REGULATION OF SUGAR TRANSPORT IN CARDIAC MUSCLE: THE EFFECTS OF OUABAIN AND FREQUENCY OF CONTRACTION.

A Thesis Presented to
The University of Manitoba

In Partial Fulfillment
of the Requirements for the Degree of
Master of Science

Just Elbrink

1969

ACKNOWLEDGEMENTS

I would like to thank Dr. Ivan Bihler for his guidance and helpful comments throughout the course of this investigation. Dr. Leslie E. Bailey has provided valuable assistance in the preparation of the manuscript for which I am most grateful.

I also wish to thank Mr. S. Vivian for his assistance in the statistical analysis, Mrs. Bernice Cook for her help in the laboratory work and Mr. R. Simpson for preparation of the illustrations.

The chemical analyses carried out by Mr. Roy Joseph and the help of Mr. J. Kiekush and his staff with respect to the animals is much appreciated.

ABSTRACT

Sugar transport in skeletal muscle is increased by such factors as contraction and ionic changes which follow the inhibition of the sodium pump by cardiac glycosides. Studies have been carried out to determine the effects of inotropic and toxic concentrations of ouabain and the frequency of contraction on the transport of non-metabolizable L-arabinose in rabbit ventricular muscle *in vitro*. Hearts were perfused by a modified Langendorff technique and were stimulated electrically at various rates or were made quiescent by cauterization of the AV-node.

The transport of L-arabinose in quiescent hearts and hearts stimulated at 180 beats/min was similar and was only increased at high frequencies of contraction (240 beats/min). An inotropic concentration of ouabain (5×10^{-7} g/ml) known to increase calcium influx, did not affect transport in quiescent hearts, increased it significantly in hearts stimulated at 180 beats/min but caused no further increase when the stimulation rate was increased to 240 beats/min. Toxic concentrations of ouabain ($0.9\text{-}1.0 \times 10^{-6}$ g/ml) which inhibit active cation transport, increased sugar penetration in both quiescent and stimulated hearts. No relationship could be demonstrated between sugar penetration and the rate of flow or the breakdown of high energy compounds.

These results suggest that ouabain increases

sugar penetration in the heart by a dual mechanism: (1) the effect of inotropic concentrations is beat-dependent and is linked to an increase in the strength of contraction, (2) the effect at high concentrations is linked to inhibition of active ion transport, a relation earlier demonstrated in skeletal muscle.

It is postulated that the activity of the sugar-carrier system in the heart is regulated by different mechanisms in systole and in diastole. In systole, the regulation of the carrier system is contraction-dependent and may involve changes in an intracellular calcium pool which is also involved in the activation of the contractile mechanism. In diastole, sugar penetration is inversely related to the activity of the sodium pump as demonstrated in skeletal muscle and may involve changes in the same calcium pool.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SECTION I

INTRODUCTION.

CARRIER HYPOTHESIS

In muscle, the rate-limiting step in the utilization of sugars is their transfer across the cell membrane as first proposed by Levine and his associates (1, 2). For metabolizable sugars, the uptake process can be divided in three stages. Diffusion across the capillary wall and the extracellular space is followed by the actual transfer across the cell membrane; in the third stage, the sugar is phosphorylated by the energy-requiring hexokinase system. Capillary transfer and diffusion across the extracellular space were found not to be rate-limiting by determinations of sorbitol distribution. This sugar alcohol can be used as an extracellular marker as it does not penetrate into the cells; its distribution will reflect the extracellular penetration of glucose. The half-time for sorbitol diffusion was found to be less than one minute and the time curves for penetration for both sorbitol and glucose were parallel (Morgan, et al., 3). Also, the concentration of glucose in the extracellular space of muscle is similar to the concentration of glucose in the blood plasma (Park, et al., 4). However, capillary and extracellular transfer can become rate-limiting <u>in vitro</u> when the substrate concentration is low. In the perfused rat heart, Morgan, et al., (3) found that in the presence of insulin the intracellular penetration of glucose was decreased by as much as 30% when the perfusate concentration was about 3mM. Bihler, Cavert and Fisher (5),

working with isolated perfused rabbit hearts, found that at perfusate concentrations below 3.5 mM of L-arabinose, the intracellular penetration of this sugar did not conform to the carrier model. Under conditions where penetration is increased, such as the presence of insulin, the hormone does not act by changing capillary and extracellular diffusion because its effect is still present in the lens of the eye where capillary transfer does not take place (Ross, 6).

Diffusion has to be excluded as the transfer process across the cell membrane and a carrier mechanism has been postulated to explain the transfer of the water-soluble sugars across the mainly lipid membrane barrier (Fisher and Lindsay, 7; Bronk and Fisher, 8). In this hypothesis, sugar molecules are thought to combine with a membrane component, the carrier, of which only a limited number are available at the outer membrane surface. Only sugars with a specific stereochemical configuration can attach to these carriers and can be transported across the cell membrane; the so-called 'responsive' sugars must have the same configuration on carbons one, two and three as D-glucose (Goldstein, et al., 9). This sugar-carrier complex diffuses across the cell membrane and at the inner cell surface, dissociation of the complex will take place. Efflux occurs by the same mechanism so that the carriers are available alternately at the outer and inner surfaces of the cell membrane. Evidence for this type of carrier-

mediated facilitated diffusion has been found in erythrocytes (Widdas, 10; Reinwein, Kalman and Park, 11), skeletal muscle (Randle and Smith, 12, 13) and myocardial muscle (Morgan, et al., 3). The carrier mechanism in muscle must have additional features to allow for its regulation by such factors as exercise and hormones.

Several lines of evidence favour the hypothesis. The hyperbolic time curve for penetration can only be satisfactorily explained by the Michaelis-Menten equation for enzyme-catalyzed reactions. The Michaelis-Menten theory assumes the formation of an intermediate enzyme-substrate complex. The dissociation of this complex into the free enzyme and the reaction products is the rate-limiting step in enzyme-catalyzed reactions. In terms of the carrier mechanism, the combination of the sugar with the carrier is indicated by the slope of the rising phase of the time curve, which is dependent upon the affinity of the sugar for the carrier. The plateau phase indicates complete saturation of the carrier. The mobilities of the free carrier and the sugar-carrier complex were thought to be identical but this assumption is now under reconsideration. Competition experiments also show compatibility with the proposed carrier mechanism (Reinwein and Park, in Park, et al., 14). When pairs of sugars with the required stereochemical configurations are added to the incubation medium, both will compete for attachment to the carrier and the one

with the greater affinity will be inhibited less than the sugar which has a smaller affinity for the carrier. The affinity of the sugar molecules for the carrier is expressed as the Km, the substrate concentration at which half-maximal velocity of cellular penetration is obtained.

The temperature coefficient for cellular penetration is too large to be explained by simple diffusion alone. This ratio of the rate of cellular penetration at a given temperature compared to the rate at a temperature ten degrees lower should be slightly higher than one for diffusion. Values of about two to three, however, are generally obtained (Park, et al., 14).

The definitive evidence is given by the occurrence of countertransport by which competition between pairs of sugars can lead to transport of one of those sugars against its concentration gradient without the expenditure of energy (Morgan and Park, 15). After perfusing an isolated heart with a non-metabolizable sugar till equilibrium is obtained, influx equals efflux, the addition of a metabolizable sugar to the perfusion medium will decrease the intracellular concentration of the non-metabolizable sugar which must leave the cells against its concentration gradient. This phenomenon can only be explained by the carrier mechanism. At the outer cell membrane surface, the two sugars will compete for the carrier while at the inner membrane surface, only the accumulated, non-phosphorylated sugar will be

available for transport. This active, uphill transport takes place without the expenditure of metabolic energy since the concentration gradient, which is set up by the addition of the metabolizable sugar to the perfusate, supplies osmotic energy for this process. Counter-transport has also been demonstrated in vivo (Goldstein, in Park, et al., 14).

The above lines of evidence favour the acceptance of the carrier mechanism for the transfer of sugars across the cell membrane.

FACTORS AFFECTING INTRACELLULAR SUGAR PENETRATION

The penetration of sugars in muscle tissue can be increased from the basal level under different physiological conditions which must affect the rate-limiting step to sugar penetration; other steps in the overall uptake process could then, of course, become rate-limiting. The effects of insulin and anoxia have been extensively investigated in isolated perfused rat hearts (Morgan, Randle and Regen, 16; Park, et al., 14) and in skeletal muscle (Randle and Smith, 12, 13, 17) and similar results have been obtained in the two types of muscle. Physiologically, insulin is the main cause for the fall in blood sugar in the postprandial state and it has been shown that insulin acts directly on the cell membrane (Levine, 1, 2).

Under anoxic conditions, when energy is supplied only by anaerobic glycolysis, sugar penetration is also in-

creased. However, this is not due to a nonspecific increase in cell membrane permeability since in anoxia, sorbitol is still excluded from the cells, insulin is still effective in increasing sugar penetration and competition between pairs of sugars still takes place (Morgan, et al., 16). Insulin and anoxia increase sugar penetration to the same extent. With insulin, however, free metabolizable sugar will accumulate within the cell, suggesting that phosphorylation has now become the rate-limiting step since glucose-6-phosphatase is not present in muscle tissue. In anoxia, the equal increase in sugar penetration must be accompanied by an increased rate of phosphorylation since there is no intracellular accumulation of sugar; cell membrane transport still remains rate-limiting. When insulin is present under anaerobic conditions, sugar penetration is even further increased and since no free sugar accumulates within the cells, the activity of hexokinase must have been increased by anoxia and cell membrane transport remains the rate-limiting factor in utilization.

In the absence of substrate, anaerobic incubation of brain slices will decrease the cellular utilization of glucose upon its subsequent addition to the incubation medium (Dickens and Greville, 18). Since free sugar accumulates within the cells, Elliot and Rosenfeld (19) have suggested that glycolysis has become impaired because of the lack of adenosine triphosphate which is required in

phosphorylation. In erythrocytes it could be demonstrated that the addition of nucleosides to the medium would restore phosphorylation (Prankerd, 20). In cardiac muscle phosphorylation may also become rate-limiting since free glucose accumulates when it is added to the medium after a period of anaerobic, substrate-free perfusion (Morgan, et al., 16).

The increased cellular penetration of sugar in anoxia is very important in the Pasteur effect, which explains the conservation of nutrients under aerobic conditions when energy production is the greatest. Under anaerobic conditions, there is only a partial oxidation of the carbohydrate substrate to lactic acid in which only about 8% of the available energy is obtained as compared to aerobic glycolysis. In anoxia, the rate of glycolysis has to be increased to meet the energy requirements of the cell. The metabolism is increased by greater availability of substrate due to stimulation of the intracellular penetration of sugar. When substrate is oxidized aerobically, its supply to the cells can be kept lower while producing equal amounts of energy. Particularly skeletal muscle, which is at least partially geared to anaerobic metabolism under conditions of high energy requirement, will benefit from anoxic increased cellular sugar penetration.

The metabolic poisons, cyanide, 2:4-dinitrophenol and salicylates, which inhibit oxidative phosphorylation,

increase the cellular penetration of glucose and its analogues as well. Because of the analogy of the above two conditions, Randle and Smith (12, 13) have suggested that energy is required to prevent cellular sugar penetration under aerobic, resting conditions.

Muscular exercise has also been shown to increase the cellular penetration of sugars (Goldstein, et al., 21; Helmreich and Cori, 22) in eviscerated nephrectomized and depancreatectomized as well as in diabetic animals (Ingle, Nezamis and Morley, 23; Ingle, Nezamis and Rice, 24). Since both metabolizable and non-metabolizable sugars are equally affected, the increased penetration cannot be explained by an accelerated intracellular sugar utilization (Ingle, et al, 24). Some workers have explained this exercise effect as a local one, caused by local hypoxia (Helmreich and Cori, 22; Dulin and Clark, 25). Experiments with the working, isolated heart preparation have, however, shown that work performed under aerobic conditions is also able to increase cellular sugar penetration (Neely, Liebermeister and Morgan, 26; Mansford, 27). Also, Goldstein, et al., (21) have conclusively shown that the intracellular sugar penetration of all resting muscle is increased when only the hindlegs of eviscerated, nephrectomized and pancreatectomized dogs are exercising very vigorously. He therefore postulated that a humoral, insulin-like factor is released during exercise. The acceptance

of this theory has been hindered by the inability to consistently isolate and show an effect of this systemic hypoglycemic factor. Goldstein has recently been able to induce hypoglycemia in recipient dogs, cross-perfused with blood or lymph from chronically depancreatectomized, exercising donor dogs (Goldstein, 28). It has to be considered that the effect of this circulating factor may be mainly a local one; a systemic plasma component could either bind or destroy the factor. The isolation of this hypoglycemic factor, with a much greater potency, has now been accomplished from exercising dogs, in which the plasma protein concentration has been drastically reduced (Goldstein, 29). That the increased cellular sugar penetration in skeletal muscle in vivo is not due to variations in blood flow because of the release of vasodilatory substances has been shown in isolated muscle preparations. Havivi and Wertheimer (30) have isolated a muscle activity factor (MAF) from cardiac, skeletal and smooth muscle which increases the cellular penetration of glucose and some of its analogues in rat hemidiaphragms in vitro. This factor can only be obtained when the stimulated muscle is allowed to contract in an aerobic incubation medium (Havivi and Wertheimer, 31). Gabel, Bihler and Dresel (32) have demonstrated the release of a material from kitten hearts which were originally perfused with a 95% O_2-5% CO_2 gaseous mixture. This material, which has some of the prop-

erties of MAF, has a positive inotropic effect on isolated, failed kitten atria and also increases the cellular penetration of D-xylose and 3-O-methyl-D-glucose in intact rat hemidiaphragms (Bihler and Dresel, 33). Frederickson, Bihler and Dresel (34) have recently separated the above mentioned effects. Material released from both resting and stimulated phrenic nerve hemidiaphragms had the cardiotonic effect, while only material released from the stimulated preparation increased cellular sugar penetration.

Certain anions, such as nitrate ions, will increase the force of contraction of stimulated frog skeletal muscle. These ions seem to sensitize the muscle cell membrane since the rheobase for electrical stimulation is reduced (Chao, 35). The threshold for contraction was not lowered by the nitrate ions since the increase in the mechanical contraction could be obtained by submaximal and supramaximal stimulation (Kahn and Sandow, 36). It is unlikely that the ions increase the force of contraction by acting directly on the contractile proteins since the increased membrane sensitivity occurs only minutes after exposure to the nitrate ions. This dual action may be due to an ion effect on one of the links of excitation-contraction coupling. The action potential is not affected while the latent period, which temporarily represents at least the initiation of the coupling process, is

slightly but persistently shortened. The increase in sugar permeability in the stimulated nitrate-treated frog muscles persists for many hours and can be measured when the period of stimulation is over. In resting muscle, nitrate ions do not affect the cellular penetration of 3-O-methyl-D-glucose-^{3}H, but when nitrate-treated muscles were stimulated, the rate of sugar penetration increased more rapidly and attained significantly higher values than was found in the controls. However, at very high frequencies (120 shocks per minute) the addition of nitrate ions had no further effect. It was also shown that in frog sartorius the increased cellular penetration was not related to the tension developed (Holloszy and Narahara, 37). As no relationship was found between the breakdown of high energy compounds and cellular sugar permeability and as stimulation is known to increase the cytoplasmic calcium concentration, it has been proposed that calcium, apart from initiating contraction, may also be involved in the regulation of cell membrane permeability. Supportive evidence was obtained by studies of contractures which have been shown to increase sugar penetration (Holloszy and Narahara, 38). In contractures induced by high extracellular potassium, ^{45}Ca from the medium will enter the muscle in significant amounts since the tension of the contracture will be maintained for longer periods of time when the calcium concentration in the medium is increased

(Frank, 39; Lorkovic, 40).

In contrast to skeletal muscle, the intracellular penetration of sugar in hearts is related to the amount of work performed. Neely and his associates (26, 41) have demonstrated in isolated perfused working rat hearts that the degree of sugar penetration was directly related to the ventricular pressure development. This relationship was also found in Langendorff preparations where the pressure-time integral was shown to increase with the perfusion pressure. This interesting difference may be due to both functional and morphological differences of the two types of muscle. The functional differences are reflected in the contraction characteristics; myocardial muscle has an appreciable resting tension which may be subject to self-regulatory controls, its contractility has a slow on-set and tetanus does not occur (Brady, in Page, 42). The heart always contracts as a unit regardless of the load imposed, while in skeletal muscle the amount of work to be performed determines the number of fiber units which will participate in the contraction. Because of these distinctions, it is not surprising that the process of excitation-contraction coupling differs in the two types of muscle.

CALCIUM AND EXCITATION-CONTRACTION COUPLING

The importance of calcium ions in excitation-contraction coupling and in the actual contractile process is well established for both skeletal and for myocardial