. All of the tissue radioactivity was found by paper chromatography to be due to unmodified galactose. All results were reported in terms of milligrams of galactose taken up from the medium per gram wet weight diaphragm muscle per period of incubation.

Time-Course of Galactose Uptake, With and Without Added Insulin, by the Isolated Diaphragm Muscle

Since galactose is not metabolized by muscle under these in vitro conditions, the total tissue volume available for galactose represents the difference between the wet and dry weight of the diaphragm preparation. Since the average dry weight of rat diaphragm muscle was found to be 24% of the wet weight (86 determinations, range 19-28%), 76% of the wet weight, or 0.76 ml. per gm. diaphragm muscle, is available for equilibration with the galactose in the medium. Since the medium contains 1.0 mg. galactose per ml., it is apparent that maximal distribution of galactose in diaphragm muscle would be equivalent to a galactose content of 0.76 mg. per gm. tissue (range 0.72 to 0.81 mg./gm.).

The galactose uptake at time intervals ranging from 15 to 60 minutes, both in the presence and absence of added insulin, was determined at 38°C. It was observed that maximal galactose uptake in the absence of insulin had not yet occurred at 60 minutes. In the presence of insulin, galactose penetration had leveled off at 30 minutes of incubation and was close to the theoretical maximum. Since galactose penetration proceeds in the absence of added insulin, it is clear that the action of insulin is best evidenced early in the incubation period (i.e., at 15 or 30 minutes) rather than at 1 hour. Thus the insulin effect at 15 and 30 minutes is 26 and 23%, respectively, while at 60 minutes the insulin effect is only 6%. These results were found to be statistically significant.

Effect of Temperature Upon Galactose Entry Into the Diaphragm Muscle Cells, With and Without Added Insulin

Inasmuch as insulin action upon the transfer of galactose can be evidenced at 30 minutes of incubation, this period of incubation was chosen to test a variety of factors. Accordingly, isolated hemidiaphragms

were incubated in KRP for 30 minutes, with and without added insulin, at temperatures ranging from 0 to 38°C. in order that the influence of temperature might be studied. It was observed that the effect of insulin on the transfer of galactose into muscle cells is remarkably sensitive to temperature in that no effect is obtainable between 0 and 10°C., and that at 20°C. the effect appears to be maximal.

The possibility that insulin does not act because of a primary inability of muscle to combine with insulin at low temperatures has been ruled out by the following experiments. Hemidiaphragms were incubated with insulin at 38° or 24°C. and then transferred to KRP plus galactose. Incubation was then carried out for 30 minutes at 10° or 0°C. No insulin effect upon galactose uptake was observed.

In marked contrast, the process which allows galactose to penetrate in the absence of insulin is, on the basis of our data relatively insensitive to temperature, as expected from a consideration of the effect of temperature upon a diffusion process.

Characteristics of Insulin Action

It was demonstrated that the uptake of galactose by isolated diaphragm muscle involves the entry of this sugar into both the extra- and intracellular components of the muscle tissue. This was based upon measurements of the extracellular volume of the diaphragm muscle tissue by means of inulin. It was found that during 30 minutes of incubation of diaphragm muscle in KRP plus galactose, at temperatures ranging from 0 to 38°C., the galactose not only enters the extracellular component but also the intracellular component of the muscle tissue. This occurs even in the absence of insulin. The rate of such entry is markedly accelerated

by the action of insulin.

The effect of insulin on the rate of entry of sugar into the muscle cells may be due to either a direct action on the rate of galactose entry per se, or a reduced rate of galactose exit from the cells. That the latter factor is not important is suggested by the observation that the galactose which entered the muscle during incubation can very readily diffuse out of the tissue when the tissue is incubated in a medium containing no galactose.

The Effect of Varying the Ionic Composition of the Medium on Galactose Uptake

Preliminary experiments indicate that the absence of K, Ca, Mg or Mg plus Ca ions from the incubation medium is without influence upon galactose entry in either the presence or absence of insulin. These data suggest that either these ions are not involved in galactose transfer or that sufficient amounts of such ions are already associated with the tissue cells.

The Effect of Several Muscle Constituents on Galactose Uptake by Isolated Diaphragm Muscle

Galactose uptake, with or without insulin, was found not to be significantly influenced by ATP, AMP or creatine hydrate added to the incubation medium.

The Effect of Several Inhibitors and Anaerobiosis on Galactose Uptake by Isolated Diaphragm Muscle

It was observed that galactose entry is not influenced in either the absence or presence of insulin by sodium cyanide or sodium fluoride at concentrations of 1×10^{-4} M. 2-4, Dinitrophenol and dinitronaphthol were

found to have a marked effect on insulin action. At a concentration of 2×10^{-4} M, these nitro compounds completely abolish insulin action upon the uptake of galactose by isolated diaphragm muscle.

It has been reported that prolonged anaerobiosis inhibits the action of insulin on galactose uptake by isolated diaphragm muscle. Experiments were performed which demonstrated that there is still an insulin effect with shorter periods of anaerobiosis. Quantitatively, however, the insulin response is much smaller.

Summary

The evidence obtained by studying the uptake of galactose by isolated diaphragm muscle in vitro seems to indicate that galactose is capable of entering the intracellular component of muscle tissue even in the absence of insulin. If a sufficient period of incubation is allowed, the final volume of distribution of galactose approximates that of a distribution in total tissue water, even in the absence of insulin. When an action of insulin on galactose uptake is manifested, it is taken to mean that insulin increases the rate at which galactose enters into the intracellular component of the muscle tissue.

It was also demonstrated that galactose entry into muscle tissue, in the absence of insulin, is not affected markedly by temperature, anaerobiosis or DNP. However, the action of insulin to facilitate galactose entry into muscle tissue is inhibited by low temperatures, prolonged anaerobiosis and DNP.

Possible explanations for these findings have been elaborated.

Autobiography

Candidate: Oscar Resnick

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Schools and
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Educational Positions:

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