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.

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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

muscle (Sandow, 43; Nayler, 44). There is also evidence that extracellular calcium may be required in the release of the neurohumoral transmitter substances, acetylcholine, noradrenaline and adrenaline, and the initiation of the action potential may therefore be calcium-dependent (Douglas and Rubin, 45; Katz and Miledi, 46). In skeletal muscle, calcium is required for the propagation of the action potential (Edman and Grieve, 47) and for the mechanical contraction (Frank, 48). Ringer (49) showed that extracellular calcium was required for contraction of heart muscle and Mines (50) demonstrated that this ion was also necessary for the coupling of the action potential with contraction. At the time of depolarization, membrane permeability is altered and sodium ions enter while potassium ions leave the cells. The exchange of calcium ions is also increased in both types of muscle (Winegrad and Shanes, 51; Frank, 48). Depolarization may increase the size of an ionic calcium pool known to participate in contraction (Bailey and Dresel, 52). When the ionized calcium concentration in the myoplasm exceeds threshold (10^{-7}M) contraction will occur (Caldwell, 53). The rate of influx of calcium in resting cardiac muscle is lower than that found in skeletal muscle. Electrical stimulation, on the other hand, increases the percentage of calcium influx much more in cardiac than in skeletal muscle (Bianchi and Shanes, 54; Winegrad and Shanes, 51; Langer and Brady, 55). It has

been shown *in vitro* that calcium is also released from the sarcoplasmic reticulum by stimulation (Lee, 56). It is not known if the calcium ions which enter cells during depolarization are directly involved in contraction or if they, in turn, release intracellular calcium from the sarcoplasmic reticulum (Nayler, 57).

The coupling process has been extensively investigated in frog skeletal muscle and is summarized by Page (42). Upon excitation, the action potential is generated along the sarcolemma and is transmitted to the interior of the cell along the transverse T-tubules which lie at right angles to the cell membrane of which they are extensions. The T-tubules lie in close proximity to the calcium storage depots of the longitudinal sarcoplasmic reticulum (Costantin, Franzini-Armstrong and Podolsky, 58) and calcium release is presumably triggered by the electrical signal. The increased cytoplasmic calcium concentration will then initiate the interaction between the contractile proteins. In skeletal muscle, these calcium depots are only found in the cytoplasm alongside the transverse tubules and calcium can only be released during the inward spread of the action potential. However, in mammalian heart muscle the morphological specializations of the sarcoplasmic reticulum corresponding to the calcium stores are also found alongside the sarcolemma (Page, 59, 60) and could conceivably be affected by the direct depolarization of the sarcolemma.

Huxley and Taylor (61) could induce local contractions in skeletal muscle myofibrils by depolarizing the surface membrane only near the opening of the transverse tubules. In trabecular muscle, Müller (62) was unable to repeat the above experiments; only widespread contractions could be induced. It has been suggested (Page, 42) that in heart muscle membrane depolarization can release calcium from the storage sites underneath the sarcolemma.

Three kinetic compartments have been demonstrated by washout studies both in skeletal and myocardial muscle, an unexchangeable fraction, a slowly exchangeable fraction and a rapidly exchangeable fraction (Winegrad and Shanes, 51; Langer and Brady, 55). The half-life of the rapidly exchangeable fraction is very similar in both types of muscle but the half-life of the slowly exchangeable fraction is much longer in skeletal than in myocardial muscle.

The above differences in calcium pools and compartments between skeletal and cardiac muscle may be responsible for the dissimilar effect caused by work on the intracellular penetration of sugar in the two types of muscle.

CARDIAC GLYCOSIDES

Cardiac glycosides consist of the pharmacologically active aglycone or genin to which from one to four sugar molecules are attached which probably increase the

watersolubility of the drug. The aglycone nucleus consists of a cyclopentaperhydrophenanthrene structure with a five or six membered lactone ring joined to C-17. Aldehyde, methyl and hydroxyl groups are attached to the nucleus in positions characteristic for specific aglycones. The drugs are therapeutically employed in the treatment of congestive heart failure because of their ability to increase the strength of contraction of the heart. They also modify intracellular sugar penetration and have been widely used to study transport systems because of their ability to inhibit the sodium pump in a variety of tissues (Schatzmann, 63; Schatzmann and Ackermann, 64; Glynn, 65; Matchett and Johnson, 66; Caldwell and Keynes, 67; Weatherall, 68).

The cellular mechanism for the cardiotonic action of this group of drugs has so far not been explained; because of the many cellular actions of the glycosides a cause-effect relationship has been difficult to separate. Sanyal and Saunders (69) postulated that cardiac glycosides only affect contracting heart muscle and Moran (70, 71) has provided evidence for a contraction-dependent rather than a time-dependent positive inotropic effect of these drugs. In isolated rabbit left atria, a correlation was found between the positive inotropic effect of ouabain and the number of contractions at different frequencies of stimulation. Vincenzi (72) has demonstrated that in guinea pig

left atria, the increased force of contraction caused by ouabain is time-dependent at low stimulating frequencies but beat-dependent at higher frequencies. His findings are supported by the work of Byrne and Dresel (73) who showed a positive inotropic response when quiescent rabbit atria, exposed for a certain period to ouabain, were stimulated after the drug had been washed off. This effect was not only temperature-dependent but also showed a linear relationship with the calcium concentration in the medium. Glucose is required as substrate for the myocardial response to glycosides as has been shown for rat ventricular strips (Berman, Masuoka and Saunders, 74) as well as for rabbit atria (Bailey and Dresel, 75). This carbohydrate substrate may have a metabolic role, although other substrates are ineffective in sustaining contraction in the presence of cardiac glycosides.

Much confusion exists about the relationship between the effect of cardiac glycosides on oxygen consumption and intermediary metabolism, and the positive inotropic effect of these drugs. Wollenberger (76) in an attempt to separate the metabolic from the cardiotonic effects, had used non-contracting, respiring, cardiac slices. An increase in oxygen consumption could be demonstrated after the addition of digitalis at concentrations as low as 2 and 3 x 10^{-7}M. The work of Lee, Yu and Bernstein (77) also indicates that the metabolic and cardiotonic effects

of the digitalis glycosides are independent. On the basis of their studies of the effects of therapeutic concentrations of digoxin (0.065 mg./kg.) on intermediary metabolism in dog hearts in vivo, Kien and Sherrod (78) have suggested that the main effect of the drug is on glucose metabolism since these effects could occur in the absence of any cardiodynamic changes. This concentration of digoxin also increased the cellular penetration of galactose which is not appreciably metabolized (Kien, Gomoll and Sherrod, 79). The glycoside accelerated glucose metabolism which was indicated by an increased rate of turnover of the intermediates of the glycolytic and tricarboxylic acid cycles. Kien and his associates have therefore postulated that the metabolic changes induced by the glycoside are a necessary prerequisite for their positive inotropic response. However, since the myocardial $^{14}CO_2$ pool remained the same, there was only a shift in the type of substrate oxidized. No evidence exists that energy production in failing hearts is different from normal ones and the extraction of substrates by these hearts is similar (Blain, et al, 80). Kreisberg and Williamson (81) have pointed out that glucose, even in the presence of insulin, contributes maximally less than 60% to the total energy supply of isolated perfused rat hearts and that endogenous substrates such as lipids are the main energy source. In working and non-working rat hearts, Neely, et al., (26, 41) have shown that glucose and glycogen

are important as substrates only when the ventricular pressure-development is high. It is doubtful whether the metabolic effect of the cardiac glycosides is indeed a primary one. Since it is now known that the glycosides have similar effects on contractility in normal and failing hearts (Bloomfield, et al., 82) the inability to detect these changes may have been due to the cardiovascular parameters recorded.

In their own studies on the isolated perfused rat heart, Kreisberg and Williamson (81) have shown that with concentrations of ouabain, which are known to give a positive inotropic response, the cellular penetration of glucose, its metabolism and respiration are all increased. However, the slow rate of glycogen turnover is not affected by the drug. The same effects on carbohydrate metabolism could be obtained by increasing the calcium concentration of the perfusate while the addition of ouabain to this medium did not have any further effects. When the perfusate calcium concentration was reduced the effects of ouabain could no longer be observed. The authors therefore postulated that the primary action of cardiac glycosides is not on carbohydrate metabolism but on calcium transport.

Although cardiac glycosides do not have any cardiotonic effects on skeletal muscle, interesting metabolic changes, very different from those in heart muscle,

have been demonstrated with these drugs. The glycosides have the ability to inhibit the sodium pump which is involved in the maintenance of ionic gradients across the cell membrane (Matchett and Johnson, 66). Clausen (83) demonstrated that inhibition of the cation pump by ouabain in rat and mouse diaphragms in the presence of glucose will decrease lactate production and increase glycogenesis. The reciprocal relationship between the fraction of glucose being deposited as glycogen and the fraction undergoing anaerobic glycolysis was shown to be dependent upon the external sodium concentration. Since the same results could be obtained by the use of a potassium-free incubation medium, which also inhibits the sodium-potassium pump, it was suggested that the observed metabolic effects were secondary to this inhibition (Clausen, 84). Clausen, however, failed to show an increase in the cellular penetration of glucose with ouabain.

By preincubating intact rat hemidiaphragms with the desired concentration of glycoside as well as 10 mM glucose, Bihler (85) has been able to affect the cellular penetration of a number of non-metabolizable sugars. The action of the cardiac glycosides was shown to be a biphasic one, low concentrations of the drug decreased penetration while high concentrations increased this process; this could be correlated with an increase and decrease in cation transport respectively. The required preincubation of

the tissue with the glycoside and a substrate suggests that transport or binding of the drug is necessary for its action.

Ho and his associates (Ho and Jeanrenaud, 86; Ho, et al., 87) have demonstrated that cardiac glycosides exert metabolic effects in adipose cells. In addition to the already discussed metabolic actions, ouabain has been shown to oppose the lipolytic and the glycogenolytic effects of adrenalinein rat diaphragm and epididymal fat pad as well as in the in vivo dog preparation (Triner, Kypson and Nahas, 88, 89; Kypson, Triner and Nahas, 90). Triner, Killian and Nahas (91) have indicated that the hypoglycemic effect of ouabain is insulin-mediated.

SECTION II

METHODS

Isolated rabbit hearts were perfused through the coronary circulation in a retrograde fashion. After a period of preperfusion (washout) a perfusate containing L-arabinose and inulin was recirculated through the system. During perfusion the hearts were stimulated at two different frequencies or were made quiescent. Ouabain was added to the preperfusion and the perfusion media in some experiments. At the end of perfusion the ventricular tissue and the perfusate were analyzed for L-arabinose and inulin content and the percentage of cellular sugar penetration determined.

PERFUSION APPARATUS

Retrograde perfusion was carried out in a modified Langendorff apparatus in which the cannula was connected, by means of a three-way valve, to separate flow-through preperfusion and recirculating perfusion units. Figure I shows a schematic diagram of the perfusion apparatus. An open perfusion technique was used and the media flowed through the heart by means of gravity from reservoirs set at 70 cm water pressure. This pressure, which was equal in both the preperfusion and the perfusion units, was increased slightly for large hearts and decreased for the small ones. The effluent from the heart chamber was either discarded ('preperfusion') or returned to the reservoir by a pump ('perfusion'). The cannula, from which the hearts were suspended, consisted of a glass T-tube (outer diameter 6 mm) of which one of the

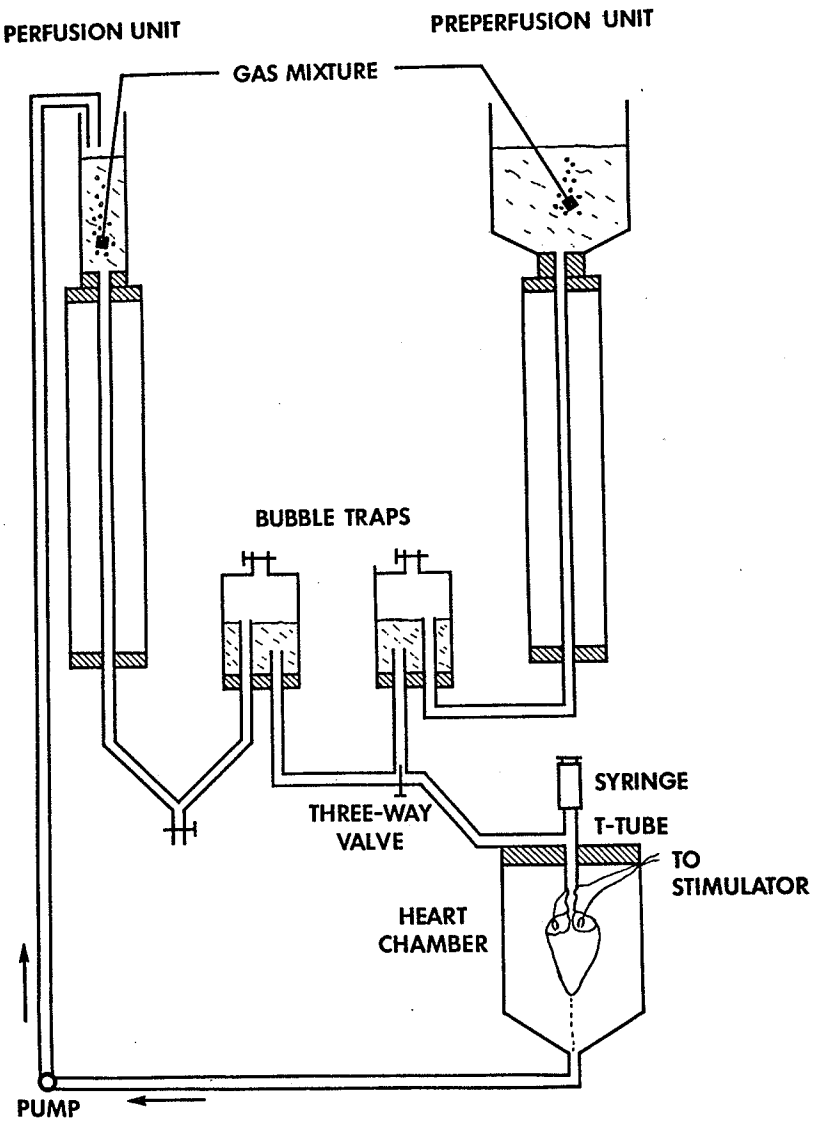


Figure I. Schematic diagram of the perfusion apparatus. See text for explanation.

straight ends was fixed through a rubber stopper which closed off the heart chamber. The tip of this straight end was ground down slightly and shortened to prevent excessive stretch of the aorta and damage to the tricuspid valve. The opposite straight outlet was connected to a small syringe which served as an auxillary bubble collector, sometimes required when switching from the preperfusion to the perfusion medium. The crosspiece of the T-tube was connected to the three-way valve. The double-walled cylindrical heart chamber (made of acrylic plastic) could be pivoted away to allow the hearts to be cannulated; its conical bottom facilitated the outflow of the perfusion medium.

The reservoir of the preperfusion unit consisted of a large container from which the medium flowed through a condensor and a jacketed bubble trap to the heart. In the experiments with the cardiac glycoside, two different preperfusion media were used and the large reservoir was replaced by an easily interchangeable glass cylinder which contained a smaller volume of medium, maintained at the same hydrostatic pressure. This facilitated the exchange of these two preperfusion media. The perfusion unit was made to recirculate 50-60 ml of medium which contained the sugar and the extracellular marker. It contained an outlet between the condensor and the bubble trap to collect perfusate samples. A pump served to return the effluent from

the heart to the elevated reservoir (Peristaltic-action pump, Aminco; Masterflex tubing pump, Cole-Palmer). Polyvinyl plastic tubing was used wherever possible in the perfusion apparatus. The temperature of the heart chamber, bubble traps and condensors was kept constant at 30°C by means of a circulating, thermostatically controlled waterbath. The two media were oxygenated with an O_2:CO_2 (95%:5%) gas mixture, equilibrated with water at room temperature during the experiments. The gas mixture was expelled through polyethylene gas dispersion tubes (Labpor), placed in the reservoirs so that as large a fluid volume as possible was exposed.

PERFUSION MEDIA

The normal perfusion medium was Krebs bicarbonate buffer (Krebs and Henseleit, 92) at pH 7.4, which contained only one half of the normal concentration of ionized calcium (2.5 mEq.Ca^{2+}/l). The final concentration of the salts (in mM) was as follows: NaCl, 118.6; $NaHCO_3$, 24.7; KCl, 4.8; $MgSO_4$, 1.2; KH_2PO_4, 1.2 and $CaCl_2$, 1.25. Two other media were used in the perfusions, one containing 1.25 mEq.Ca^{2+}/l, the other 0.625 mEq.Ca^{2+}/l. These will be referred to as half calcium and low calcium buffer respectively. Reagent grade chemicals were used for the solutions. Stock solutions were prepared as suggested by DeLuca and Cohen (93). The $NaHCO_3$ stock solution, which was stored in the cold, was equilibrated with

TABLE I - CHEMICALS AND THEIR SOURCE OF SUPPLY

L-arabinose	Mann Research Lab. Inc.
Sodium pyruvate	Sigma Chemical Co.
D-glucose	Fisher Scientific Co.
Ouabain	Nutritional Biochemicals Corp.
Inulin	Mann Research Lab. Inc.
Insulin	Connaught Medical Research Lab.
EDTA-Ca (ethylenedinitrilo) tetra-acetic acid dicalcium salt	Eastman Organic Chemicals

O_2:CO_2(95%:5%) for several hours to prevent the formation of insoluble calcium salts (Young, 94). Fresh buffer solution was made daily. Sodium pyruvate was added to the preperfusion medium to a final concentration of 4 mM in the control experiments. This substrate served as a nutrient to prevent the deterioration of the mechanical function of the heart with its subsequent increase in intracellular sugar penetration (Zachariah, 95). The pyruvate was replaced by 10 mM of D-glucose when ouabain was present in this medium; this sugar is necessary for the maintenance of contractility of the heart in the presence of cardiac glycosides (Bailey and Dresel, 75) and also is required for the action of these drugs on sugar penetration (Bihler, 85). The final concentration of ouabain in the preperfusion and perfusion media was 5×10^{-7} g/ml ('inotropic' concentration) or $0.9\text{-}1.0 \times 10^{-6}$ g/ml ('toxic' concentration). The stock solution of ouabain dissolved in distilled water to 1 mg/ml was kept in the refrigerator and was replaced every four weeks. The perfusion medium contained 15 mM of L(+)arabinose, the non-metabolizable test sugar, 4 mM of sodium pyruvate (both in the presence and in the absence of ouabain), 1% (w/v) pyrogen-free inulin serving as the extracellular marker and 0.5 mM EDTA-Ca to improve the stability of the hearts, possibly by chelation of heavy metals present in the reagent chemicals (Neely, et al., 41). In a few preliminary experiments, insulin at a final concentration of

1 m-u/ml was used.

STIMULATION

A Grass model SD5 stimulator was used to induce contraction. Mammalian heart clip electrodes (C.F. Palmer, Ltd.) were attached to the atria and the hearts were stimulated with a supramaximal, square wave pulse. The stimulus had a duration of 10 msec and the voltage was adjusted to 1.5-2 times threshold. During the 30 minutes of preperfusion, the hearts were stimulated at 120 beats/min. Hearts which were made quiescent were stimulated at the same frequency for the first 20 minutes of preperfusion, after which the heart block was induced. During perfusion, stimulation was carried out at either 180 or 240 beats/min. Since at high frequencies of stimulation AV-nodal blocks often occurred, the frequency of contraction of those hearts was carefully inspected during the course of an experiment.

INDUCTION OF HEART BLOCK

The technique of Pruett and Woods (96) and MacDonald (97) to obtain complete heart block in dogs was modified to facilitate this procedure in the isolated heart of the rabbit. After 20 minutes of preperfusion, the stimulator was turned off and the electrodes removed from the atria. The right atrial chamber was exposed by an incision started at the tip of the atrium and extended along its posterior edge. The flow of preperfusate was stopped and the fluid remaining in the atrial chamber

drawn off with filter paper. The fine tip of a preheated soldering-gun (Simpson-Sears, 240/325 watts) was then applied to the area of the AV-node at the center of the base of the septal cusp, distal to the ostium of the coronary sinus. The Bundle of His which winds around the medial half of the base of this cusp also was destroyed. To prevent accidental damage to the septum, the areas of the conducting system were only lightly burned, which sometimes necessitated a second application of heat.

EXPERIMENTAL PROCEDURE

Healthy male and female rabbits (1.5-4.5 kg) of mixed breeds, kept on a stock laboratory diet, were used in the experiments. The animals were fasted overnight before being sacrificed. In a few experiments this was not done but no differences were noted in the extracellular space or in the intracellular penetration of L-arabinose in hearts of animals which were fasted or those which had free access to food. The animals were killed by a blow on the head and bled for a few seconds; the chest was opened by a longitudinal cut, starting in the abdomen to prevent accidental damage to the heart. The rib cage was pulled away, the heart was quickly removed by severing the distal vessels and immediately placed into cold isotonic saline ($4^{\circ}C$). Contractions generally stopped within a few seconds. After the heart was chilled, the aorta was clamped to two small hemostats and could easily be slipped over the grooved

cannula of the perfusion apparatus. Stimulation and preperfusion with the warm medium were started immediately and all visible blood was removed with the first few milliliters of medium. All experiments were carried out at 30°C to decrease the occurrence of ventricular ectopic foci in the quiescent hearts. Any extraneous tissue left on the heart was then removed and the heart chamber swung into place. An important requirement for successful liquid perfusion is to rapidly slow the metabolic processes of the excised heart so as to prevent damage due to anoxia. The heart should be placed in the cold isotonic saline within 30 seconds after death of the animal, the actual time of cannulation then becomes of secondary importance. Preperfusion served to equilibrate the heart to the lowered temperature and to wash out endogenous substances. The length of the period of preperfusion has to be selected carefully. Bihler, et al., (5) and Zachariah (95) have shown that the duration of the preperfusion period influences the cellular sugar penetration in rabbit and rat hearts respectively; when preperfusion is started, an initial fall in sugar penetration has been shown and is related to the washout of endogenous insulin (Zachariah, 95). The washout of other substances such as adrenalineand Goldstein's 'hypoglycemic' factor (21) also may have contributed to the initial decrease in cellular penetration. A preperfusion period of 30 minutes was found to be sufficient

to wash out endogenous substances and the intracellular penetration of sugar was stable for the following 60 to 75 minutes (Zachariah, 95). The addition of EDTA-Ca will further have improved the stability of the preparation (Neely, et al., 41).

Because of the postulated frequency dependence of the action of ouabain, as discussed in the introduction, the hearts were stimulated during the preperfusion period. Glucose was added to the preperfusion medium in the ouabain experiments since it had been shown that this substrate was required for the maintenance of contractility in the presence of cardiac glycosides (Bailey and Dresel, 75) as well as for the action of the drugs on intracellular sugar penetration (Bihler, 85). Control hearts, where the glycoside was not present in the preperfusion medium, were also stimulated to eliminate variations due to different heart rates. The chosen stimulation frequency of 120 beats/min was an approximate average of the spontaneous rate of the isolated hearts.

The preperfusion procedure was varied according to the type of experiment performed (Table II). In the control experiments, the hearts were preperfused as described above; when the hearts were made quiescent, the period of stimulation was decreased to 20 minutes. The AV-node and Bundle of His were then destroyed and preperfusion continued for a further ten minutes without stimu-

TABLE II

EXPERIMENTAL DESIGN

CONTROL EXPERIMENTS

	QUIESCENT		STIMULATED	
	Time	Medium contains	Time	Medium contains
Preperfusion	20 min	sodium pyruvate	30 min	sodium pyruvate
	Cauterization			
Preperfusion	10 min	sodium pyruvate		
Perfusion	30 min	L-arabinose inulin sodium pyruvate	30 min	L-arabinose inulin sodium pyruvate

OUABAIN EXPERIMENTS

Preperfusion	20 min	ouabain glucose	20 min	ouabain glucose
	Cauterization			
Preperfusion	10 min	sodium pyruvate	10 min	sodium pyruvate
Perfusion	30 min	L-arabinose sodium pyruvate ouabain inulin	30 min	L-arabinose sodium pyruvate ouabain inulin

lation. In the ouabain experiments, the stimulated hearts were preperfused with a medium which contained the same concentration of ouabain as was used during the actual perfusion. However, 10 mM glucose was substituted for the sodium pyruvate. This was followed by a further washout period for ten minutes. In the quiescent hearts treated with ouabain the same procedure was followed, except that after the initial preperfusion with the ouabain-containing buffer, the heart was made quiescent. With the above procedures, consistency in the frequency of contraction, in the exposure to ouabain and in the length of the washout period under different conditions was maintained.

When switching to the perfusion medium, which contained the sugar and the extracellular marker, the first 15-20 ml of medium passing through the heart were discarded to prevent dilution of the recirculating perfusate by preperfusate remaining in the system and in the coronary circulation. During perfusion the hearts were either left quiescent or were stimulated at 180 or 240 beats/min. The period of perfusion was 30 mins. This time was of necessity a compromise: with short perfusion times, cellular penetration would be too small to detect differences due to changes in experimental conditions; with long perfusion times, differences again would be difficult to detect since a state close to equilibrium would be reached under all conditions.

The perfusate temperature was checked five minutes after perfusion was started. All experiments were done aerobically. The rate of flow was measured at the end of the perfusion period by collecting the coronary effluent from the outlet of the heart chamber in a graduated cylinder; it is expressed as ml/min. The rate of flow, perfusion pressure and the general appearance of the hearts were carefully observed; all unsatisfactory hearts were excluded.

PREPARATION OF SAMPLES

At the end of the perfusion period, the heart was removed from the cannula. The heart was cut transversely at the level of the circumflex artery and the ventricular tissue divided into two parts and lightly blotted on hardened filter paper (Whatman, No. 50), weighed and stored in the deep freeze. A perfusate sample was collected in a plastic test tube and also stored in the deep freeze. One tissue sample was dried to constant weight at 80°C under vacuum for the determination of total tissue water. The other tissue sample was partly thawed at room temperature and finely minced with scissors. This mince was transferred to conical glass centrifuge tubes and extracted in boiling water for ten minutes. After cooling, the extract was deproteinized with $Ba(OH)_2$-$ZnSO_4$ (Somogyi, 98), diluted to a specific known volume and centrifuged. Suitable dilutions were made of the supernatant

for the separate determinations of L-arabinose and inulin. Samples of the perfusate were also deproteinized with the barium-zinc method and diluted for analysis. The prepared samples were covered with Parafilm and kept overnight in the cold before analysis was made.

The analysis was carried out with a Technicon Autoanalyzer for which the resorcinol method for inulin (Roe, Epstein and Goldstein, 99) and the bromoaniline method for L-arabinose (Roe and Rice, 100) were adapted. A series of known standards was analyzed with each group of samples. With the above chemical determinations, as opposed to a radioactivity assay, a correction has to be made for tissue blanks (Bihler, et al., 5). A number of heartswere perfused routinely but without L-arabinose and inulin in the perfusion medium. Chemical analysis was then performed and an average tissue blank for the two substances determined.

CALCULATIONS

The results are expressed as percentage penetration of sugar into the intracellular water, which indicates the fraction (in percent) of the intracellular water which is equilibrated with the sugar in the perfusate. The extracellular space and total tissue water were determined individually for each heart to eliminate variations due to tissue handling and blotting. The distribution of inulin in the tissue water is an estimate of the extracellular

space since this large substance is known not to pass into the cells. The extracellular space can be calculated by the ratio of the concentration of inulin in the perfusate to that of the concentration in the tissue. The concentration of inulin in the tissue was corrected for dry weight and for the predetermined tissue blank, found to be 137 ± 23 ugm/ml tissue water. The total sugar space, the volume of the total tissue water equilibrated with the sugar, was calculated in the same way. The tissue blank for L-arabinose was found to be 112 ± 16 ugm/ml tissue water. When the total sugar space is greater than the extracellular space, intracellular penetration of sugar will have occurred. The intracellular water, fully equilibrated with the perfusate sugar concentration, expressed as a percentage of total tissue water, can be determined by subtracting the extracellular space from the total sugar space. The percentage of penetration is 100 x the ratio of the fully equilibrated intracellular tissue water to the total intracellular tissue water. The following are the formulae used:

$$\text{Inulin space (extracellular space)} = \frac{\text{Inulin/g. tissue} \times \%\ \text{water in tissue}}{\text{Inulin/ml. perfusate}} \times 100$$

$$\text{Total sugar space (virtual space)} = \frac{\text{Sugar/g. tissue} \times \%\ \text{water in tissue}}{\text{Sugar/ml. perfusate}} \times 100$$

$$\%\ \text{penetration (intracellular sugar space)} = \frac{\text{Total sugar space} - \text{Inulin space}}{(1 - \text{Inulin space})} \times 100$$

SECTION III

RESULTS

CONDITIONS OF PERFUSION

The stability of the isolated hearts was greatly improved by the addition of sodium pyruvate to the perfusing media. This substrate prevents the deterioration of the mechanical function of the heart and the subsequent increase in cellular sugar penetration (Zachariah, 95). The force of contraction, heart rate and flow are not affected by the addition of pyruvate (Bihler, et al., 5). EDTA-Ca added to the perfusate also improved the stability of the preparations (Neely, et al., 41).

The technique used for the induction of heart block proved to be quite successful in making the hearts completely quiescent. However, in some hearts it was not possible to produce complete atrioventricular dissociation. In such hearts, the beats were counted at 5, 15 and 28 minutes after perfusion was started and if the average exceeded 50 beats per minute, the experiment was excluded from consideration. No difference was seen in sugar penetration between hearts which were completely quiescent during the entire perfusion period and those which were beating at rates up to 50 beats per minute.

The size of the hearts, the sex of the rabbit from which the hearts were obtained, or overnight fasting of the animals before use did not affect the penetration of L-arabinose either in the experimental series or in the determination of the blank values for L-arabinose and inulin.

In preliminary experiments the functional state of the isolated hearts was determined by their physiological response to insulin. The addition of 1 m-u/ml of insulin to the perfusion medium, nearly doubled the percentage of sugar penetration in five spontaneously beating hearts to 72.1 ± 2.6% as compared to their controls. Interestingly, the same increase in sugar penetration to 72.2 ± 3.4% was found when five quiescent hearts were perfused with the same concentration of insulin in the perfusate. The physiological competence of the preparation was thus established. Since the percentage of sugar penetration of the controls as well as those obtained with insulin compared favourably to that found by other workers for the isolated rabbit heart (Bihler, et al., 5), the perfusion technique was judged to be satisfactory.

EXPERIMENTS

Three series of experiments were conducted. In the first, sugar penetration was determined in three groups of hearts beating at different frequencies. One group of hearts was made quiescent by the destruction of the AV-node and the Bundle of His while the other two groups were stimulated at either 180 or 240 beats/min. Because of the postulated involvement of calcium in sugar penetration and the well known role of calcium ions in cardiac contraction each group in turn included hearts perfused with normal calcium, half calcium and low calcium buffers. In the second

series of experiments, the effect of ouabain on the intracellular penetration of sugar was determined in the three frequency groups. Ouabain was added to both the preperfusion and the perfusion media as outlined in the Methods section. Again perfusions were carried out with the three different calcium buffers. The concentration of ouabain (5×10^{-7} g/ml) has been shown to give a positive inotropic response in isolated rabbit atria (Bailey and Dresel, 75; Byrne and Dresel, 73). This concentration of ouabain also increased the penetration of sugar under appropriate experimental conditions. A lower concentration of ouabain (2×10^{-7} g/ml) was found to be insufficient to obtain reliable results. In the third series, the effect of toxic concentrations of ouabain ($0.9\text{-}1.0 \times 10^{-6}$ g/ml) was determined on a few hearts. These concentrations always induced contracture at the conclusion of the perfusion period. Perfusions were only carried out with the normal calcium buffer.

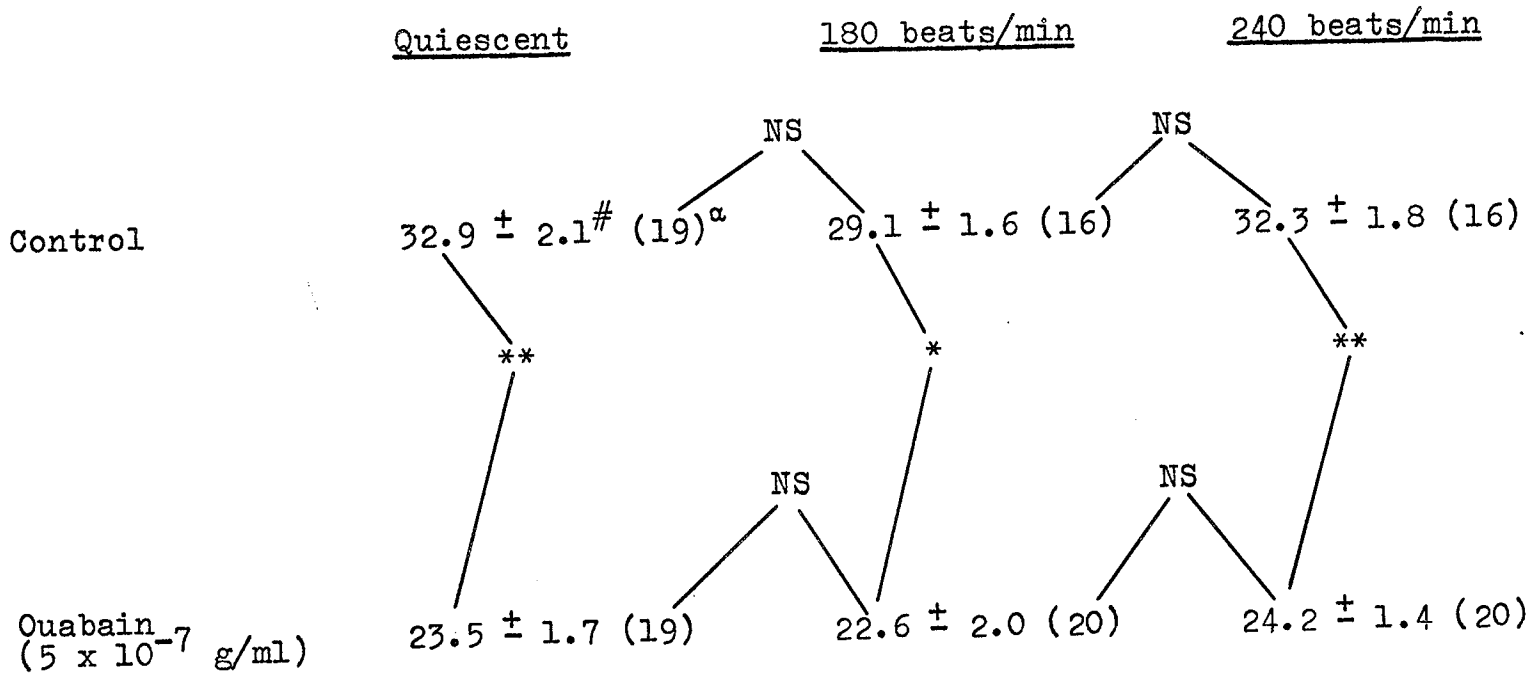
FLOW

As shown in Table III, the rate of flow, although highly variable between individual experiments, showed no differences between hearts which were quiescent and those which were stimulated at a given frequency whether with or without ouabain. However, the addition of ouabain decreased the flow from 22% to 29% in all three groups of hearts. This difference was statistically significant. The toxic concentrations of the cardiac glycoside always induced

TABLE III

THE EFFECTS OF FREQUENCY OF CONTRACTION AND OF OUABAIN ON THE RATE OF FLOW IN ISOLATED PERFUSED RABBIT HEARTS

Perfusion Flow (ml/min)



#Mean ± SE

αNumber of experiments

*P<0.05

**P<0.01

contracture at the conclusion of the perfusion period. However, flow up to the time of contracture appeared to be comparable with that found in hearts perfused with the inotropic concentration of ouabain.

EXTRACELLULAR SPACE

The extracellular spaces obtained under the different experimental conditions are shown in Table IV. In the control experiments, the extracellular space of the quiescent hearts was somewhat larger than of those stimulated at 180 beats/min or at 240 beats/min. With inotropic concentrations of ouabain the extracellular space of the quiescent hearts was also larger than those of the stimulated hearts. With toxic concentrations of ouabain, the extracellular spaces were reduced, probably because of the induced contractures. It should, however, be understood that the apparent extracellular space is an operational value, in part reflecting variations in blotting procedure. It was determined individually for each heart and used in the calculation of the percentage of intracellular sugar penetration.

SUGAR PENETRATION IN CONTROL EXPERIMENTS

The results of the control experiments are summarized in Table V. The cellular penetration of L-arabinose in hearts perfused with normal calcium buffer showed an increase roughly parallel to the frequency of contraction. The low percentage of sugar penetration in

TABLE IV

THE EFFECTS OF FREQUENCY OF CONTRACTION AND OF OUABAIN ON THE APPARENT EXTRACELLULAR SPACE IN ISOLATED PERFUSED RABBIT HEARTS.

Extracellular space (percentage of total tissue water)

	Quiescent	180 beats/min	240 beats/min
Control	39.4 ± 1.5[#] (20)[α]	37.9 ± 0.7 (16)	37.7 ± 1.0 (17)
Ouabain (5×10^{-7} g/ml)	42.7 ± 1.3 (19)	33.6 ± 0.9 (19)	34.1 ± 1.1 (21)
Ouabain ($0.9\text{-}1.0^{-6}$ g/ml)	29.8 ± 5.4 (19)	29.4 ± 0.5 (2)	31.3 ± 6.9 (2)

[#]Mean ± SE

[α]Number of experiments

TABLE V

THE EFFECT OF FREQUENCY OF CONTRACTION ON THE INTRACELLULAR PENETRATION OF L-ARABINOSE IN ISOLATED RABBIT HEARTS PERFUSED WITH DIFFERENT MEDIA.

Percentage Intracellular Penetration

Calcium concentration in the perfusion medium (mEq. Ca^{2+}/l)	Quiescent	180 beats/min	240 beats/min
2.5	33.6 ± 3.5# (8)α	39.4 ± 5.2 (6)	57.4 ± 5.8 (6)
1.25	44.0 ± 4.7 (6)	43.2 ± 7.3 (6)	57.0 ± 9.3 (5)
0.625	38.6 ± 6.2 (6)	48.2 ± 2.4 (4)	47.3 ± 4.9 (6)

#Mean ± SE

αNumber of experiments

quiescent hearts, 33.6 ± 3.5% increased to 39.4 ± 5.2% in hearts stimulated at 180 beats/min and this was further increased to 57.4 ± 5.8% when the frequency of stimulation was raised to 240 beats/min. Although the increase in sugar penetration between quiescent hearts and those stimulated at 180 beats/min was small, the great increase found in hearts stimulated at 240 beats/min seemed to indicate a beat-dependency. Sugar penetration in hearts perfused with half calcium buffer again showed a similar relationship between penetration and the frequency of contraction when the frequency was raised from 180 to 240 beats/min. The effect of halving the calcium concentration of the perfusate was to increase penetration in both the quiescent hearts and those stimulated at 180 beats/min while the penetration of hearts contracting at 240 beats/min was no different from that in the normal calcium medium. Hearts perfused with the low calcium medium showed a different relationship between cellular sugar penetration and the frequency of contraction. Sugar penetration in hearts stimulated at 180 beats/min was increased over that found in quiescent hearts but remained about the same when the frequency of contraction was increased to 240 beats/min.

OUABAIN EXPERIMENTS

The results of the ouabain experiments are summarized in Table VI. When the hearts were perfused with normal calcium buffer to which ouabain had been added, the

TABLE VI

THE EFFECTS OF FREQUENCY OF CONTRACTION AND OF OUABAIN ON THE INTRACELLULAR PENETRATION OF L-ARABINOSE IN ISOLATED RABBIT HEARTS PERFUSED WITH DIFFERENT MEDIA.

Percentage Intracellular Penetration

Calcium concentration in the perfusion medium (mEq. Ca^{2+}/l)	Quiescent	180 beats/min	240 beats/min
2.5	25.4 ± 1.2[#] (7)[α]	57.2 ± 5.7 (10)	62.5 ± 4.9 (11)
1.25	28.3 ± 2.3 (4)	59.1 ± 7.3 (5)	58.4 ± 8.2 (5)
0.625	43.0 ± 6.8 (8)	59.8 ± 10.0 (4)	72.1 ± 1.4 (5)

[#]Mean ± SE

[α]Number of experiments

sugar penetration in hearts stimulated at 180 beats/min, 57.2 ± 5.7%, was about twice that found in the quiescent hearts, 25.4 ± 1.2%. Little further increase in penetration was seen when the frequency of contraction was increased to 240 beats/min (62.5 ± 4.9%). This same relationship was seen when the hearts were perfused with the half calcium buffer. The sugar penetration in hearts stimulated at 180 beats/min was again about twice that found in the quiescent hearts. When the stimulation frequency was increased to 240 beats/min, sugar penetration remained the same. Sugar penetration in hearts perfused with the low calcium buffer showed a different response to the frequency of stimulation. The sugar penetration in quiescent hearts was increased, as compared to that found with the other calcium buffers, and showed a further increase with contraction.

Comparison of the control experiments with the ouabain experiments showed that the intracellular penetration of L-arabinose in quiescent hearts was decreased by the cardiac glycoside, while penetration was increased by the drug when the hearts were stimulated at 180 beats/min but remained approximately the same at 240 beats/min. This response was only seen when the hearts were perfused in normal calcium and half calcium buffer. However, in hearts perfused with the low calcium buffer, the addition of ouabain increased sugar penetration in all three groups of hearts.

The toxic concentrations of ouabain (in normal calcium medium) increased sugar penetration in quiescent as well as in stimulated hearts to an equal extent (Table VII).

STATISTICAL ANALYSIS

Duncan's New Multiple Range Test (Steel and Torrie, 101) was used to determine if statistically significant differences existed between the means of the percentage of sugar penetration in hearts perfused under different experimental conditions. In this test, the means are ranked in order of magnitude and then analyzed for significant differences. The test requires homogeneity of the variances of all the results. When analyzing the homogeneity of the data by Bartlett's test (Steel and Torrie, 101) it was found that the experimental Chi-square was highly significant, indicating that the variances of the different groups of hearts were not homogeneous. Thus, the Multiple Range Test was invalid and no meaningful statistical comparisons could be made on the data in their present form. Bartlett's test was also applied to the pooled results obtained from the normal, and half calcium buffers only. However, the variances again were found to be nonhomogeneous. Covariance analysis was done to remove extraneous sources of variation. Variables which were so analyzed for the groups of hearts perfused under different experimental conditions were the rate of flow, heart weight

as reflected by body weight, perfusion pressure and extracellular space. No correlation between any of the above parameters, or combinations of them, and intracellular sugar penetration could be found. Therefore, the results which were obtained with each group of hearts perfused with the three different calcium buffers were pooled, since the large standard errors indicated that statistically significant differences between them were unlikely to have occurred. Bartlett's Test now showed that the variances of the pooled data were homogeneous. Thus the differences between the means could be analyzed by Duncan's Test. Consequently, the results presented below refer to pooled data at all three calcium concentrations. When the data are pooled statistical analysis of the results obtained with the three calcium buffers is, of course, no longer possible.

Of the 18 different types of experiments the following four groups had variances which were not homogeneous with the variances of all experimental groups and which necessitated the pooling of the data obtained at the three calcium concentrations.

(1) Quiescent hearts, treated with ouabain, and perfused with normal calcium buffer;

(2) Quiescent hearts, treated with ouabain, and perfused with half calcium buffer;

(3) Hearts stimulated at 180 beats/min, perfused with low calcium buffer;

(4) Hearts stimulated at 240 beats/min, treated with ouabain, and perfused with low calcium buffer.

In the analysis of the apparent extracellular space obtained under the different experimental conditions the same problems were encountered, and since no meaningful statistical comparison could be made, no significance can be attached to the observed differences in the means.

The results obtained with the pooled data are given in Table VII. In the control experiments sugar penetration in the quiescent hearts was not significantly different ($P>0.05$) from that found in hearts stimulated at 180 beats/min. However, the percentage of sugar penetration in hearts stimulated at 240 beats/min was significantly greater than in the quiescent hearts ($P<0.01$) and in hearts stimulated at 180 beats/min ($P<0.05$).

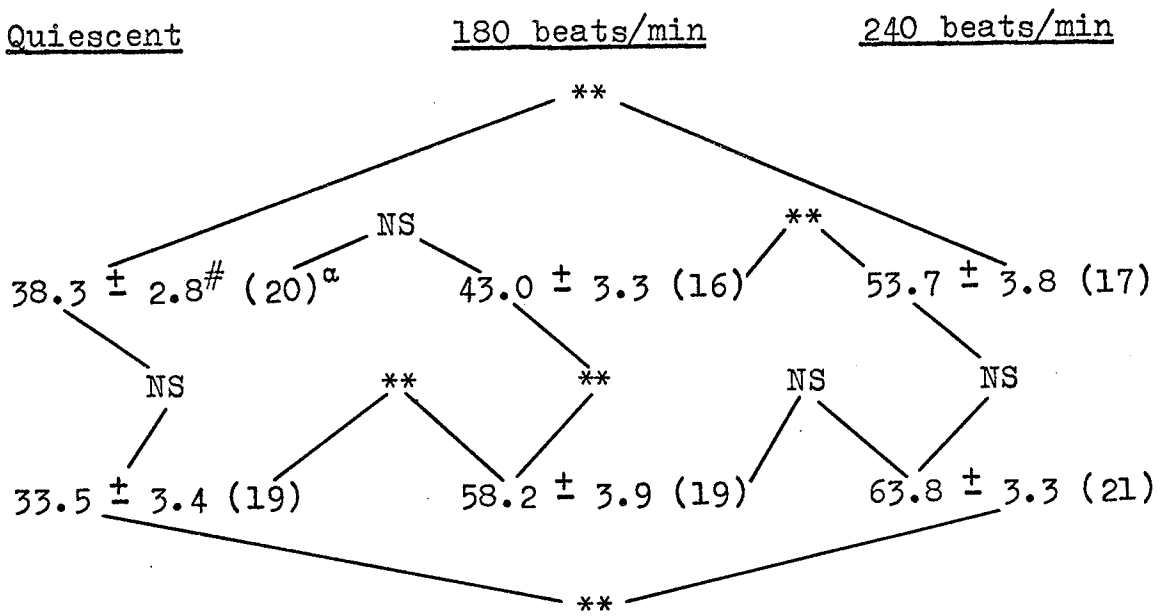
In the ouabain experiments, sugar penetration of hearts stimulated at 180 beats/min was significantly increased over that found in the quiescent hearts ($P<0.01$). However, the increased sugar penetration in hearts stimulated at 240 beats/min did not differ from those stimulated at 180 beats/min ($P>0.05$).

When the control experiments were compared with the ouabain experiments, the decrease in sugar penetration found in the quiescent hearts treated with ouabain was not

TABLE VII

THE EFFECTS OF FREQUENCY OF CONTRACTION AND OF OUABAIN ON THE INTRACELLULAR PENETRATION OF L-ARABINOSE IN ISOLATED PERFUSED RABBIT HEARTS.

Percentage Intracellular Penetration



Ouabain (0.9-1.0 x 10^{-6} g/ml)	63.9 ± 4.3 (2)	69.0 ± 7.8 (2)	65.6 ± 3.4 (2)

#Mean ± SE αNumber of experiments NS - Not significant

* P<0.05 ** P<0.01

significant ($P>0.05$). However, the increase in sugar penetration due to ouabain in hearts stimulated at 180 beats/min was highly significant ($P<0.01$). On the other hand, the effect of ouabain in hearts stimulated at 240 beats/min was not significant ($P>0.05$).

These pooled results indicate that sugar penetration is increased in the stimulated hearts only at high frequencies of contraction. Penetration shows some dependence upon the calcium concentration of the perfusate. The addition of ouabain had no effect on sugar penetration in quiescent hearts but increased this significantly at the low stimulation frequency of 180 beats/min. No further increase in penetration was observed when the stimulation frequency was increased to 240 beats/min (Figure II).

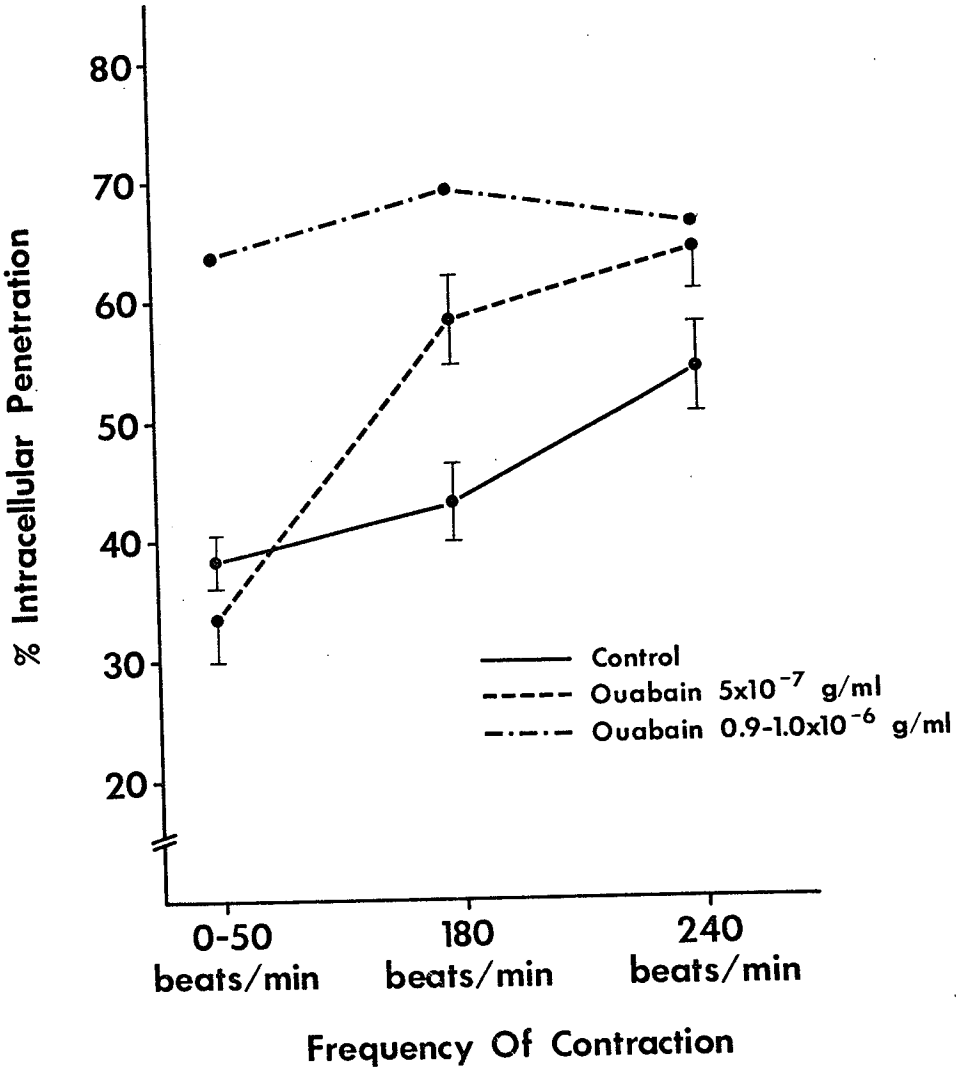


Figure II. The effects of the frequency of contraction and of ouabain on the intracellular penetration of L-arabinose in perfused rabbit hearts. The vertical bars represent the SE.

SECTION IV

DISCUSSION

The experimental results will be discussed in light of the carrier hypothesis in which a limited number of membrane sites are postulated to be available for the intracellular penetration of the hydrophyllic sugar molecules. Kinetic studies have indicated that the formation and breakup of the sugar-carrier complex on the membrane surfaces is not the rate-limiting factor in sugar penetration, but that the diffusion of the sugar-carrier complex or the carrier alone determines the rate of penetration (Morgan, et al., 102). Some aspects of the regulation of the sugar-carrier system can be studied even though the nature of the binding sites or of the carrier is unknown. Morgan, Regen and Park (103) have shown that both insulin and anoxia specifically stimulate the carrier system and so increase sugar penetration without opening accessory pathways for free diffusion. In skeletal muscle, it has been found that the intracellular penetration of sugar is also increased by contraction (Holloszy and Narahara, 104) and by ionic changes which follow the inhibition of the sodium pump by cardiac glycosides (Bihler, 85). The purpose of this study was to determine if similar mechanisms can also activate the sugar-carrier system in cardiac muscle. We have now studied the effects of the frequency of contraction and of ouabain on the transport of L-arabinose in rabbit ventricular muscle, <u>in vitro</u>.

The pentose, L-arabinose, was used because it

had been shown that this sugar utilizes the same carrier mechanism as D-glucose and since this sugar is not metabolized its intracellular penetration can be studied without complications from subsequent metabolism. Morgan and Park (15) have demonstrated competition between L-arabinose and D-glucose in the isolated perfused rat heart, while Morgan, et al., (16) have shown by countertransport experiments in the same preparation that the transport of L-arabinose is freely reversible. The kinetics of the carrier-mediated transport of this pentose have been worked out by Bihler, et al., (5).

The isolated, perfused heart was used for the study of the intracellular penetration of substrates. The differences in the degree of intracellular sugar penetration under various experimental conditions are indicative of carrier regulation in vivo since the substrate reaches the cardiac muscle cells via the normal coronary circulation. In addition, the physiological condition of the preparation can be monitored by means of such parameters as the rate of flow, perfusion pressure, contractility and oxygen consumption to ensure that the hearts have not deteriorated during the course of an experiment.

RELATIONSHIP BETWEEN SUGAR PENETRATION AND FLOW

The coronary circulation in the rabbit is similar to that of other mammals and although differences in the distribution of the coronary vessels are seen, intraspecies

differences are generally greater than interspecies differences. The rate of coronary flow in isolated perfused hearts is partly determined by the aortic perfusion pressure of the coronary arteries. It is further regulated by the mean diameter of the coronary bed and the phasic action of the ventricular muscle in the contraction-relaxation cycle. In systole, flow in the left coronary artery will come to a stop because of compression of the intramyocardial vessels by the ventricular muscle. The subsequent increase in the rate of flow during this period is due to perfusion of the epicardial anastomosing vessels. In diastole, the perfusate will flow through the intramyocardial vessels and will then slowly decline during this period. Flow in the right coronary artery follows the aortic pressure curve closely (Gregg, 105).

In the control experiments, no differences were noted in the rate of flow between the quiescent and the stimulated hearts. This interesting observation warrants clarification. In stimulated hearts the time spent in systole is increased proportionally more than the time spent in diastole when the frequency of contraction is increased. As contraction is known to decrease the rate of flow, hearts stimulated at high frequencies should show a decreased rate of flow. This was not observed and dilatation of the coronary vessels must have occurred, which may have been due to the increased metabolic activity and the release

of metabolites, such as lactic acid and CO_2, associated with greater tension development per unit time in the stimulated hearts. This observation suggests that no direct relationship exists between the rate of flow and the intracellular penetration of sugar in these experiments, since with equal flow rates, highly significant differences in penetration between quiescent hearts and those stimulated at 240 beats/min could be found.

In the presence of ouabain, the rate of coronary flow was reduced in the quiescent as well as in the stimulated hearts compared to the controls, which was surprising since cardiac glycosides increase the rate of tension development and so decrease the time hearts spend in systole (Sonnenblick, et al., 106; Mason and Braunwald, 107). Thus, an increase in the rate of flow would be expected. However, it has been shown that ouabain has a direct vasoconstrictor action (Braunwald, et al., 108; Page, et al., 109) which explains the approximately equal decrease in flow rates in both the quiescent and the stimulated hearts. Although a slight, but not statistically significant decrease in sugar penetration was seen in the ouabain treated quiescent hearts, as compared to their controls, the highly significant increase in sugar penetration in hearts stimulated at 180 beats/min also indicates that flow was not a limiting factor in sugar penetration in the ouabain treated isolated perfused hearts.

RELATIONSHIP BETWEEN SUGAR PENETRATION AND BREAKDOWN OF HIGH ENERGY COMPOUNDS

The possibility that the breakdown of high energy compounds during contraction is associated with intracellular sugar penetration must be considered. The load on the muscle, i.e., the total energy requirement for a certain period of time, consists of two components: the energy required for the mechanical work in ejecting the stroke volume and the energy required for maintenance. The latter factor, called the time-tension integral, is proportional to the tension developed and is at least 30 times greater than the energy required for mechanical work. The time-tension integral is increased by such factors as an increase in heart rate or an increase in blood pressure (Burton, 110). Van Citters, et al., (111) have measured the maintenance metabolism, as reflected by the oxygen consumption, in non-working dog hearts stimulated at various rates in a modified Langendorff preparation. Variations in heart rate in this preparation should neither affect the diastolic volume nor the amplitude of contraction and should give an estimate of the time-tension integral. Heart rate and oxygen consumption showed a linear relationship and by extrapolation of the measurements, a value for cardiac oxygen consumption in quiescent hearts was obtained. The increase in oxygen consumption in this preparation with increased frequencies of stimulation was very low. Theoretically, hearts perfused by the Langendorff technique do

not exert any mechanical work but energy will be expended during contraction to create tension in the ventricular wall. However, Neely, et al., (41) have shown that in this preparation the ventricular pressure as well as the aortic pressure increased with each contraction and fluid was ejected during each systole. Their study on the observed pressure changes suggested that intraventricular filling took place during diastole and was either caused by incompetence of the tricuspid valve due to its distortion by the cannula or by the deep coronary drainage. The contribution of the atrioluminal vessels, the atriovenous anastomoses and the Thebesian veins to intraventricular filling is not known, although its importance in failing hearts has been emphasized by Bartelstone, et al., (112). High potassium losses have been observed during the course of experiments with isolated hearts (Areskog, Arturson and Grotte, 113) which are probably in some failure because of the lack of sympathetic tone. The observed intraventricular filling and subsequent ejection of perfusate in our experiments may have contributed to the total energy expenditure of the stimulated hearts.

The relationship between oxygen consumption and heart rate in working hearts, as determined in a heart-lung preparation, shows a steeper slope than in non-working hearts (Van Citters, et al., 111), thus, the difference in energy expenditure between quiescent hearts

and those stimulated at 180 and 240 beats/min may have been greater than expected for a Langendorff preparation. Since no significant increase was found in sugar penetration between quiescent hearts and those stimulated at 180 beats/min, the highly significant increase in penetration when hearts were stimulated at 240 beats/min cannot be due to an increase in the breakdown of high energy compounds associated with higher contraction rates.

This conclusion is consistent with that of Holloszy and Narahara (37). In skeletal muscle, they dissociated the breakdown of high energy compounds, as expressed by tension development, from the degree of intracellular sugar penetration. When the maximum peak tension obtained by stimulation of frog muscle in a nitrate incubation medium was decreased to below that of the control muscles, sugar penetration was still greater than that measured in the controls. This suggested that the development of tension and therefore the energy expenditure was not associated with the increased sugar penetration.

SUGAR PENETRATION IN QUIESCENT HEARTS

In the absence of any factors such as insulin, contraction and anaerobiosis, which affect the sugar-carrier system, the intracellular penetration of sugar in the heart is similar to that found in skeletal muscle. In quiescent 'intact' rat hemidiaphragms, inhibition of the sodium pump by high concentrations of cardiac glycosides or aglycones

or by a potassium-free incubation medium have been shown to increase the penetration of 3-O-methyl-D-[^{14}C] glucose. The increased penetration was mediated by the sugar-carrier system since it was inhibited by phlorizin and was not additive to maximal penetration induced by insulin (Bihler, 85). Diphenylhydantoin and low concentrations of cardiac glycosides which were shown to stimulate the sodium pump inhibited the intracellular penetration of sugar in the same preparation (Bihler, 114). It has yet to be determined if the change in the intracellular concentration of either sodium or potassium or of both ions is responsible for the altered carrier activity. The sugar-carrier system could, of course, also be regulated by some other ion, whose concentration in a particular intracellular compartment is dependent upon the sodium pump. Since the sodium pump determines the intracellular sodium concentration, it also affects the concentration of calcium ions. Baker and coworkers (Baker, et al., 115; Baker and Blaustein, 116) have shown in nerve axons that calcium influx is enhanced when the intracellular sodium concentration is increased, while Kahn (117) has suggested that sodium ions can displace calcium ions from the sarcoplasmic reticulum in heart muscle. The relationship between cardiac glycosides and ion transport was first demonstrated by Schatzmann (63) who found that digitalis inhibited the active transport of sodium and potassium through the cell membrane of ery-

throcytes. It was later shown by other workers that cardiac glycosides also affected active ion transport in a variety of tissues. Repke (118) has found that low concentrations of ouabain stimulate the transport ATP-ase activity of guinea pig heart muscle while high concentrations inhibit this enzyme. Palmer, Lasseter and Melvin (119) demonstrated the same relationship between inhibition and stimulation of the ATP-ase and the concentration of ouabain. It is generally accepted that this ATP-ase is associated with the sodium pump and the maintenance of the ionic gradients across cell membranes (Skou, 120, 121).

Although no comparable study has been made with heart muscle, our experiments with ouabain suggest that a similar mechanism for the regulation of the sugar-carrier system by the sodium pump exists in the heart during diastole. The decrease in sugar penetration found in quiescent hearts in the presence of low inotropic concentrations of ouabain, although not significant, suggests that the glycoside may have decreased the activity of the sugar-carrier system, while the high toxic concentrations stimulated the carrier system, increasing the intracellular penetration of the sugar.

The relationship between the positive inotropic effect of cardiac glycosides and their effect on ion transport remains a controversial issue. The increased force

of contraction induced by the glycosides has been correlated with a net loss of myocardial potassium (Hajdu and Leonard, 122). However, the work of Vick (123) and of Brown and Grupp, (in Kahn, 117) has shown that a reduced potassium reentry is not an obligatory requirement for the positive inotropic effect of the glycosides. The latter investigators demonstrated a net gain in cardiac potassium in the dog heart-lung preparation with inotropic concentrations of dihydro-ouabain. Toxic concentrations of dihydro-ouabain produced a net potassium loss in this preparation. Sherlag, et al., (124) have shown that the same positive inotropic effect with digitalis can be obtained in dogs in situ after pretreatment with diphenylhydantoin which decreased the rate of myocardial potassium efflux. Palmer and his associates (119), on the basis of their own work and that of Okita, et al., (125) have suggested that in vivo, the positive inotropic effect of cardiac glycosides may be causally related to stimulation of ion transport rather than to inhibition of the sodium pump. Therefore it seems that although potassium loss does occur during the course of experiments with isolated hearts, it is doubtful that this loss is greater in ouabain treated than in the control preparations. Although the mechanism of action for the inotropic effect of cardiac glycosides is unknown, low concentrations of the drug will increase the strength of contraction of the heart.

Since contractile force cannot be affected in quiescent hearts, inotropic concentrations of ouabain will only stimulate the sodium pump while toxic concentrations will inhibit ion transport. It can therefore be postulated that during diastole, the activity of the sugar-carrier system is regulated by the activity of the sodium pump in the same manner as in skeletal muscle.

SUGAR PENETRATION IN STIMULATED HEARTS

Holloszy and Narahara (104) have shown that the increased sugar penetration found with exercise is not primarily due to changes in blood flow. In isolated frog sartorius muscle, they demonstrated a direct relationship between intracellular sugar penetration and the frequency of stimulation, within a specific range. The degree of sugar penetration was approximately proportional to the stimulation frequency.

In the heart, a similar beat-dependent relationship between sugar penetration and the frequency of contraction was not found in this study. No significant difference in intracellular sugar penetration in quiescent hearts and those stimulated at 180 beats/min could be seen, although at stimulation rates of 240 beats/min sugar penetration was significantly increased. The absence of a beat-dependent activation of the sugar-carrier in cardiac muscle could be explained if sugar penetration in the quiescent hearts is higher than expected for basal, non-

stimulated uptake. The values obtained were, in fact, higher than those found by other workers for isolated spontaneously beating rabbit hearts (Bihler, et al., 5). Morgan, et al., (102) have studied the effects of ventricular tension development on glucose uptake in isolated working rat hearts. Their extrapolated value of glucose uptake at zero tension was extremely low, about 4% of the value found under anaerobic conditions at the same glucose concentration (16 mM). Correspondingly low values for intracellular sugar penetration in our quiescent hearts would have made the difference in penetration between quiescent hearts and those stimulated at 180 beats/min significant. Therefore, a direct relationship between the percentage of sugar penetration and the frequency of stimulation, similar to that measured in skeletal muscle, would then be obtained. As Morgan suggests, any measurable concentration of intracellular metabolizable sugar may indicate that the carrier system has been activated. Although some penetration and accumulation of L-arabinose is to be expected, since this sugar is not metabolized, the high values obtained in quiescent hearts suggest that carrier activation may have taken place. In the quiescent hearts, perfusion may have been less efficient, leading to some hypoxia and a subsequent increase in sugar penetration.

Lowering of the temperature of the isolated hearts and the perfusion media from body temperature will slow down

all energy-requiring processes. Although sugar penetration has a high temperature coefficient (Park, et al., 14) this process is little affected in isolated, perfused, spontaneously beating rabbit hearts when the temperature is lowered from 37°C to 30°C (Bihler, unpublished results). Schatzmann (63) has shown that the sodium pump is indeed inhibited when cooled, thereby decreasing the ionic gradients across the plasma membrane. Inhibition of the sodium pump has been correlated with an increase in sugar penetration and the low temperature at which the experiments were carried out may to some extent be responsible for the high degree of sugar penetration in the quiescent hearts. However, these direct and indirect opposing effects on intracellular sugar penetration caused by a decrease in temperature may counteract each other and the net result would be uncertain to predict.

POSTULATED MECHANISM FOR CARRIER ACTIVATION IN STIMULATED HEARTS

An extremely interesting contradiction is seen with respect to the effect of the sodium pump on sugar penetration. Toxic concentrations of ouabain increase sugar penetration in quiescent and in stimulated hearts, whereas low concentrations, which produce a positive inotropic effect, have a tendency to decrease sugar penetration in quiescent hearts. This may be related to the activation of the sodium pump. In contrast, in stimulated

hearts the sodium pump is also activated since it must cope with sodium influx during each depolarization, and still the intracellular penetration of sugar is not decreased, but increased. These results suggest that the activity of the sodium pump and consequently the concentrations of potassium and sodium ions in a particular cellular compartment are not directly involved in the regulation of intracellular sugar penetration in stimulated hearts.

Therefore, events associated with the positive inotropic effect induced by the glycoside must be responsible for the increased sugar penetration in stimulated hearts. Kreisberg and Williamson (81) have linked stimulation of intracellular sugar penetration to the increased force of contraction caused by ouabain. In frog skeletal muscle, Holloszy and Narahara (37) have demonstrated that muscle potentiators which increase the force of contraction, also increase the rate and degree of sugar penetration. Furthermore, when potassium contractures were elicited in a high calcium medium which increased the tension development, sugar penetration was correspondingly increased (Holloszy and Narahara, 38). Since no relationship exists between the breakdown of high energy compounds in contraction and sugar penetration, one or more of the events in excitation-contraction coupling may be involved in the activation of the sugar-carrier system.

From the previous discussion, it appears that two mechanisms are involved in the regulation of the sugar-carrier system in the heart. In diastole, which in quiescent hearts is the entire perfusion period, the activity of the sodium pump may regulate the degree of sugar penetration as in skeletal muscle. During systole, a second contraction-dependent mechanism activates the sugar-carrier system and much higher degrees of intracellular sugar penetration were obtained in contracting hearts. This mechanism is therefore probably of greater physiological importance.

THE EFFECT OF OUABAIN ON SUGAR PENETRATION IN STIMULATED HEARTS

A possible explanation for the mechanism which activates the sugar-carrier system in contracting cardiac muscle has been obtained by the use of ouabain. The ouabain-treated hearts were stimulated during preperfusion in the presence of the cardiac glycoside since the drugs are ineffective in non-contracting preparations (Moran, 70). Glucose was added to the preperfusate as an energy source for a possible binding or transport step required for its action (Bailey and Dresel, 75; Bihler, 85). Ouabain increased the intracellular penetration of sugar in hearts stimulated at 180 beats/min to the same degree as was found in control hearts stimulated at 240 beats/min. In the control experiments, the percentage of sugar pene-

tration in hearts contracting at a rate of 180 beats/min was not significantly different from the quiescent hearts, while in the ouabain experiments this increase in sugar penetration was highly significant.

Of the known actions of the cardiac glycosides on the heart, the electrophysiological effects, such as changes in refractory period and conduction velocity, are unlikely to affect the sugar-carrier mechanism.

Apart from its action on the sodium pump, ouabain also affects the flux of other ions. In 1918, Loewi suggested that the cardiac glycoside strophanthin sensitized the heart to calcium. Wilbrandt and Caviezel (in Holland and Sekul, 126) subsequently showed that K strophanthoside prevented the loss of ^{45}Ca from the frog ventricle. The work of Holland and Sekul (126) and Sekul and Holland (127) on isolated rabbit atria has further shown that ouabain increases the influx of ^{45}Ca but has no effect on efflux. However, this is only found when the atria are stimulated and depolarization is a necessary requirement for this action of cardiac glycosides on calcium flux. Although ouabain induces a positive inotropic effect and affects calcium influx, it is at present unknown whether this altered ion flux is a primary cause for the increased strength of contraction or whether the effects of the drug on this ion are a consequence of the increase in contractile force. It has also

been shown that cardiac glycosides, apart from increasing the calcium flux through the plasma membrane (which is already increased during excitation-contraction coupling) are capable of causing the release of calcium from the sarcoplasmic reticulum in vitro (Lee, 128; Lee, Yu and Struthers, 129). Because the activation of the sugar-carrier system is beat-dependent and since ouabain has been shown to increase calcium influx in contracting heart muscle, it is reasonable to suggest that the regulation of the sugar-carrier system may be influenced by a change in an intracellular calcium pool or compartment. Kreisberg and Williamson (81) have determined that the effects of ouabain on sugar penetration are dependent upon changes in calcium transport or on the myoplasmic calcium concentration. They found that inotropic concentrations of ouabain had the same effect on several parameters of myocardial metabolism in the isolated perfused rat heart as an increase in the extracellular calcium concentration. However, when the calcium concentration in the perfusate was reduced by half, the addition of the same concentration of ouabain had no effect on the penetration of glucose or on myocardial metabolism, possibly because a positive inotropic effect could not be obtained due to insufficient calcium. The magnitude of calcium influx has been shown to be a function of the external calcium concentration (Langer and Brady, 55) which in turn

determines the strength of contraction (Nayler, 44). The observed changes were most likely due to the same mechanism, an increase in the force of contraction associated with a change in the myoplasmic calcium concentration. Some of the literature on the relationship between the metabolic effects of cardiac glycosides and the positive inotropic effect of the drug is difficult to evaluate (Wollenberger, 76; Lee, et al., 77). Coleman (130) found no difference in the oxygen consumption in quiescent dog papillary muscle in the presence and absence of acetyl-strophanthidin. An increase was shown during contraction, indicating that the increased metabolism may be a consequence of the greater energy expenditure associated with the positive inotropic effect of the cardiac glycosides. This had previously been demonstrated by Kreisberg and Williamson (81) and Kien and Sherrod (78).

The hypothesis that an increased myoplasmic concentration of calcium is responsible both for contraction and for the activation of the sugar-carrier is very attractive. It suggests that myocardial muscle can regulate the force of contraction as well as the supply of nutrients required to produce the necessary energy by a single, common mechanism.

In our experiments, a correlation between the simultaneous increase in intracellular calcium and in sugar penetration can be seen. In quiescent hearts, the

addition of ouabain, which was found to be without effect on calcium transport in unstimulated preparations, did indeed not show an increase in the percentage of sugar penetration when compared to the quiescent controls. The ineffectiveness of the drug may be due to the absence of contraction, since cardiac glycosides can only exert their positive inotropic effect on contracting heart muscle. In hearts stimulated at 180 beats/min, the addition of ouabain, which increases the intracellular calcium concentration, also increased the percentage of sugar penetration when compared to controls. The absence of a significant increase in sugar penetration when hearts were contracting at 240 beats/min compared to 180 beats/min can be explained in two ways. Either the sugar-carrier system was maximally stimulated by the postulated change in a specific intracellular calcium pool or compartment or this change was insufficient to stimulate the carrier system to a greater extent. In the control experiments a similar relationship between the degree of intracellular sugar penetration and the myoplasmic calcium concentration can be observed. In the quiescent hearts, the high degree of penetration may have been induced by increased tension of the ventricular wall with subsequent elongation of the muscle fibres. Since calcium is required to maintain tension, its intracellular concentration may have so increased with concomittant activation of the sugar-carrier

system. In hearts contracting at 180 beats/min the intracellular calcium concentration may have been only slightly increased compared to the quiescent hearts, so that no significant increase in the degree of sugar penetration could be demonstrated. When the rate of contraction was increased, thereby further increasing the intracellular calcium concentration, the sugar-carrier system was stimulated as seen by the significantly increased degree of sugar penetration. In comparing the control with the ouabain experiments, ouabain-treated hearts stimulated at 180 beats/min should have a greater intracellular calcium concentration and stimulation of the sugar-carrier system. This was indeed seen in the significantly increased sugar penetration in the ouabain-treated hearts. At 240 beats/min no significant increase in penetration was seen between control and ouabain-treated hearts. This interpretation of our results suggests that the dual role for calcium also exists in myocardial muscle.

In skeletal muscle, a similar correlation between intracellular sugar penetration and the myoplasmic calcium concentration has been put forward. Holloszy and Narahara (38) have summarized their evidence as follows: contraction has been shown to increase the intracellular penetration of sugar (Holloszy and Narahara, 104) but depolarization is not necessarily required for this since low concentrations of caffeine induce contractures and also increase sugar

penetration without depolarizing the plasma membrane (Axelsson and Thesleff, 131). The influx of calcium during potassium contractures is related to the extracellular calcium concentration (Lorkovic, 40). A direct relationship between the extracellular calcium concentration and the degree of sugar penetration induced by potassium contractures could also be shown. Their work with nitrate ions (Holloszy and Narahara, 37) provides further evidence. These ions potentiate twitch tension and also increase sugar penetration in skeletal muscle. Ebashi, Otsuka and Endo (132) have reported that these muscle potentiators can prevent the reaccumulation of calcium ions by the sarcoplasmic reticulum, thereby increasing the myoplasmic calcium concentration while Bianchi and Shanes (54) demonstrated increased calcium influx in frog sartorius muscle when nitrate was present in the incubation medium. It was also found that nitrate ions could not increase the degree of sugar penetration above that obtained by high stimulation rates. It is interesting to note that nitrate ions have no effect on the force of contraction in myocardial muscle (Nayler, 133). The above evidence strongly suggests that the intracellular calcium concentration is also involved in the activation of the sugar-carrier system in skeletal muscle.

However, in frog skeletal muscle, it is unlikely that the myoplasmic calcium pool is directly involved in

carrier activation since the increase in myoplasmic calcium concentration after depolarization is only of short duration (Sandow, Taylor and Preiser, 134) while sugar penetration remains elevated for a considerable time after a period of stimulation. The calcium ions may trigger another reaction with a slower time course which in turn activates the carrier (Holloszy and Narahara, 38). The presence of this secondary slow step in skeletal muscle is not necessarily present in myocardial muscle and may reflect differences in the regulation of the sugar-carrier between the two types of muscle. It should also be considered that this slow step may be a difference in carrier regulation between coldblooded and warmblooded species.

The interpretation of the pooled data seems to favour a change in an intracellular calcium pool in the regulation of the sugar-carrier system. However, the results obtained in hearts perfused with the three different calcium media, which could not be analyzed statistically, did not show the same relationship between sugar penetration and the tested parameters.

In hearts perfused with the half calcium buffer an increase in sugar penetration was observed between hearts stimulated at 180 and at 240 beats/min, but penetration in quiescent hearts was the same as that found in those stimulated at 180 beats/min and was much higher than found with the normal calcium buffer. In the presence of

ouabain, sugar penetration was similar in hearts perfused with normal calcium or half calcium buffer at the three contraction frequencies. Kreisberg and Williamson (81) were also unable to show differences in cardiac metabolism when the calcium concentration of the perfusate was halved to 0.635 mM while the addition of ouabain with this calcium concentration had no effect.

With the low calcium buffer an increase in sugar penetration was observed between quiescent hearts and those stimulated at 180 beats/min. No further increase was noted when the contraction frequency was increased to 240 beats/min. With ouabain, sugar penetration was related to the frequency of contraction. In quiescent hearts and those stimulated at 240 beats/min the values obtained were, however, higher than found with the other calcium media. With this buffer, the calcium concentration may have been too low to maintain the hearts in their proper functional state so that the results obtained did not show the normal physiological relationship between sugar penetration and changes in an intracellular calcium pool.

SUMMARY

The results suggest that in cardiac muscle the activity of the sugar-carrier system is regulated by two different mechanisms which affect the carriers during diastole and systole respectively. In diastole, the rate of activity of the sodium pump is inversely related to

the degree of sugar penetration. Inhibition of the sodium pump will activate the sugar-carrier system while stimulation of the sodium pump will inhibit the sugar-carriers. This mechanism appears to be similar to that found in skeletal muscle and may involve changes in an intracellular calcium pool or compartment. It probably plays a minor role in the regulation of intracellular penetration of sugar in the heart. The second mechanism is contraction-dependent and regulates the degree of sugar penetration during systole. The increase in the intracellular calcium concentration during each depolarization which activates the contractile mechanism may also affect the sugar-carrier system. The specific intracellular calcium pool which is so affected may be similar to the one postulated to be involved in carrier regulation during diastole. This second mechanism could be responsible for the increased extraction of carbohydrate substrate required with an increased work load.

SECTION V

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BIBLIOGRAPHY

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