MEMBRANE TRANSPORT OF SUGARS IN VARIOUS MAMMALIAN TISSUES AND THE INTEGRATION OF TRANSPORT WITH CELLULAR METABOLISM.

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ABSTRACT

The pathway by which various monosaccharides enter the lens of the eye in rats has been investigated. This organ is avascular and very suitable for in vitro studies. Using non-metabolized 3-O-methyl-D-glucose (3-O-MG) as test sugar, it was shown that even after 20 hours of incubation no active accumulation of sugar against its concentration gradient had taken place; the time curve was typical for an equilibrating process. The concentration dependence of transport was determined by measuring the initial rate of influx. This rate was shown not to be linearly related to the concentration. The transport process was therefore inconsistent with diffusion but conformed to the kinetics of mediated transport (facilitated diffusion). The V_{max} and K_m of transport were determined by various graphic procedures and by the method of Bliss and James (1966). Depending upon whether it is assumed that the sugar concentration in the extracellular space is in equilibrium with that in the incubation medium, minimum and maximum estimates for these constants were determined. The mean of these values was V_{max}, 28 μmole.ml^{-1}.15 min^{-1}. and K_m, 88 mM. The chemical specificity of the transport system was determined by comparing the penetration of various sugars and by determining their inhibitory effect on the penetration of 3-O-MG. The specificity pattern was very similar to that found in other tissues where mediated transport of sugar takes place. The apparently active transport of D-xylose and D-galactose was shown to be due to the accumulation of radioactive metabolites rather than of free sugar. The kinetics, chemical specificity and competition between pairs of sugars are all consistent with mediated transport as

the pathway for sugar entry; inhibition of sugar penetration by phlorhizin and phloretin also agrees with this interpretation. Phloretin was found to act in the lens as a non-competitive inhibitor with a K_i of approximately 0.6 mM.

Insulin did not stimulate the transport of 3-O-MG *in vivo* and *in vitro* or after decapsulation of the lenses by trypsin and collagenase. These enzymes by themselves also had no effect. In contrast to skeletal muscle, inhibition of the sodium pump or uncoupling of oxidative phosphorylation did not increase sugar penetration while the decrease observed in the absence of external sodium ions appeared to be due to a nonspecific effect on cellular membranes. The effect of sulfhydryl group reagents was also nonspecific. The absence of any regulatory mechanism suggests that the sugar transport system in the lens of the eye is similar to that found in the mature mammalian erythrocyte.

The results obtained with the lenses together with data published for other tissues suggest that the regulation of sugar transport is correlated with the metabolic requirements of tissues. As a general rule, in tissues where glucose utilization is stable, free intracellular glucose is present and the transport system has a more than sufficient capacity to supply the required substrate. Tissues which belong in this category are the mature mammalian erythrocyte, the lens of the eye and the central nervous system. Transport is also not rate-limiting in the liver where the metabolic rate is variable; this may be related to the large capacity of the hepatic transport system required for the release of glucose into the bloodstream. On the other

hand, in tissues where glucose utilization is variable, its transport across the cellular membrane is rate-limiting. Examples are skeletal, cardiac and smooth muscle, adipose tissue and polymorphonuclear leukocytes. In these tissues the sugar transport system is regulated by the same factors which alter cellular metabolism. These include modulators of functional activity, such as muscular contraction, and of the deposition of energy reserves. This classification of activity and storage regulation applies to muscle but not to adipose tissue whose main activity is storage of reserves.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SECTION I

INTRODUCTION

The cell membrane must meet two important requirements to ensure cellular integrity and function. First of all, it must be selectively permeable to substances which are required to maintain the specific intracellular aqueous medium. Secondly, substrates required in cellular metabolism must be able to penetrate rapidly enough to ensure their adequate supply under all necessary conditions; conversely, metabolic waste products must be able to leave the cells at a sufficiently rapid rate to prevent their accumulation to toxic levels. Although the nature of the cell membrane has been the subject of intense investigation, the detailed structural arrangement of its various components has not yet been elucidated. With regard to the entry of hydrophilic molecules, the membrane may be considered as a lipid barrier with other, presumably proteinaceous regions. Substances may cross cellular membranes by various pathways such as diffusion through the membrane matrix or aqueous channels. In addition, the existence of specialized transport processes has been postulated to account for the characteristic kinetics and other properties which suggest that some substances interact with a membrane component. These transport systems permit a much faster entry of certain hydrophilic solutes than could be achieved by diffusion. From the point of view of energy requirements, two types of specialized transport can be distinguished, active transport and facilitated diffusion. The active transport systems can translocate substances against their electrochemical gradient and require metabolic energy. An active transport system for sugars is present in the intestine and kidney,

while amino acids are actively transported in many other tissues as well. On the other hand, facilitated diffusion leads only to equilibration of the substrate concentrations across the cell membrane and is independent of metabolic energy. This appears to be the process by which simple monosaccharides cross cellular membranes in those tissues in which active transport does not take place. This thesis deals with the facilitated diffusion of sugars in various tissues. This process is of particular interest because of the importance of glucose as a metabolic substrate.

Diffusion, whereby a substance moves passively from a region of high concentration to one of lower concentration, is described by Fick's equation (Fick, 1855). It predicts that the rate of penetration into the cell is directly proportional to the concentration gradient of the sugar across the cell membrane, regardless of whether diffusion takes place through aqueous channels or through the membrane substance. The permeability coefficients should be independent of the gradient and should not vary with the concentration. It is also to be expected that the rates of entry for sugars with similar physicochemical properties should be very close to each other. Early studies already showed that diffusion could not account for the entry of sugars into erythrocytes. Neither the chemical specificity of sugar transport in human erythrocytes observed by Kozawa (1914) nor the dependence of the permeability constants for glucose entry on the sugar concentrations (Ege, 1919, as quoted by Bang and Ørskov, 1937) conform to the laws of diffusion. Further work by LeFevre (1948) suggested that sugar transport across the erythrocyte membrane occurs

in both directions across the cell surface. These observations, as well as other work, led to the development of the concept of facilitated diffusion of sugar, which is characterized by saturation kinetics, reversibility and chemical specificity. These properties are accounted for by the carrier hypothesis, first proposed by LeFevre (1948) and extended, amongst others, by Widdas (1952, 1954) and Rosenberg and Wilbrandt (1952). Although only equilibration of concentrations across the cell membrane occurs, the rate of penetration of the hydrophilic sugar molecules is much faster than would be expected by diffusion. According to the carrier hypothesis, sugar molecules are thought to combine with membrane components, carriers, of which only a limited number are available at the outer membrane surface. The carrier-sugar complex (CS complex) is then thought to diffuse across the cell membrane and to dissociate again at the inner surface. Efflux is thought to occur by the same mechanism, and the carriers therefore operate reversibly; whether the carriers indeed move across the cell membrane is not known but their alternate availability at the two membrane faces is an essential characteristic of the hypothesis.

The kinetic treatment of carrier-mediated transfer is based on the assumption that the rate of diffusion of the carriers and the CS complexes through the cell membrane is very much slower than the formation and dissociation of these complexes. This step is therefore rate-limiting in facilitated diffusion. For human erythrocytes, this assumption was verified by the findings of LeFevre (1962) and Regen and Morgan (1964). In addition, it is assumed that the carriers are present at the extracellular and the intracellular interfaces, for

a time long enough for equilibration with the substrate. It follows that the rate of unidirectional flux is related to the number of loaded carriers moving in one direction across the cell membrane. Net transport will therefore be directly related to the difference in the number of loaded carriers moving in one direction and those moving the opposite way (Widdas, 1952, 1954). Apart from the number of carriers involved, unidirectional and net flux will, of course, also depend upon their mobility. This term describes the rate of diffusion of the carriers and the CS complexes within the membrane between the two interfaces.

The equilibrium between carrier and substrate at the membrane interfaces can be described by Langmuir's Adsorption Equation (1916) which has essentially the same form as the equation describing the chemical combination between enzymes and substrate; in either case one component, the surface area or the amount of enzyme, determines the reaction velocity. It is not surprising, therefore, that the kinetics of carrier-mediated transport are formally identical to the classical kinetics of enzyme reactions. However, this treatment should not imply that enzymes are involved in transport. In the words of Christensen and Palmer (1967): 'Had transport kinetics been investigated earlier, we might now be expressing surprise that enzymes show transport kinetics'.

Based on the suggestion of Henri (1902) that enzymes catalyze biological reactions through the formation of an intermediary enzyme-substrate complex (ES complex), Michaelis and Menten (1913) formulated the mathematical expression for enzyme-catalyzed reactions. This equation, $v = V_{max} \frac{S}{S + K_m}$, is known as the Michaelis-Menten equation. The two constants, the V_{max} and the K_m, quantitatively describe the enzyme-catalyzed

reaction. The V_{max} is the maximum reaction velocity at infinite substrate concentration. This parameter represents the capacity and is dependent upon the amount of enzyme present. In contrast, the K_m is an inherent characteristic of the enzyme and is independent of concentration; it indicates the affinity of enzyme for substrate. In the derivation of the Michaelis-Menten equation it was assumed that the rate of dissociation of the ES complex into free enzyme and product was negligible compared to its dissociation into the original reactants. This is the equilibrium assumption and under these conditions, the K_m would then have the special meaning of being the dissociation constant, K_s, of the ES complex. However, Briggs and Haldane (1925) proposed a steady-state approximation: After a very short initial period, the change in the concentration of the ES complex would be extremely small compared with the changes in concentrations of the substrate and product. The final rate equation derived on the basis of this assumption is formally identical with the Michaelis-Menten equation but the K_m is no longer equal to the K_s. Since the ES complex can dissociate into free enzyme and product as well as into free enzyme and substrate, it includes a term with the rate constants for the transformation of substrate to product.

When these concepts are applied to the kinetics of carrier transport, the capacity term, the V_{max}, is a function of the number of carriers present, and their mobility.

The K_m describes the magnitude of the interaction between carrier and substrate for transport. This constant is formally analogous to the affinity term of an enzyme-mediated reaction but is not quite identical; its meaning depends upon the assumption of the model for

facilitated diffusion. With the equilibrium assumption, when the rate of diffusion of the free carriers and the CS complexes is rate-limiting, the K_m is equivalent to the dissociation constant of the CS complex. This interpretation appears to be valid for certain transport processes such as that for monosaccharides in rabbit erythrocytes (Kotyk and Janáček, 1970). On the other hand, under the steady-state assumption, where the movement of the carriers and CS complexes is not necessarily rate-limiting, the physical meaning of K_m becomes very complex. Since CS complex formation is followed by translocation of the complex through the cell membrane, this term will include a contribution of this factor. Fisher and Gilbert (1970a, 1970b) have shown that the affinity term is a function not only of association and dissociation but also of the mobility of the CS complex. The term 'apparent affinity' is best used to describe this parameter, which is best defined operationally as that substrate concentration at which transport proceeds at half-maximal velocity. Although the kinetic parameters are extremely useful to describe facilitated diffusion, they are not sufficient to indicate the physical processes which take place in the mediated transport of sugars.

When the initial velocity is plotted against substrate concentration, a hyperbola is obtained which is characteristic for mediated transport and for enzyme-catalyzed reactions. The dependence of the initial velocity upon the substrate concentration changes with increasing concentrations. When the sugar concentration is low in relation to the K_m, the process approaches first order kinetics as indicated by an initial linear section, while in the curved part the

velocity is related to concentration as described by the Michaelis-Menten equation. In the plateau phase, the substrate concentration is very high with respect to the K_m and the rate is independent of concentration. The failure to distinguish diffusion from mediated transport in some tissues may have been due to the choice of experimental conditions. When the sugar concentrations used are low compared to the K_m, the measurements will take place in the initial, virtually linear portion of the curve. To observe saturation of the transport sites the concentrations of test sugar have to be sufficiently high in relation to the K_m (Wilbrandt and Rosenberg, 1961).

So far, the sugar transport system has been discussed in terms of the original mobile carrier mechanism. This simple model is, however, no longer in complete agreement with the more recently obtained precise quantitative data. Miller (1968a, 1968b) has summarized the objections to the carrier model. These include unexpected discrepancies between the rate of net transport and the rate of exchange; the K_m values were found to be smaller than the K_s, and a change in temperature had anomalous effects on these parameters. In addition, the theoretically predicted values for countertransport did not quantitatively agree with experimental data. New models have been proposed to account for these observations and while these differ in important details from the mobile carrier hypothesis they are also characterized by a limited number of membrane sites to which sugars can be attached. The concept of a shuttling membrane component has, however, been abandoned. One important feature which has been incorporated is that the mobilities of the free and complexed carriers need not necessarily be equal, and

experimentally two maximum velocities have indeed been distinguished for sugar transport in human erythrocytes. Since the CS complex diffuses faster than the free carrier, the greatest velocity is obtained when substrate is present at both membrane interfaces (Levine et al., 1965; Levine and Stein, 1966). The newer models also differ from the carrier hypothesis in that sugar is supposed to interact with more than one binding site while crossing the cell membrane. In the model of Lieb and Stein (1971) two types of binding sites, of low and high affinity for the sugar, respectively, are present at each of the interfaces; within the membrane transfer of the sugar is thought to take place from a site at one interface to a site at the other interface. A similar type of arrangement has been suggested by LeFevre (1971) but in his model only one type of binding site is required at each of the membrane interfaces. On the other hand Naftalin (1970) proposes that several binding sites are present within a 'channel' across the membrane.

As far as has been determined, most of these models are able to satisfy some but not all of the objections to the carrier hypothesis and it is at present not known whether all the inconsistencies can be explained. Because of these developments, the term carrier will be replaced in this discussion by 'transport site' and the membrane transport process will be described as either mediated transfer (transport) or facilitated diffusion.

Experimental evidence for mediated transport

Historically, the concept of facilitated diffusion was first developed to explain sugar transport in erythrocytes and was subsequently found to apply to the transport of various substances in many tissues.

The evidence for facilitated diffusion of sugars in mammalian tissues will be briefly reviewed below.

Kinetic evidence

Saturation kinetics for sugar transport have been demonstrated in a large number of tissues including skeletal and cardiac muscle, erythrocytes of various species, ascites tumour cells, fibroblasts and others (see reviews by LeFevre (1961) and Stein (1967)). The kinetic parameters have been determined for many of these tissues in several species and have been listed by Stein (1967). Although the V_{max} and K_m are useful in comparing the characteristics of transport systems for sugars in various tissues, the numerical values, particularly those of the K_m, are difficult to interpret. The problem in the interpretation of the K_m as an equilibrium constant or a steady-state constant has already been mentioned. The presence of a second transport system or of an additional diffusional component would further complicate their evaluation. There are also experimental difficulties in obtaining the correct values for the sugar concentration in the interstitial space; this is discussed further below. In addition, the physical existence of membrane components associated with facilitated diffusion has so far not been demonstrated. Thus, it seems safest to consider the K_m only as that substrate concentration at which sugar transport proceeds at half-maximal velocity.

Specificity of the transport system

Another observation inconsistent with diffusion as the transfer mechanism for simple monosaccharides in erythrocytes is the preference for certain sugars over others with similar physicochemical

properties. As pointed out by Park et al. (1959), diffusion through membrane 'pores' is therefore very unlikely. It is interesting that the specificity of intestinal active transport differs from that of facilitated diffusion in erythrocytes and other tissues. The transport system in the intestine has a strict specificity which includes glucose and galactose (Crane, 1960), the two sugars most important in animal metabolism. In contrast, the specificity of the facilitated diffusion systems in other tissues is much broader, and many hexoses and pentoses excluded from intestinal active transport can penetrate into these tissues (LeFevre, 1961; Wilbrandt and Rosenberg, 1961; Stein, 1967). It appears that the tissues in the interior of the body are protected by the selectivity of the intestinal transport system and do not require such a strict specificity of their own.

Competition experiments

Competition for transport will occur if two sugars both have an affinity for the same transport site and when two transported sugars share the same site, they will inhibit each other's penetration if present simultaneously. Competition between pairs of sugars has long been recognized as good evidence for mediated transport (LeFevre, 1955). Fisher and Zachariah(1961) had therefore rejected the slow diffusion hypothesis of Bronk and Fisher (1957) which was unable to satisfactorily explain competition for entry between pairs of sugars in isolated perfused rabbit hearts. This type of inhibition is analogous to competitive inhibition of enzyme reactions; it can be overcome by raising the concentration of the test sugar; the V_{max} will remain unchanged while the apparent K_m is increased. Competition experiments are extremely useful

in demonstrating facilitated diffusion of sugars with very low affinity for the transport site (Whittam, 1964) and they have been the main tool for deriving the specificity pattern of transport systems.

Countertransport

The most definitive evidence for mediated sugar transport is the demonstration that competition between pairs of transported sugars can lead to transport of one of these sugars against its own concentration gradient. This process is called countertransport or counterflow. Countertransport can be induced by the addition to the medium of a second sugar, after a first non-metabolized sugar has equilibrated between tissue and medium, i.e. when its influx equals efflux. Competitive inhibition will decrease the influx of the first sugar but, at least initially, not its efflux. This sugar therefore leaves the cells against its concentration gradient. Counterflow can best be demonstrated when the second sugar is metabolized by the tissue, keeping its intracellular concentration at a low level. Countertransport has been demonstrated in erythrocytes (Rosenberg and Wilbrandt, 1957a), in isolated perfused rat hearts and in rat diaphragm and gastrocnemius muscle *in vivo* (Morgan *et al.*, 1964) and, more recently, in the brain (Buschiazzo *et al.*, 1970; Cutler and Sipe, 1971).

Counterflow against the concentration gradient takes place without the expenditure of metabolic energy; the energy for driving the first sugar 'uphill' is derived from the simultaneous 'downhill' influx of the second sugar.

Temperature coefficient

The ratio of penetration rates at two temperatures differing

by ten degrees is usually slightly above one for diffusion. For sugar transport the values of Q_{10} are generally between two and three suggesting that, as opposed to diffusion, sugar transport involves reactions with a fairly high energy of activation. However, a large Q_{10} might also result from certain types of interactions between the solvent and a diffusing substrate (Davson, 1960).

The above lines of evidence favour the acceptance of facilitated diffusion as the mechanism for transfer of sugars across many types of cellular membranes. In summary, the transport of monosaccharides is much faster than would be expected from their physicochemical characteristics and takes place by a system which displays saturation kinetics and chemical specificity. Except in the intestine and kidney, only equilibration of the sugar across the cell membranes takes place and metabolic energy is not required.

Even though all the properties of facilitated diffusion can best be explained by transient binding of the sugar to a membrane component, attempts to isolate and identify these sites in animal tissues have not been very successful (LeFevre et al., 1964; Bobinski and Stein, 1966; Baker, 1967). Very recently, Kahlenberg et al. (1971) have reported selective binding of D-glucose to isolated human erythrocyte membranes. In bacteria, however, binding proteins which play a role in the transport of certain simple sugars, amino acids and sulfate, have been isolated and characterized (for review see Kaback, 1970).

Regulation of the activity of the sugar transport system

For a sugar such as glucose the overall uptake process can conveniently be divided into three separate steps: Diffusion from the

capillaries to the cell membrane, the actual penetration through the membrane and metabolic transformation, e.g. phosphorylation by hexokinase. Diffusion of sugars from the vascular space to the cellular membranes does not appear to be limiting under normal physiological conditions. As outlined by Park et al. (1959) there normally is no restriction of glucose movement through the capillary wall and the extracellular space. This step can, however, become rate-limiting when low perfusate concentrations of glucose are used, as has been found in isolated perfused rat and rabbit hearts (Morgan et al., 1961; Bihler et al., 1965). In skeletal and cardiac muscle the rate-limiting step in the utilization of glucose usually is the transfer across the cell membrane, and in these tissues no appreciable free intracellular glucose pool is present. In these muscles, and in adipose tissue, any change in glucose utilization necessitates an alteration in the activity of the transport system; many hormonal, metabolic and other factors are capable of doing so. When glucose penetration is maximally stimulated by insulin, appreciable amounts of free glucose will be found in tissues such as the heart, while with an identical increase in sugar penetration induced by anoxia, no free sugar will be present. These results are interpreted to mean that stimulation of sugar transport by factors such as anoxia is accompanied by an increase in the activity of hexokinase; with factors such as insulin, free intracellular glucose will be found and phosphorylation now becomes the rate-limiting step in glucose utilization (Morgan et al., 1961).

From a consideration of the kinetics of facilitated diffusion, it follows that activation of the sugar transport system requires either

an increase in V_{max} or a change in K_m. As mentioned in the preceding section, the first possibility could involve either an increase in the number of sites mediating penetration or an increase in mobility. Depending upon the model used, mobility can be interpreted as the ability of the transport site to change its conformation (flip-flop) or in the classical model, in the rate of diffusion of the CS-complex. De novo synthesis of transport sites may play a role in the effect of sex hormones on some of their target tissues but is unlikely with other factors which stimulate sugar transport, including insulin (Weis and Narahara, 1969).

Although suggested by Rosenberg (1961) it is unlikely that a stimulatory factor selectively changes either the V_{max} or the K_m. In experiments in vitro, a specific change in either of these parameters has often been demonstrated but this appears to depend both upon the conditions of the experiment and the assumptions made when determining the kinetic parameters. For instance, Morgan et al. (1961) have shown that insulin stimulates glucose transport in isolated perfused rat hearts by increasing both the K_m and V_{max}. Similarly, Bihler et al. (1965) and Fisher and Gilbert (1970a, 1970b) have reported that in isolated perfused rabbit and rat hearts, respectively, the effect of insulin could best be explained by an increase in the mobility of the transport sites which was expressed as an increase in both V_{max} and K_m.

Among the numerous unrelated agents and conditions which increase the rate of sugar penetration in tissues such as muscle, there are many which act by a specific effect on the sugar transport system rather than by a nonspecific effect on membrane permeability. When the

transport system is stimulated the stereospecific selectivity of the system remains the same and saturation kinetics can still be demonstrated (Morgan et al., 1964; Ilse, 1971). Furthermore, a stimulatory agent will not increase the rate of sugar penetration when the transport system is already maximally stimulated by another factor; on the other hand, submaximal stimulation of sugar transport by two different agents is generally additive. Thus, in frog sartorius muscle, insulin could not increase the rate of penetration of 3-O-methyl-D-glucose (3-O-MG) when the muscle was electrically stimulated to contract at a high frequency, a treatment which by itself activates sugar transport. At a lower frequency of stimulation, the addition of insulin caused a further increase in the rate of sugar transport, up to the maximum obtained by electrical stimulation (Holloszy and Narahara, 1965). While electrical stimulation will depolarize, insulin will tend to hyperpolarize the muscle membrane; the same transport system may be stimulated by agents which have otherwise very different actions on the cell membrane.

The terminology used in this thesis is as follows: The terms penetration and transport will be restricted to describe the movement of sugars across the cell membranes by facilitated diffusion. Uptake is operationally defined as disappearance from the medium and includes penetration as well as intracellular utilization; this term is analogous to penetration only when specifically indicated. When stimulation of the transport system is discussed, this should be taken as an increase from the control level under the conditions of the experiment. A true basal value is difficult to obtain because of the possible presence of

various stimulatory and inhibitory factors in isolated preparations.

The effect of hormones

INSULIN

Historically, in the study of insulin, attention was mainly focussed on the effect of this hormone on carbohydrate metabolism. It is now known, however, that insulin is a general anabolic hormone which affects a number of metabolic processes. Glycogenesis is increased by activation of glycogen synthetase in liver and muscle, and lipogenesis is also stimulated in liver and adipose tissue. In fat tissue there is also an antilipolytic effect which appears to be associated with a decrease in the level of cyclic AMP but it is at present disputed whether insulin causes this change by decreasing the activity of adenyl cyclase or by increasing the activity of phosphodiesterase (Sutherland and Robison, 1969). In addition, insulin induces the de novo synthesis of glycolytic enzymes and depresses the activity of enzymes involved in gluconeogenesis. The hormone also stimulates the transport of amino acids and enhances protein and RNA synthesis. Most of these effects are independent of the stimulatory action on sugar utilization (for review see Rieser, 1967). In this thesis we will only be concerned with this latter action of insulin.

The original work of Levine and his associates (1949, 1950) showing that insulin stimulated the transport of sugars into cells, a step preceding metabolic conversion, has been confirmed in a number of tissues (Wilbrandt and Rosenberg, 1961). The effect of this hormone on the activity of the sugar transport system can best be studied independently from its metabolic actions by the use of non-metabolized sugars.

The stimulation of sugar transport by insulin has the following characteristics: The membrane site with which insulin interacts appears not to be identical with a component of the actual transport system; both influx and efflux of sugars are equally stimulated (Park et al., 1959), while the chemical specificity of the transport system is maintained. Protein synthesis does not appear to be involved in this effect of insulin and de novo synthesis of additional transport sites may be excluded. The time course for insulin-stimulated sugar efflux from isolated perfused rat hearts indicated that the effect of the hormone was nearly instantaneous (Park et al., 1959). The evidence suggests that the effects of insulin are mediated through an interaction of the hormone with the cell membrane. Under certain conditions, enzymes such as trypsin and phospholipase, which probably are excluded from cells, have been shown to inactivate the cellular component with which insulin interacts (Blecher, 1965). Supporting evidence also comes from the work of Cuatrecasas (1969) who showed that in fat cells hormone-induced lipolysis could be inhibited by insulin covalently bound to large agarose polymers which were unable to penetrate across the cell membranes. More recently, Cuatrecasas (1971) showed an excellent correlation between the binding curve of insulin and the dose-response curve for the oxidation of glucose in rat adipocytes. Even though insulin has been shown to decrease the cellular levels of c-AMP, there is no evidence that the effect of this hormone on the sugar transport system is related to the intracellular concentration of this nucleotide. In rat adipocytes, it has been shown that some of the metabolic effects of insulin may be due to the insulin-induced assembly of microtubules (Soifer et al., 1971).

However, these structures were not associated with the stimulating action of insulin on glucose transport.

ADRENALINE

Less is known about the effect of this catecholamine on the activity of the sugar transport system. This has been investigated by following the transport of the non-metabolized sugar, 3-O-methyl-D-glucose (3-O-MG). Saha et al. (1968) have shown that in quiescent frog sartorius muscle, adrenaline stimulates the penetration of this glucose analogue, and that this could be inhibited by the β-antagonist pronethalol. In rat hemidiaphragm no consistent effects of adrenergic agents on the penetration of this same sugar were seen (Bihler, unpublished data). In cardiac muscle, the positive inotropic effect of adrenaline makes an evaluation of its effect on sugar transport very difficult since the facilitated diffusion of monosaccharides is stimulated when the force of contraction is increased (see below). The adrenaline-induced increase in glucose uptake and oxidation in isolated perfused rat hearts was explained by Williamson (1964) on the basis of the chronotropic and inotropic effects of this drug. Challoner (1970) was unable to show stimulation of sugar transport when he tried to separate the inotropic effect of adrenaline from that on sugar transport by arresting rat hearts with a high potassium medium. In these quiescent hearts the transport of 3-O-MG was greater than in beating ones, and the effect of adrenaline may have been obscured under these conditions. In rat adipocytes, adrenaline stimulates the oxidation of glucose, for which its penetration is rate-limiting, as well as the transport of the non-metabolized sugar, L-arabinose (Bray and Goodman, 1968).

Much older work indicates that adrenaline decreases glucose utilization *in vitro* (Walaas and Walaas, 1950) and *in vivo*. The latter effect could be due to several factors, such as the release of free fatty acids which will depress glucose transport and utilization. In tissues such as skeletal and cardiac muscle, the increased intracellular concentration of glucose-6-phosphate due to adrenaline-stimulated glycogenolysis will also have the effect of inhibiting hexokinase. The inhibition of insulin release by this hormone may also contribute to the effect. The mechanism by which adrenaline decreases glucose uptake in skeletal muscle *in vitro* is not well established but may also be caused by an indirect effect.

OTHER HORMONES

The stimulation of sugar penetration by insulin is without any doubt of the greatest physiological importance but other hormones appear also to affect sugar transport. Sex hormones have been shown to stimulate the transport of non-metabolized sugars *in vivo* in certain tissues. Pretreatment of rats with testosterone increased the penetration of D-xylose in the levator ani muscle but the addition of the hormone *in vitro* had no effect (Bergamini, 1969). Similarly, estrogen injection *in vivo* stimulated the transport of 3-O-MG in rat uterus while estradiol *in vitro* had no effect (Roskoski and Steiner, 1967). It has been concluded that the effects of these hormones are limited to their respective target tissues and involve *de novo* synthesis of transport sites. Under some experimental conditions, growth hormone also appears to have an insulin-like effect (Bornstein *et al.*, 1969).

The effect of electrical stimulation and contractile activity

The sugar transport systems of skeletal, cardiac and probably smooth muscle are also stimulated by factors which increase or modulate their contractile activity.

SKELETAL MUSCLE

Dulin and Clark (1961) proposed that the increase in sugar penetration during muscular exercise could be due to hypoxia. However, in vitro experiments have shown that in muscle incubated in a well-oxygenated medium, where limitations in blood flow cannot play a role, sugar transport could still be stimulated by contraction. When resting frog sartorius muscles were incubated anaerobically, the penetration of 3-O-MG was enhanced only after a considerable lag period; since the increase in sugar penetration with stimulation occurred more rapidly, this was further evidence that hypoxia was not responsible. In these muscles, the penetration of 3-O-MG could be directly correlated with the duration and frequency of stimulation. When the sartorii were stimulated at the same frequency with different work loads, the rate of penetration of sugar remained unchanged although with a higher work load lactate production increased and creatine phosphate content decreased to a greater extent. These experiments indicate that there is no correlation between the activity of the sugar transport system and the breakdown of high energy compounds or tension development (Holloszy and Narahara, 1965). Rather, these results suggest that one or more of the events associated with excitation-contraction coupling are involved in regulating the activity of the sugar transport system. Supportive evidence comes from studies with muscle treated with nitrate, an agent which

increases contractile force. In its presence the penetration of 3-O-MG was increased in stimulated but not in resting frog sartorius muscle. This link between excitation-contraction coupling and sugar transport suggests that calcium ions may be involved (Holloszy and Narahara, 1967a, 1967b). Nitrate ions can decrease the calcium-binding capacity of the sarcoplasmic reticulum (Ebashi et al., 1962) and in addition have been shown to increase the calcium influx in frog sartorius muscle (Bianchi and Shanes, 1959).

Holloszy and Narahara (1967a) obtained further evidence on a possible regulatory role of calcium, by determining the rate of sugar penetration in frog muscle in which potassium contractures were induced in media containing different calcium concentrations. Sugar transport was stimulated to a greater extent in those muscles exposed to the higher calcium concentration. During potassium-induced contractures, there occurs a calcium influx which is related to the concentration of this ion in the incubation medium (Lorković, 1962). The influx of extracellular calcium ions is, however, not essential for activation of the transport process; when a contracture was induced with a low concentration of caffeine (which did not depolarize the membrane) the transport system was also activated. Since caffeine appears to act by releasing calcium ions from storage sites within the muscle (Weber and Herz, 1968) the evidence on the location of the calcium pool involved in regulation of sugar transport is far from clear. A direct effect of calcium ions is also difficult to reconcile with the observation that in frog sartorius muscle, activation of sugar transport persisted long after electrical stimulation had ceased. In addition, in muscle where

transport was stimulated by potassium-contractures the rate of sugar penetration continued to increase considerably for some time after the contracture and remained at this elevated level for several hours (Holloszy and Narahara, 1967a). This was also seen after short periods of stimulation at high frequencies (Holloszy and Narahara, 1965). In contrast, the increase in the cytoplasmic free calcium pool involved in contraction lasts only for a short time after depolarization (Sandow et al., 1965).

These observations would suggest that even though calcium ions may be involved in regulating the activity of the transport system, they probably act by triggering another reaction with a much slower time course, which in turn stimulates sugar penetration. It is also apparent that the increase in activity of the transport system is initiated but need not necessarily be completed during contraction. This delayed increase in sugar transport suggesting a secondary slow step is not characteristic for skeletal muscle of coldblooded species only, since it has also been demonstrated in the gastrocnemius muscle of rats in situ (Helmreich and Cori, 1957).

CARDIAC MUSCLE

The isolated perfused heart has been a favourite preparation in transport studies since its physiological condition can be monitored continuously during the course of an experiment. One particular advantage is that the substrate will reach the cardiac cells via the normal coronary circulation. The non-working Langendorff preparation has been used extensively and, more recently, working heart preparations have also been used in the study of the effects of cardiac work on sugar

transport. In contrast to skeletal muscle, the rate of sugar penetration in cardiac muscle is related to both frequency of stimulation and to load. In isolated working rat hearts, Morgan et al. (1965) demonstrated that an increase in cardiac work accelerated the rate of glucose uptake. Work was determined either as cardiac output (summation of aortic and coronary flow) or as aortic or ventricular tension development. While phosphorylation was also increased to some extent, intracellular glucose could not be detected and sugar transport remained the rate-limiting step in the uptake process in these experiments (Neely et al., 1967). An increase in the penetration of the non-metabolized pentose, L-arabinose, was also demonstrated in these working hearts. As in frog skeletal muscle, sugar transport in cardiac muscle is also activated by an increase in the stimulation frequency; in isolated non-working rabbit hearts, the penetration of L-arabinose was increased when the frequency of contraction was changed from 180 to 240 beats per minute (Elbrink, 1969). However, since the contractile force of cardiac muscle is also related to the stimulation frequency, the mechanism whereby this increase occurs is not clear. Additional support for the relationship between the activity of the sugar transport system and contractile force comes from studies in which the cardiac glycoside, ouabain, was used. In stimulated rabbit hearts, an inotropic concentration of ouabain increased the rate of sugar penetration compared to control hearts stimulated at the same moderate frequency. The same concentration of the drug, however, did not affect the penetration of L-arabinose in quiescent hearts or in hearts stimulated at the highest frequency (Elbrink, 1969). This suggests that the events in cardiac muscle are

very similar to those in skeletal muscle.

In the study of Kreisberg and Williamson (1964) on glucose uptake and oxidation in guinea-pig and rat hearts, it was also found that sugar transport and contractile force closely paralleled each other when the isolated hearts were exposed to inotropic concentrations of ouabain or alterations in the perfusate calcium concentration.

The effect of cellular metabolism

Even though the effects of muscular work on sugar penetration are not related to hypoxia, this latter condition can also increase sugar transport in skeletal and cardiac muscle. Randle and Smith (1958a, 1958b) have demonstrated that sugar penetration in the isolated resting rat diaphragm was greatly accelerated in an oxygen-deficient medium or by the addition of inhibitors of oxidative phosphorylation. In these studies, glucose transport was rate-limiting in the uptake process and no free intracellular sugar could be detected; the penetration of the non-metabolized sugar, D-xylose, was equally accelerated. As the increase in sugar penetration occurred simultaneously with a decreased supply of high energy phosphate compounds, Randle and Smith postulated that under aerobic resting conditions, glucose penetration is restrained by an energy-requiring mechanism. In the isolated perfused rat heart, Morgan and coworkers (1961) also showed that under anaerobic conditions sugar penetration was increased and remained the rate-limiting step.

In a tissue where the transport of sugar is rate-limiting for its metabolism, an increase in sugar penetration during anoxia is essential for the operation of the Pasteur effect. This well known stimulation of glycolysis during anoxia may lead to increased utilization

of glucose only if the rate-limiting step, i.e. transport, is also stimulated. Since glycolysis is very inefficient in providing ATP, the rate of sugar utilization has to be increased to supply sufficient high energy phosphates to the cells. This regulatory mechanism therefore is correlated with the metabolic requirements. This concept is well demonstrated in cardiac muscle, where the insulin-sensitivity of the transport system is greatly increased under anaerobic conditions (Morgan et al., 1965).

Even though the effect of anoxia on sugar penetration is well documented, the mechanism by which facilitated diffusion is stimulated remains unsettled. The suggestion of Randle and Smith (1958a, 1958b) that under aerobic conditions, sufficient ATP is available to phosphorylate the sugar carrier to a less active form is of great interest but has so far not been substantiated by experimental data. The results obtained with avian erythrocytes do not support this hypothesis (Wood and Morgan, 1969).

Another proposal to explain the effect of anoxia relates to inhibition of the sodium pump and consequent alterations in cation levels (Bihler, 1968). This will be discussed below.

The effect of changes in the extracellular and intracellular cation levels

Although the intracellular ion composition will to a large extent depend upon the concentration of the extracellular ions, for simplicity the effects of their alterations will be discussed under separate headings.

EXTRACELLULAR IONS

Much evidence has been obtained about the requirement for extracellular sodium in sugar active transport in the intestine (for review see Crane, 1968). It has been suggested that in the equilibrating mediated sugar transport system of other tissues, this ionic dependence has been maintained (Letarte and Renold, 1969). However, the work reviewed below indicates that since influx and efflux are equally affected (Clausen, 1969; Bihler and Sawh, 1971a) this hypothesis cannot explain the effect of ions on the facilitated diffusion of sugars. The early work of Bhattacharya (1961) indicated that the basal uptake of glucose in quiescent rat hemidiaphragm and the penetration of the non-metabolized sugar, D-xylose, required extracellular sodium but not potassium. These experiments may be criticized because of the very unphysiological composition of the incubation media. Clausen (1965) has also shown that glucose uptake by the rat hemidiaphragm was directly proportional to the external sodium concentration. The total or partial replacement of sodium by lithium also increased glucose uptake (Clausen, 1968a, 1968b). On the other hand, incubation of the rat hemidiaphragm in a potassium-free medium failed to affect glucose uptake (Clausen, 1966) although lactate production was decreased and glycogenesis was increased. In contrast, Bihler (1968) using the same preparation, but employing a non-metabolized sugar, 3-O-MG, demonstrated a significant increase in penetration after incubation in potassium-free medium, and a decrease when the tissues were incubated in a high-potassium medium (Bihler and Sawh, 1971b). These effects were explained by inhibition and activation of the sodium pump, respectively, as will be discussed

below. Also in rat hemidiaphragms, Ilse and Ong (1970) found that with progressive replacement of sodium by D-mannitol, the penetration of 3-O-MG in the presence of insulin was greatly increased when approximately 65 mM NaCl had been replaced but was inhibited when sodium was completely omitted. These authors explained their observation by a change in the fixed charge distribution on the membrane.

Although some of these studies suggest that the ionic composition of the extracellular medium may affect the activity of the sugar transport system, it should be realized that partial or total replacement of physiologically important ions may constitute a severe insult to the tissues. Such ionic changes may affect cellular metabolic processes (Clausen,1966) and intracellular ionic levels (see below). Their effects on sugar transport are therefore difficult to interpret on the basis of specific effects on the sugar transport system.

INTRACELLULAR IONS

Recently, the ionic dependence of facilitated diffusion of sugars in skeletal muscle and adipocytes has been studied by interfering with the energy-requiring sodium pump which controls the intracellular levels of sodium and potassium.

Clausen (1966) demonstrated that inhibition of the sodium pump by ouabain had the same effect on the metabolic fate of glucose as incubation in a potassium-free medium. Bihler (1968) demonstrated an increased penetration of non-metabolized sugars in the presence of a variety of cardiac glycosides and aglycones. In these experiments the tissue was preincubated with these drugs in the presence of glucose since it was shown that in the heart a specific binding or transport

step is required for cardiac glycosides. It was concluded that whenever the sodium pump was inhibited, either directly by cardiac glycosides, high concentrations of 5,5-diphenylhydantoin or potassium-free incubation, or indirectly through anoxia or uncoupling of oxidative phosphorylation, sugar transport was stimulated. Conversely, when the sodium pump was stimulated by high potassium in the incubation medium, moderate concentrations of diphenylhydantoin or very low concentrations of cardiac glycosides, the penetration of 3-O-MG was depressed (Bihler, 1969b; Bihler and Sawh, 1971a, 1971b, 1971c). These effects were not related to the activity of the sodium pump at the time of transport but were quantitatively correlated with the intracellular concentrations of sodium and potassium. This negative feedback from the sodium pump might explain the stimulation of sugar transport by anoxia. Since in muscle this pump has been shown to depend largely on aerobic metabolism, its inhibition under anoxic conditions could provide the necessary stimulus for increasing the supply of glucose for anaerobic glycolysis. On the basis of this and other evidence, the above authors suggested that the effect of these alterations in intracellular cations is indirect, presumably via a specific pool of divalent cations, such as calcium. This ion has been implicated by Holloszy and Narahara (1967a, 1967b) in skeletal muscle, and the data of Baker et al. (1967) and Baker and Blaustein (1968) in nerve and Reuter (1970) in the heart on the existence of sodium-calcium exchange in the cell membrane provide a possible link between these ions. It is, however, unlikely that intracellular calcium is involved, and preliminary experiments are more consistent with the participation of a membrane bound calcium pool (Bihler and Sawh, 1970). On the other

hand, it has been suggested by Gould and Chaudry (1970) that magnesium also plays an important role, particularly in maintaining the insulin-responsiveness of the transport system in skeletal muscle.

Statement of the problem

There is still a need for systematic studies of sugar transport in various tissues using modern methods. Three tissues are of particular interest: Atrial muscle would be very useful since much is known about calcium and sodium fluxes in this tissue, and the role of these ions in regulating the activity of the sugar transport system could be studied. Smooth muscle is of interest since excitation-contraction coupling in this tissue differs from that in skeletal muscle. Insight into the role of these events in the regulation of sugar penetration might be obtained. The lens of the eye is an avascular organ, very suitable for in vitro studies. Previous work had shown that sugars probably enter by facilitated diffusion (Patterson, 1965), but this process is not well characterized. This tissue is non-excitable, contains free intracellular glucose but, surprisingly, was reported to be sensitive to insulin.

The aims of the present study were threefold: Firstly, by using a variety of monosaccharides, it was attempted to better characterize the transport system for sugars in these three tissues. Secondly, it was of particular interest to determine whether the activity of the sugar transport system in the lens could be regulated by some of the factors and conditions known to stimulate sugar penetration in other tissues. Finally, it was attempted to correlate the characteristics of sugar transport systems and metabolic requirements of various tissues.

SECTION II

METHODS

The lens

The lens of the eye is very suitable for studies of sugar transport *in vitro*. Since the lens has no vascular supply, substrates will follow the normal physiological route to enter this tissue during incubation. In addition, the lenses can be removed from the globe without apparent damage and are able to survive *in vitro* for an appreciable time. Several parameters can be used to determine whether the lenses remain in good condition during the course of an experiment: Since the weights of the two lenses from the same animal are extremely close, this can give an indication about shrinkage or swelling (see below). The extracellular space, measured with an appropriate marker substance, is also a good indicator of the physiological condition of the lens, as is the loss of transparency or damage to the capsule, both of which can easily be observed.

The lens is a biconvex, transparent structure which is located behind the iris and the pupillary aperture. It is kept in place by the zonular ligaments which are attached to the acellular capsule on either side of the equator and are in turn connected to the ciliary body. Posteriorly, the lens is bound by the vitreous body, a gell-like structure, and anteriorly by the posterior chamber. This chamber is connected to the anterior chamber through the pupillary aperture, both chambers being filled with aqueous humour. The lens obtains nutrients from this humour, into which waste products are discharged.

The lens itself consists of several components. It is entirely surrounded by an elastic capsule which consists of collagen-like material in a proteinaceous matrix. The anterior surface of the lens is covered

by an epithelial layer whose cells become elongated towards the equator and are, in the course of growth, transformed into lens fibers which move into the actual lens substance, the cortex. The young fibers, hexagonal in cross section, still contain a nucleus which is lost when they move deeper into the cortex. The older fibers are compressed into the lenticular nucleus which increases in size with age.

Experimental animals and preparation of the lenses

Male Sprague-Dawley albino rats of about 150-200 g (Canadian Breeding Laboratories) were used. The animals were kept in our animal house for at least 24 hours before use and had free access to a stock laboratory diet and water. The animals were killed by cervical dislocation, and the globes removed immediately and kept in ice-cold normal buffer. The lenses were dissected out from the posterior side: A small slit was made near the entrance of the optic nerve and was widened by iris scissors. The lens could then be placed on a loop of stainless steel wire by gently pushing with the blunt end of a glass rod on the outer surface of the globe. The lenses were carefully rolled on wet filter paper to remove the adhering humour and then incubated. The complete dissection took approximately two minutes for a pair of lenses. The weight of these lenses was between 25 and 30 mg.

Incubation procedure

The lenses were routinely preincubated for 45 minutes in a medium devoid of test sugar and subsequently incubated for varying periods with the test sugar, an extracellular marker and other additions, as required. These incubations were carried out with gentle agitation

in a Dubnoff shaking incubator at 37°C in individual 25 ml erlenmeyer flasks containing two milliliters of medium, which were equilibrated with O_2:CO_2 (95% : 5%).

Incubation media

The normal medium for incubation was Krebs bicarbonate buffer (Krebs and Henseleit, 1932) at pH 7.4, containing only one-half of the specificd calcium concentration. 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. Reagent grade chemicals were used for the solutions. The $NaHCO_3$ stock solution, which was stored in the cold, was equilibrated before mixing with CO_2 for several hours to prevent the formation of insoluble calcium salts (Young, 1968). Fresh buffer solution was made daily and equilibrated with O_2:CO_2 (95% : 5%). The medium for preincubation was normal Krebs-Henseleit solution with the addition of D-glucose, 5.5 mM (final concentration) to serve as metabolic substrate. This addition altered the osmolarity of the medium by less than 1% only and could therefore be ignored. The incubation medium consisted of the same Krebs-Henseleit buffer with the addition of test sugar (usually 5.0 mM), inulin as marker for the extracellular space, and other additions as required. The incubation medium routinely contained 1.0 mM D-glucose as metabolic substrate. This low concentration of glucose would not be expected to change the results to any significant extent (see RESULTS and DISCUSSION). The volume of buffer in the incubation flasks was adjusted in such a way that after the additions of the test sugar etc., the ion concentrations and osmolarity were very close to those of the original buffer. At the same time the

actual concentration of test sugar deviated slightly from the nominal concentration but this was constant within any one experiment. This deviation was corrected for in the calculation of the kinetic constants. In those experiments where the concentration of test sugar ranged from 0.5 to 25 mM, from 25 to 100 mM or from 0.5 to 100 mM, the sodium concentration of the buffer was decreased appropriately and was kept constant within each experiment. The osmolarity was maintained by the addition of sucrose.

Choice of test sugar, marker for the extracellular space and incubation times

For this investigation, 3-O-methyl-D-glucose was chosen as test sugar in most experiments. This glucose analogue is not metabolized in the intestine (Csáky and Wilson, 1956) and skeletal muscle (Narahara and Özand, 1963; Kohn and Clausen, 1971); Csáky and Glenn (1957) have shown that after administration in vivo the unchanged sugar could be nearly completely recovered. In frog sartorii, the transport characteristics of this sugar are very much the same as those of glucose (Narahara and Özand, 1963). In the lens of the eye Kern (1965) showed that the affinity of the transport sites for these two sugars is the same. The incubation medium contained the required concentration of test sugar, i.e. a mixture of ^{14}C-labelled (0.125 µc/ml) and unlabelled 3-O-methyl-D-glucose.

The use of a non-metabolized sugar has the obvious advantage that results can be entirely interpreted on the basis of changes in the activity of the sugar transport system, and complications due to metabolic transformation of the sugar are avoided.

The choice of inulin as marker for the extracellular space (ECS) requires some comment. This space in the lens of the eye consists of the spaces between the lens fibers of the cortex and nucleus and between the epithelial cells on the anterior surface. The water space of the acellular capsule also contributes significantly to this space. The two main properties for an ideal marker are firstly, that it should be sufficiently small to be able to enter the total ECS and secondly, that it should be excluded from the cells. The choice of inulin was a compromise. This molecule is of fairly large size but does not penetrate across cellular membranes. Although mannitol is of smaller size and is often used to determine the ECS in other tissues, this sugar alcohol is thought to be distributed in the intracellular water of the lens (Thoft and Kinoshita, 1965). The larger size of inulin does not appear to prevent its equilibration in most of the extracellular space. Paterson (1970) has reported that after hyaluronidase treatment of ox lenses, the ECS was increased. However, even though this increase was statistically significant, it was extremely small (from $5.23 \pm 0.13\%$ to $5.47 \pm 0.11\%$). It should be mentioned that inulin obtained from different commercial sources gave very different estimates of the ECS in rabbit lenses (Paterson, 1968), and for this reason inulin acquired from only one source was used throughout this study. The ^{3}H-labelled inulin was present in the incubation medium in tracer amounts (0.625 μc/ml) and the ECS was determined for each lens since this parameter depends to a certain extent upon the handling of the tissue.

There appears to be a great species difference with respect to the time required for equilibration of inulin with the ECS; in

rabbit lenses approximately six hours are required (Paterson, 1968) while complete equilibration in rat lenses takes about one hour (Thoft and Kinoshita, 1965). The average value for the ECS obtained with inulin in over fifty lenses used as controls in our study, 6.78 $\pm$ 0.36%, is very close to that found in other laboratories (Thoft and Kinoshita, 1965). Although in most experiments the ECS was very close to this value, in a few others higher estimates were obtained which may have been due to incomplete removal of the vitreous humour. However, even when the ECS was somewhat larger, it was highly reproducible within any single experiment. The sugar penetration for each lens was calculated using the inulin space determined in the same tissue.

A preincubation period of 45 minutes was chosen to allow for equilibration of the lenses with the medium and to give sufficient time to dissect the required number of lenses for a complete experiment. A one hour incubation period was used routinely, except when otherwise indicated in the text. This appears to be the minimum time required for the complete equilibration of the extracellular space with the marker. During this period, appreciable penetration but not complete equilibration of the test sugar takes place, so that differences in sugar transport with various treatments can be noted. This fairly long incubation time would appear to be required to ensure equilibration of the test sugar and the marker in the ECS throughout the lens.

Chemicals

All chemicals were of reagent grade and were acquired from commercial sources. The radioisotopes were obtained from Amersham/ Searle (D-arabinose-1-^{14}C; α-methyl-D-glucoside-^{14}C(U); D-xylose-^{14}C);

New England Nuclear (L-arabinose-1-^{14}C; inulin-^{3}H(G); L-glucose-1-^{14}C); Cal Atomic (D-galactose-U-^{14}C) and International Chemical and Nuclear Corp. (3-O-methyl-^{14}C-D-glucose). The insulin was a solution of crystalline zinc insulin (40 units/ml) from Connaught Medical Research Lab., Toronto, and diluted to 2,500 milliunits/ml (milli-u/ml) with distilled water. This stock solution was stored at 5°C, and appropriate dilutions were made daily. Simultaneous experiments with rat hemidiaphragm and detrusor muscle in our laboratory indicated that the hormone remained fully active under these conditions. The activity of trypsin preparations from bovine pancreas (Sigma Chemical Comp.) and from hog pancreas (Nutritional Biochemicals Corp.) was stated to be roughly the same by the suppliers. Two collagenase preparations were obtained from Worthington Biochemical Corp. One was chromatographically purified and the other was a crude enzyme which contained a peptidase and a trypsin-like proteinase; the enzyme activity of these two preparations was stated to be approximately equivalent.

Preparation of samples and determination of radioactivity

After incubation the lenses were quickly washed in ice-cold buffer, blotted on hardened filter paper (Whatman, No. 50), rapidly weighed on a torsion balance to the nearest 0.1 mg and placed in a glass scintillation vial. The tissues were then digested with shaking in 0.3 ml NCS Solubilizer (Amersham/Searle), a quarternary ammonium base, for 1 - 2 hours at 50°C in the dark. Aliquots of incubation medium were counted without further treatment.

The radioactivity of the samples was determined by double-label scintillation counting with a Phillips Liquid Scintillation

Analyzer. To each medium sample, 10 ml of scintillator solution were added which contained 5.6 g of 2,5-diphenyloxazole (PPO), 0.1 g of 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP), 250 ml of ethylene glycol monomethyl ether and toluene to a total volume of one liter. The tissue digests were first neutralized to pH 7.0 with 9 N acetic acid to prevent chemiluminescence of the alkaline solutions. Then 10 ml of a scintillation solution containing 6.0 g of PPO per liter of toluene were added. Quenching was determined by the external standard channels ratio method; quench curves were obtained with ^{14}C and ^{3}H toluene as standards and picric acid as quenching agent. The degree of quenching was constant and was the same in tissue and medium samples. Therefore, no corrections were necessary. Suitable blanks for ^{14}C and ^{3}H were counted with each series of samples. Samples were counted for a time sufficient for the counting error to be less than 3% and the activity of the samples was at least ten times background. For the calculations, the radioactivity was expressed as counts per minute (cpm).

Determination of D-xylose

For the chemical determination of D-xylose the lenses were transferred to conical glass centrifuge tubes and extracted for ten minutes with distilled water in a boiling water bath. After cooling, the extract was deproteinized with $Ba(OH)_2$-$ZnSO_4$ (Somogyi, 1945), diluted to a known volume and centrifuged. Samples of the incubation media were similarly deproteinized and diluted. The analysis was carried out by the bromoaniline method of Roe and Rice (1948) for pentoses adapted to the Technicon Autoanalyzer. A series of known standards was analyzed with each group of samples.

Determination of sodium and potassium ions

After incubation, blotting and weighing, the lenses were extracted in glass distilled water for 30 minutes in a boiling water bath. Polypropylene tubes were used to avoid contamination by ions extracted from glass; in preliminary experiments this was found to be appreciable. After cooling, the extract was deproteinized with trichloroacetic acid, centrifuged for 10 minutes and the supernatant decanted. The media were centrifuged, treated with trichloroacetic acid, centrifuged again and diluted. The ions were determined with a Perkin-Elmer Atomic Absorption Spectrophotometer (Model 303). Standards and blanks were read with each series of samples. All dilutions were made with glass distilled water and glassware was carefully rinsed to prevent contamination. The results are expressed as mmole/l intracellular water.

Determination of tissue water content

The water content was determined in separate lenses, preincubated and incubated as described above. After weighing, the lenses were dried to constant weight at 80°C under vacuum. The total tissue water in eight lenses, expressed as percent wet tissue weight was $62.50 \pm 1.30\%$. This value compared well with that found in other laboratories (Paterson, 1970) and was used in all the calculations.

Calculations

The results are expressed as percentage penetration which indicates the sugar concentration in the intracellular water as a percentage of the concentration in the medium. This term can also be interpreted to indicate the fraction (in percent) of the intracellular water which is equilibrated with the sugar concentration in the medium.

The calculations are based on the assumptions that the total extracellular space was equilibrated with the sugar concentration and that all the intracellular water was accessible to the sugar. The extracellular space was calculated from the ratio of the concentration of inulin in the total tissue water and its concentration in the medium. The total sugar space, i.e. the volume of total tissue water equilibrated with the sugar, was calculated the same way; this space is often called the virtual sugar space. Hence, the excess of total sugar space over the extracellular space equals the portion of intracellular water space which is fully equilibrated with the sugar, expressed as a percentage of total tissue water. 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:

$$\text{Inulin space (extracellular space)} = \frac{{}^{3}H(\text{cpm/g tissue})/\%\ \text{TTW}}{{}^{3}H(\text{cpm/ml medium})} \times 100$$

$$\text{Total sugar space (virtual space)} = \frac{{}^{14}C(\text{cpm/g tissue}/\%\ \text{TTW}}{{}^{14}C(\text{cpm/ml medium})} \times 100$$

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

where % TTW standards for total tissue water as percentage of total tissue weight.

The results, expressed as percent penetration, are an indication of the rate of sugar transport across the cell membrane. Since

sugar penetration is a reversible process, the net rate of transport will be the difference between influx and efflux which are determined by the extracellular and intracellular sugar concentrations, respectively. By using a relatively large volume of incubation medium, the external sugar concentration and therefore influx may be assumed to remain constant throughout the duration of the experiment. However, the intracellular concentration increases with time, thereby increasing the contribution of efflux. Therefore, the percent penetration tends to underestimate the true unidirectional influx. For most purposes, such as demonstrating inhibition or the effect of stimulatory factors, such a semiquantitative expression is sufficient. For the determination of kinetic constants a shorter incubation period was chosen, so that influx rates were closely approximated.

Experimental design and statistical analysis

In preliminary experiments, a paired experimental design was used with one lens of each pair serving as control. Within any one experiment, the values for controls were extremely close, and to prevent the unnecessary use of large numbers of lenses for controls, a completely random design was adopted (Steel and Torrie, 1960). A random number table was used to assign the various treatments within an experiment. With few exceptions, each treatment group consisted of a minimum of four lenses. In the experiments in which sugar penetration and the intracellular cation levels were measured, one lens of a pair was used to determine ions, the other for 3-O-methyl-^{14}C-D-glucose.

Duncan's New Multiple Range Test (Steel and Torrie, 1960) was used to determine the statistical significance of differences between

the means of the percent sugar penetration in the various treatment groups. With this method, comparisons can be made not only between control and treatment groups but between all the treatment groups. This test was also used in the evaluation of intracellular ion concentrations under different experimental conditions. When no significant difference between two treatments was found, the result is usually reported as the difference between the means $\pm$ the standard error of this difference.

SECTION III

RESULTS AND DISCUSSION

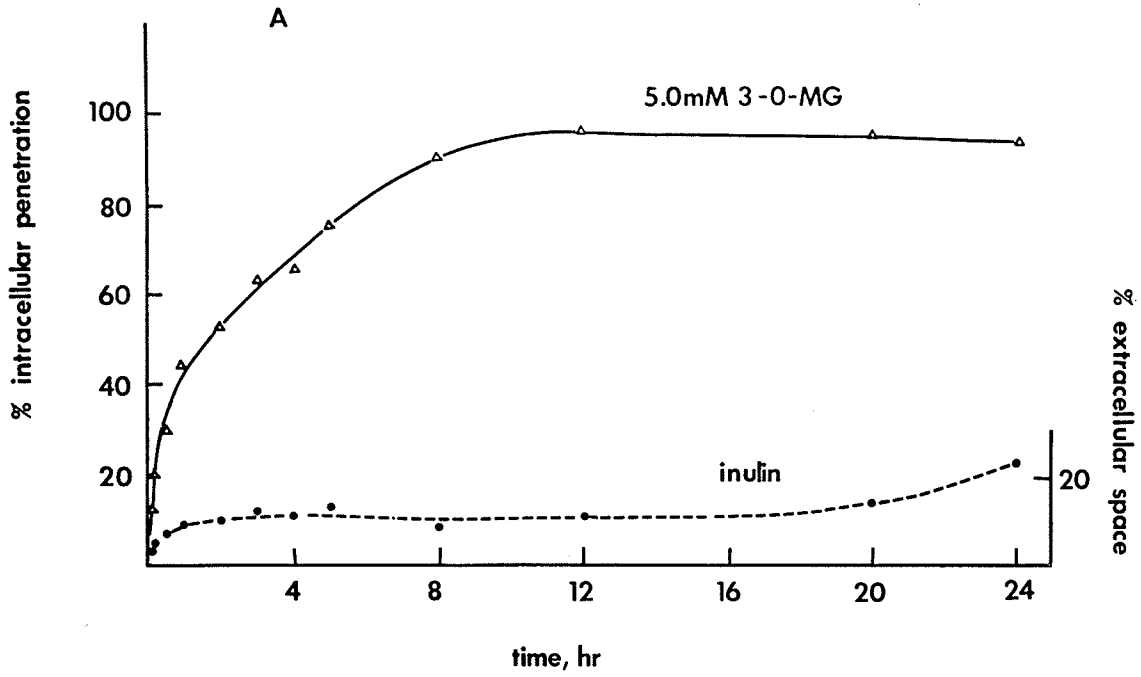
The lens of the eye

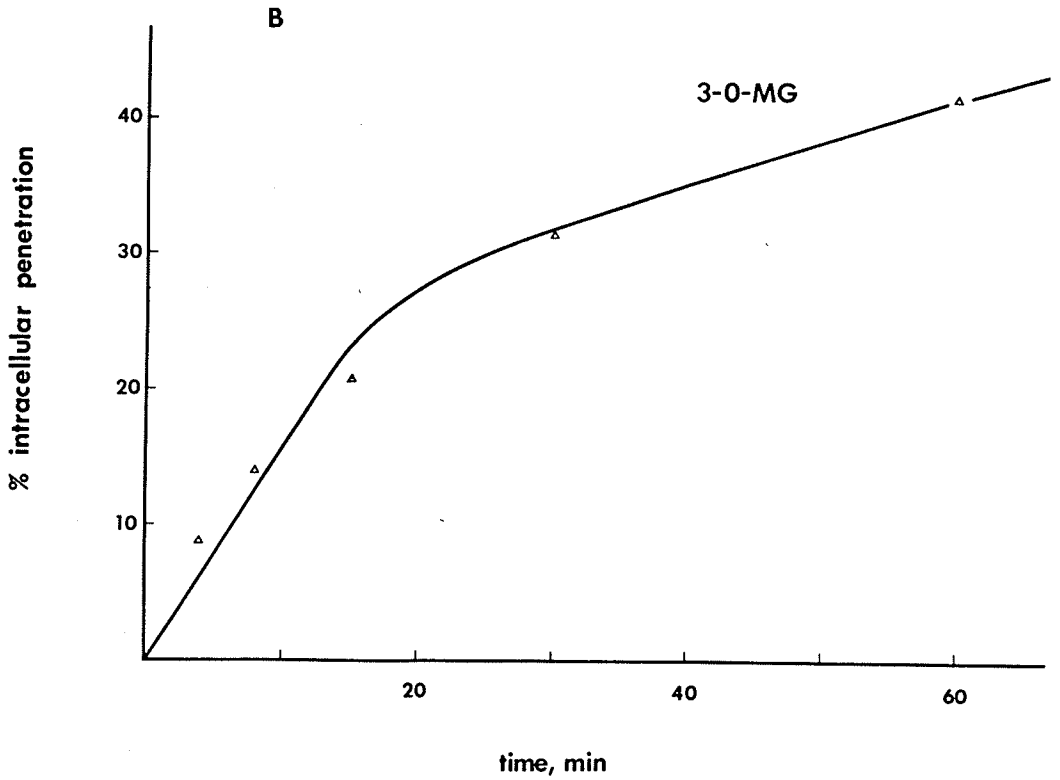
Time course

The time course of sugar penetration was determined in lenses incubated with 5.0 mM 3-O-MG for up to 24 hours. To provide sufficient metabolic substrate, the glucose content of the incubation media was increased above the usual 1.0 mM for lenses incubated for more than four hours. The amount of glucose was proportional to the duration of incubation and was calculated from the data on glucose consumption of Levari et al. (1961). As seen in Fig. 1A, the time curve can be broken down into three distinct phases, a rising straight part, a curved part and a horizontal linear portion. This type of curve is typical for an equilibrating process and can be explained by the different contributions of influx and efflux with incubation time. Initially, the rate of net influx was constant, suggesting that the intracellular concentration of the sugar was insufficient to cause significant efflux. In the rising curved section, influx was greater than efflux, while after 12 hours the sugar concentration had equilibrated across the cell membranes and influx equalled efflux. Between 12 and 20 hours of incubation, the penetration was close to 100%. This suggests that nearly all the intracellular water of the lens was accessible to the sugar, as has been found in most other tissues, although some divergent results have been obtained (Morgan et al., 1961). This observation also indicates that no active accumulation of 3-O-MG against its concentration gradient had taken place. During this long incubation period, the lenses remained in good condition; they were transparent and the extracellular space stayed low and constant. The lack of active transport could therefore

Fig. 1. A) The effect of incubation time on the intracellular penetration of 5.0 mM 3-O-methyl-D-glucose and on the inulin space. Lenses which were incubated for more than four hours received additional D-glucose (see text) and fresh O_2 - CO_2 was blown into the flasks several times during the course of the experiment. For other details see METHODS. The standard errors have been omitted as they were smaller than the symbols of the data points.

B) The time course of penetration of 3-O-MG during the initial 60 minutes of incubation; this is a magnified portion of the experiment shown in panel A.


A
5.0mM 3-O-MG
inulin
% intracellular penetration
% extracellular space
100
80
60
40
20
20
4
8
12
16
20
24
time, hr


B
3-O-MG
% intracellular penetration
40
30
20
10
20
40
60
time, min

not be ascribed to tissue deterioration. The viability of the preparation decreased sometime after 20 hours of incubation as seen by the increase in the extracellular space and, concomittantly, a small decrease in the intracellular concentration of 3-O-MG occurred.

In studies on active sugar transport in hamster small intestine, the degree of accumulation against the concentration gradient was shown to be related to the ratio of the extent of sugar binding to the transport sites at the inner and outer membrane surfaces. Depending on these values, active transport of certain sugars could only be demonstrated when low substrate concentrations were used (Bihler, 1969a). When lenses were incubated with a much lower concentration of 3-O-MG, 0.1 mM, only equilibration of the test sugar across the cell membranes had taken place after 20 hours of incubation. Thus, active transport does not appear to take place in the lens. In other tissues it has been shown that the methylated glucose analogue is not a substrate for any of the existing metabolic pathways. The possibility that 3-O-MG would have been reduced by aldose reductase in the lens seems improbable. The accumulation of radioactivity in the form of unchanged sugar together with that of its polyol metabolite(s) should then exceed a value of 100% after the long incubation periods; this has indeed been found both for D-xylose and D-galactose which are metabolized by the reductase (see below).

A time course similar to the above has been obtained in rabbit lenses with glucose (Harris et al., 1955; Giles and Harris, 1959). With this hexose, a steady-state was reached after about 24 hours of incubation, as opposed to the 12 hours required to achieve equilibration

with 3-O-MG in rat lenses. The steady-state concentration of glucose appeared to be well below equilibrium with sugar in the incubation medium. Since glucose is metabolized by the lens, its concentration at steady-state is determined not only by influx and efflux but by metabolic disposal as well. These results should therefore not be taken to indicate that less intracellular water is available to the sugar in rabbit lenses than in rat lenses although in this respect species differences may exist.

The penetration values obtained when lenses were incubated for long periods of time are the resultant of net fluxes. However, to determine the kinetic parameters of the transport system, the rate of unidirectional flux, either influx or efflux, has to be determined. Experimentally this can easily be done by incubating the tissue for such a short period of time that the intracellular concentration will be insufficient to cause significant efflux. As can be seen from Fig. 1B, for the first 15 min of incubation the net rate of influx was constant and thus the effect of efflux appeared negligible. Unidirectional influx was therefore approximated when the percentage intracellular penetration remained below about 25 percent.

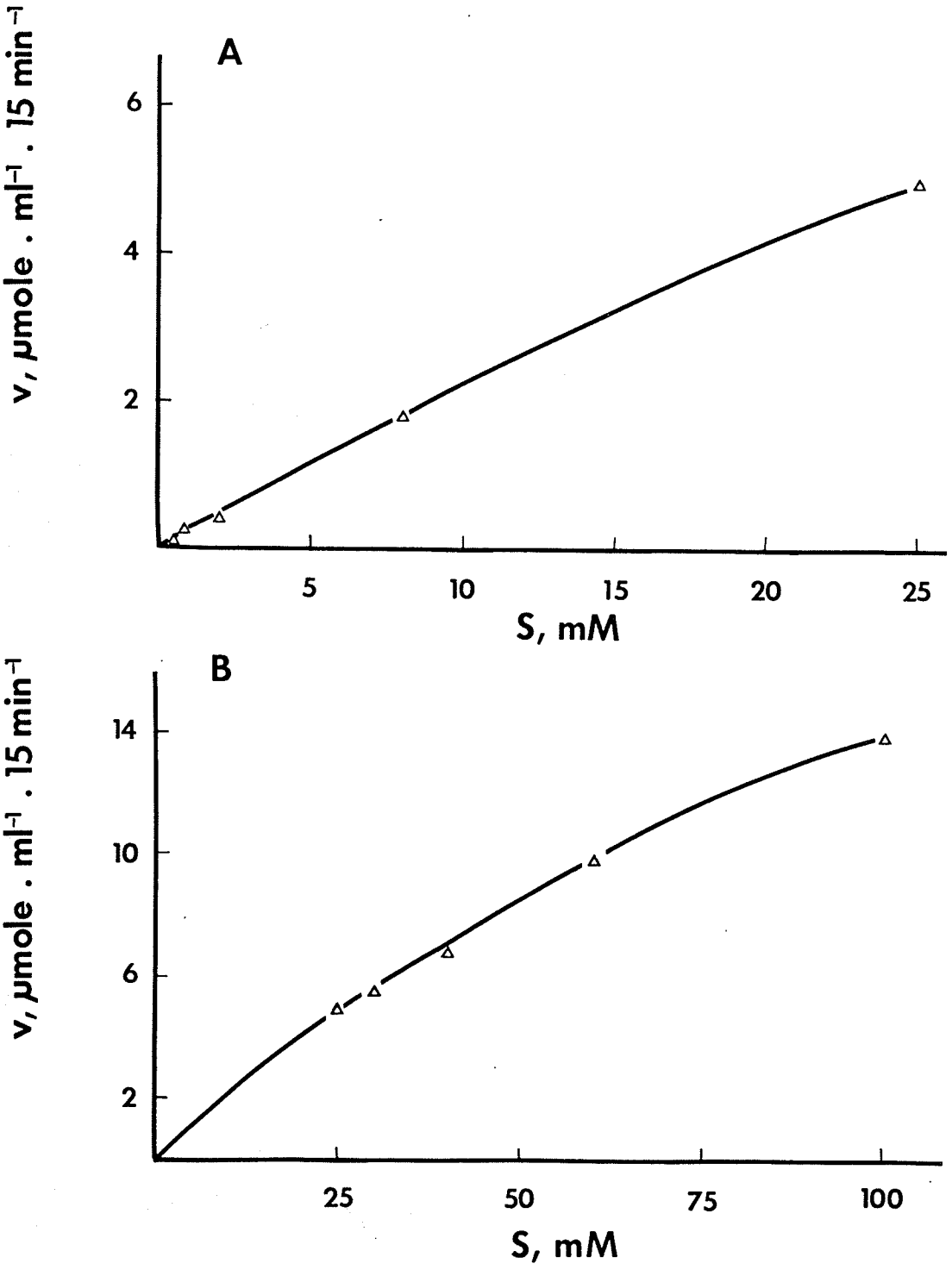
Concentration dependence

The concentration dependence of the transport process was determined by measuring the rate of penetration at different sugar concentrations. Lenses were incubated for 15 minutes with concentrations of 3-O-MG ranging from 0.5 to 25 mM. The results expressed as initial velocity rather than percent penetration are plotted against the substrate concentrations (Fig. 2A). The deviation from linearity indicated

Fig. 2. The effect of the concentration of 3-O-methyl-D-glucose on its unidirectional influx in the lens of the eye. Incubation time, 15 minutes. The data have been transformed and standard errors are not shown.

A) Concentrations of substrate from 0.5 mM to 25 mM.

B) Concentrations of substrate from 25 mM to 100 mM.


A
v, µmole . ml⁻¹ . 15 min⁻¹
6
4
2
5
10
15
20
25
S, mM
B
v, µmole . ml⁻¹ . 15 min⁻¹
14
10
6
2
25
50
75
100
S, mM

that sugar transport was inconsistent with diffusion. It therefore appeared likely that the sugar penetrated across the cell membrane by facilitated diffusion as is the case in most other tissues studied. To obtain better evidence for saturation the experiment was repeated and the concentration range extended to 100 mM. At the same time a similar experiment was done using only high concentrations of test sugar, 25 mM to 100 mM. As seen in Fig. 2B, the line curved appreciably when the results of this latter experiment were plotted as v against s but complete saturation was still not obtained. Experiments with still higher substrate concentrations were not attempted because of complications which might arise from excessive sodium chloride removal required to maintain constant osmolarity of the medium.

The absence of saturation could be explained if the sugar also crossed the cell membrane by an additional pathway or if the affinity of the transport site for the sugar was extremely low. Traditionally, incomplete saturation has been explained by a diffusion component in parallel with the specific transport process. An attempt was made to determine the possible contribution of diffusion by incubating lenses in the presence of phloretin. This agent is a specific blocker of facilitated diffusion of sugar in many tissues (Rosenberg and Wilbrandt, 1957b). Later on it will be shown that phloretin behaves as a non-competitive inhibitor in the lens but, regardless of its kinetics, this agent acts as a specific inhibitor of sugar transport. Assuming complete inhibition of the transport system, the residual transport of 3-O-MG would then be entirely due to diffusion. A relatively low concentration of inhibitor, 0.5 mM, was chosen, as high concentrations of this aglycone

and its glycoside, phlorhizin, are known to affect the activity of some enzymes (Kalckar, 1936; Keller and Lotspeich, 1959). In two experiments on consecutive days the lenses were incubated for 15 minutes with various concentrations of 3-O-MG in the presence of 0.5 mM phloretin. The range of sugar concentrations used was from 0.5 to 25 mM and from 25 to 100 mM, respectively. Close agreement in the percent penetration was obtained at the concentration of 25 mM on the two days. The inhibitor reduced the initial velocity by about 50% at high substrate concentrations (Fig. 3). In experiments to be described below, the K_i of phloretin was found to be approximately 0.60 mM. A linear relationship between initial velocity and substrate concentration would be consistent with diffusion but can also be obtained when the affinity of the saturable process for the sugar is very low (Widdas, 1954). In the presence of phloretin, there still was some indication for saturation since higher concentrations of phloretin (1.0 mM and 2.0 mM) caused a further inhibition in the transport of 3-O-MG (Fig. 3). Additional evidence for the incomplete inhibition of the transport system with 0.5 mM of the aglycone is presented in the section on competition experiments (see below). It therefore appears that the contribution of diffusion to the transport process, if at all present, is extremely small.

The alternative explanation for the apparent absence of saturation would be that the K_m of the sugar is so high that saturation could not be demonstrated under the conditions of the experiment. Simple calculation shows that when the sugar concentration is, say, five times the K_m, v is only 83.3% of V_{max}. Even when the ratio between the substrate concentration and the K_m is as high as ten, the initial velocity

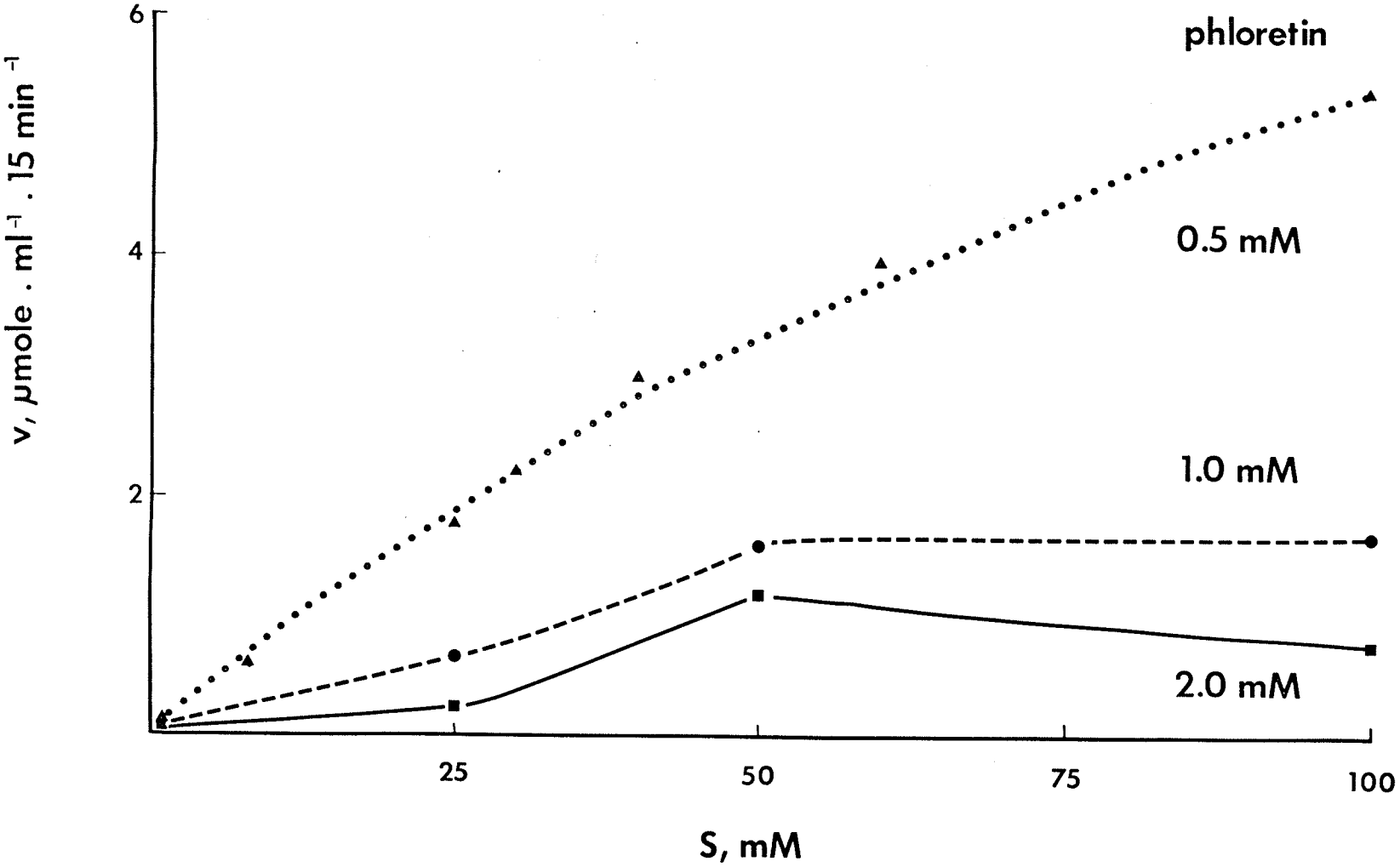


Fig. 3. The effect of various concentrations of phloretin on the initial velocity of influx of 3-O-methyl-D-glucose at concentrations from 0.5 mM to 100 mM. Incubation time, 15 minutes. The data have been transformed and standard errors are not shown.

is only 90.9% of V_{max}. The data in the next section will show that the K_m of the transport sites for 3-O-MG in the lens is so high that with a 100 mM substrate concentration only about one half saturation could be achieved (Fig. 2B). This therefore would adequately explain why saturation was not obtained with the sugar concentration used. In addition, it is also possible that more than one transport system for sugar is present in the lens; a second system with very low affinity for the substrate would also account for the failure to observe saturation. Indeed, Christensen and Liang (1966) have shown that amino acid transport in Erhlich ascites tumour cells takes place by two specific transport systems, one having a low and the other a high affinity for substrate. However, there is no evidence for the presence of more than one transport system with overlapping specificities.

Determinations of V_{max} and K_m

The initial velocity data described above were used to calculate the kinetic parameters. During the 15 minute incubation time, the inulin marker failed to equilibrate completely with the extracellular space; values of approximately 4% were obtained, instead of the usual 6 to 7% obtained with longer incubation and taken to indicate complete equilibration. The initial velocities were calculated with the estimates of the ECS obtained in the actual experiments because the difference between these values and those for complete equilibration is negligible with respect to the intracellular water available for sugar distribution. However, if it is assumed that only part (4/6th) of the ECS was equilibrated with the test sugar, the values of the kinetic parameters should be considered as maximum estimates rather than exact values. On the

other hand, it is also possible and even likely that the sugar concentration in the ECS of the lens is not uniform and gradually decreases from periphery to nucleus. These different possible interpretations are a general problem in transport studies in all tissues, and the true situation is very complex. For this reason, both maximum and minimum estimates of the V_{max} and K_m have been calculated, assuming complete or partial equilibration of the sugar with the extracellular space, respectively.

Three sets of parameters were calculated separately for the results obtained with the different ranges of substrate concentrations. The 0.5 to 25 mM will be referred to as low, 25 to 100 mM as high and 0.5 to 100 mM as the complete range of concentrations. In the first two groups, five different substrate concentrations were used, while for the last group there were six. The V_{max} and K_m were initially determined by the method of Lineweaver and Burk in which $1/v$ is plotted against $1/s$. Straight lines could be fitted by eye through the data points and the intercepts could be extrapolated. As is the usual custom, the regression lines were calculated by the method of least squares, even though this procedure is not truly applicable to this type of data. However, since the lines transected the coordinates very close to the origin, more accurate estimates could be obtained this way. The values of V_{max} and K_m obtained by using the three sets of experimental data differed greatly (Table I). With the low and complete range of substrate both the maximal velocity and the apparent affinity were about 50% lower than the values for these parameters when the high substrate range was used in the calculations. Because of this great discrepancy, the three sets of data were also plotted according to the methods of Hofstee

TABLE I

Maximum estimates for V_{max} and K_m by the three most common graphical procedures

		Range of substrate concentrations (mM)		
		0.5-25	25-100	0.5-100
Lineweaver-Burk (1934)	K_m	55.42	119.84	59.14
	V_{max}	13.99	33.17	15.39
Hofstee (1959)	K_m	155.45	128.55	111.61
	V_{max}	28.70	35.07	28.22
Woolf (1932)	K_m	135.87	137.21	113.19
	V_{max}	33.50	36.86	28.54

K_m - mM

V_{max} - $\mu mole.ml^{-1}.15\ min^{-1}$

(v versus v/s) and Woolf (s/v versus s). The various plots are presented in Fig. 4. The intercepts and slopes of the lines were obtained as described above. With both these methods much better agreement between the values of the V_{max} and K_m from the three sets of data was found. The parameters calculated from the high substrate concentration range were again the highest but differed much less from the other estimates when compared to the results obtained with the Lineweaver-Burk transformation. With these three methods, only the maximum values were calculated.

In addition to these procedures, the method of Bliss and James (1966) was used, which has the advantage that the data do not have to be transformed. This method was adapted to a PDP-8 Digital computer (Digital Equipment Corp.) and the data were fitted to a hyperbola by the procedure of maximum likelihood. The values for the V_{max} and K_m with this program also showed differences depending upon the concentration range of substrate used in the calculation. Both the low and high substrate range gave values which were higher than with the complete substrate range (Table II). The maximum estimates obtained with the complete substrate range compared well with those by the methods of Hofstee and of Woolf. In addition, the 99% confidence intervals around these estimates were the smallest and these values are probably the most reliable. The most likely maximum estimate for the V_{max} is therefore 28 μmole. ml^{-1}. 15 min^{-1} and for the K_m, 110 mM. These values are also very close to the estimates given by the Lineweaver-Burk method when the high substrate range was used in the calculations.

Although the Lineweaver-Burk plot is most commonly used, it

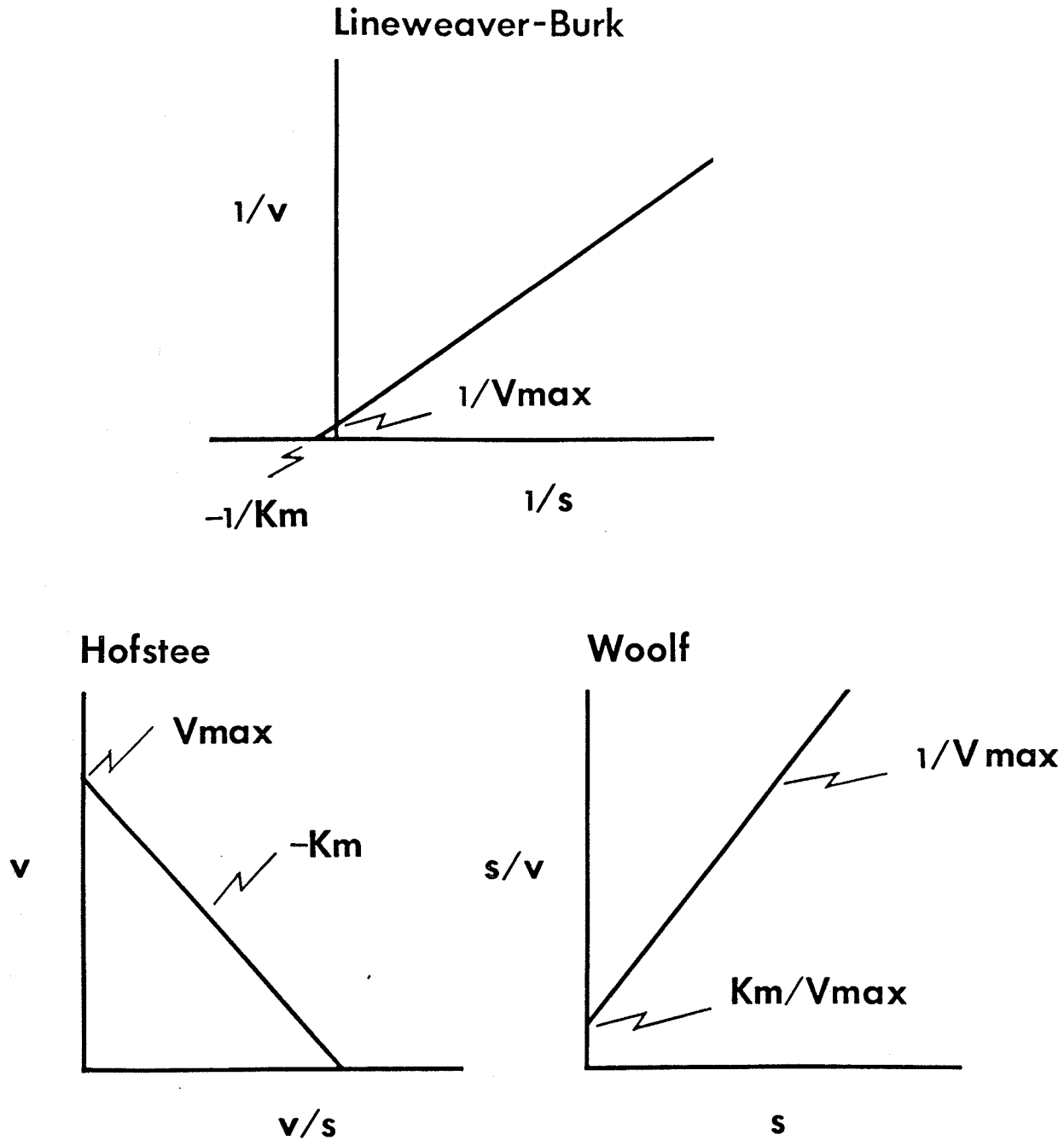


Fig. 4. The three most common graphical methods for the determination of kinetic parameters.

TABLE II

Estimates for V_{max} and K_m by the method of Bliss and James (1966)

		Range of substrate concentrations (mM)		
		0.5-25	25-100	0.5-100
Maximum estimates	K_m	142.63	142.62	110.20
	CI	101.56-224.93	84.11-284.71	88.09-139.99
	V_{max}	34.99	37.90	28.05
	CI	23.29-46.69	22.01-53.78	23.85-32.25
Minimum estimates	K_m	97.51	69.46	67.16
	CI	69.63-152.97	40.97-138.61	53.69-85.33
	V_{max}	34.96	37.90	28.05
	CI	23.38-46.54	22.02-53.78	23.85-32.26

CI, 99% confidence intervals
K_m, mM
V_{max}, μmole.ml^{-1}.15 min^{-1}

appears to suffer from a serious disadvantage. The data obtained with the lowest substrate concentrations are most prone to experimental error and since the reciprocals of these results are used, the slope of the line is to a large extent determined by these least reliable values (Dowd and Riggs, 1965; Christensen and Palmer, 1967). Particularly, when the apparent affinity of the transport system is low, small changes will greatly affect the calculated values of the kinetic parameters. Our results support this contention since entirely different estimates of the V_{max} and K_m were found when the data included the results obtained with the low substrate concentrations. While Dixon and Webb (1964) prefer the method of Lineweaver and Burk, Dowd and Riggs (1965) favour either the Hofstee or Woolf plots. These authors have analyzed the accuracy of the three different methods and found the Lineweaver-Burk plot the least reliable. Our results support their conclusion. The minimum estimates were only determined by the method of Bliss and James and are also given in Table II. Since the true situation with respect to the substrate concentration in the extracellular space is probably somewhere in between the assumptions on which the maximum and minimum estimates are based, the average of these values obtained with the complete substrate range probably are the most likely values: V_{max}, 28 μmole.ml^{-1}.15 min^{-1} and K_m 88 mM.

Chemical specificity

The chemical specificity of the sugar transport system was determined in two types of experiments. In the first series, the rate of penetration of various sugars was compared after four hours of incubation. The long incubation period in these experiments seemed most

suitable to distinguish between the rates of penetration of the various sugars; the concentration of substrate used in each case was 5.0 mM. In some preliminary trials it had been noted that, after one hour of incubation, the transport of 3-O-MG was greatly exceeded by that of D-xylose; the penetration of the former was 35.87 $\pm$ 0.60% and that of the latter 84.78 $\pm$ 3.32% and the differences became even larger with longer incubation times. The results presented in Fig. 5 indicate that three groups of sugars can be distinguished. The sugars of the first group, L-glucose and α-methyl-D-glucoside entered the lens at the slowest rate. Since, as will be shown later, these sugars did not compete with 3-O-MG, they probably do not share the same transport system. The second group were those which entered the cells by facilitated diffusion but which were not metabolized by the lens. Of the sugars tested, only 3-O-MG appeared to belong in this category. The penetration of D-arabinose was much higher than would be expected from diffusion. But, as will be shown below, this non-metabolized sugar did not compete with 3-O-MG, and is unlikely to share the same transport system. The results obtained with the last group of sugars, those which are known to be metabolized, were rather surprising. When the lenticular content of radioactivity was measured, it appeared as if D-xylose and D-galactose were actively accumulated against their concentration gradients. Experiments were therefore carried out with ^{14}C-D-xylose to delineate further this apparently active accumulation. The time course of penetration was determined in the same way as with 3-O-MG (Fig. 1); the lenses were incubated for up to 24 hours with 5.0 mM D-xylose (Fig. 6). The initial portion of the curve was much

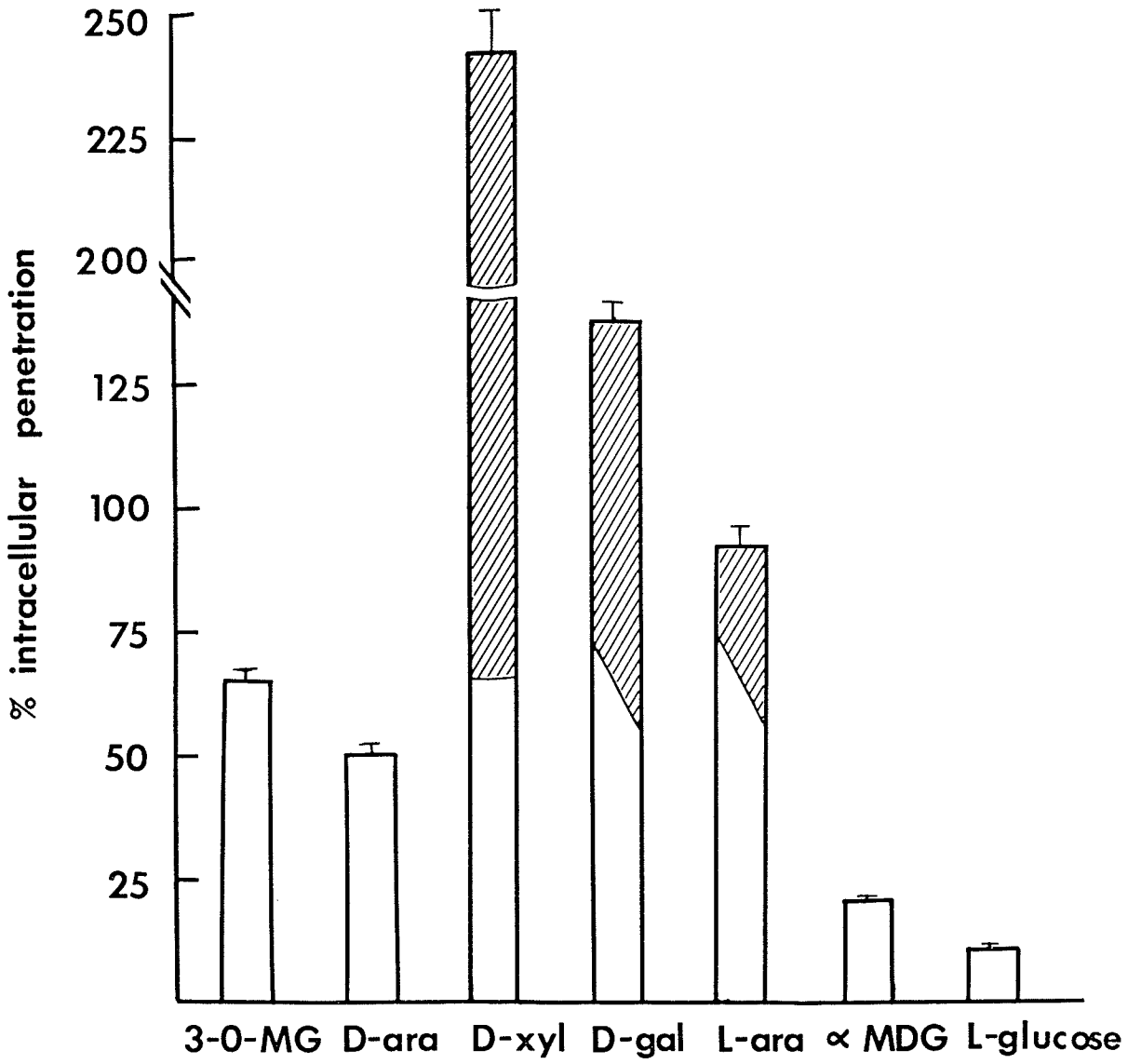


Fig. 5. The intracellular penetration of various sugars (5.0 mM) after four hours of incubation. The vertical lines represent one standard error. The clear portion of each bar represents unchanged sugar; the hatched portion indicates radioactive metabolites, determined for D-xylose and estimated for D-galactose and L-arabinose.

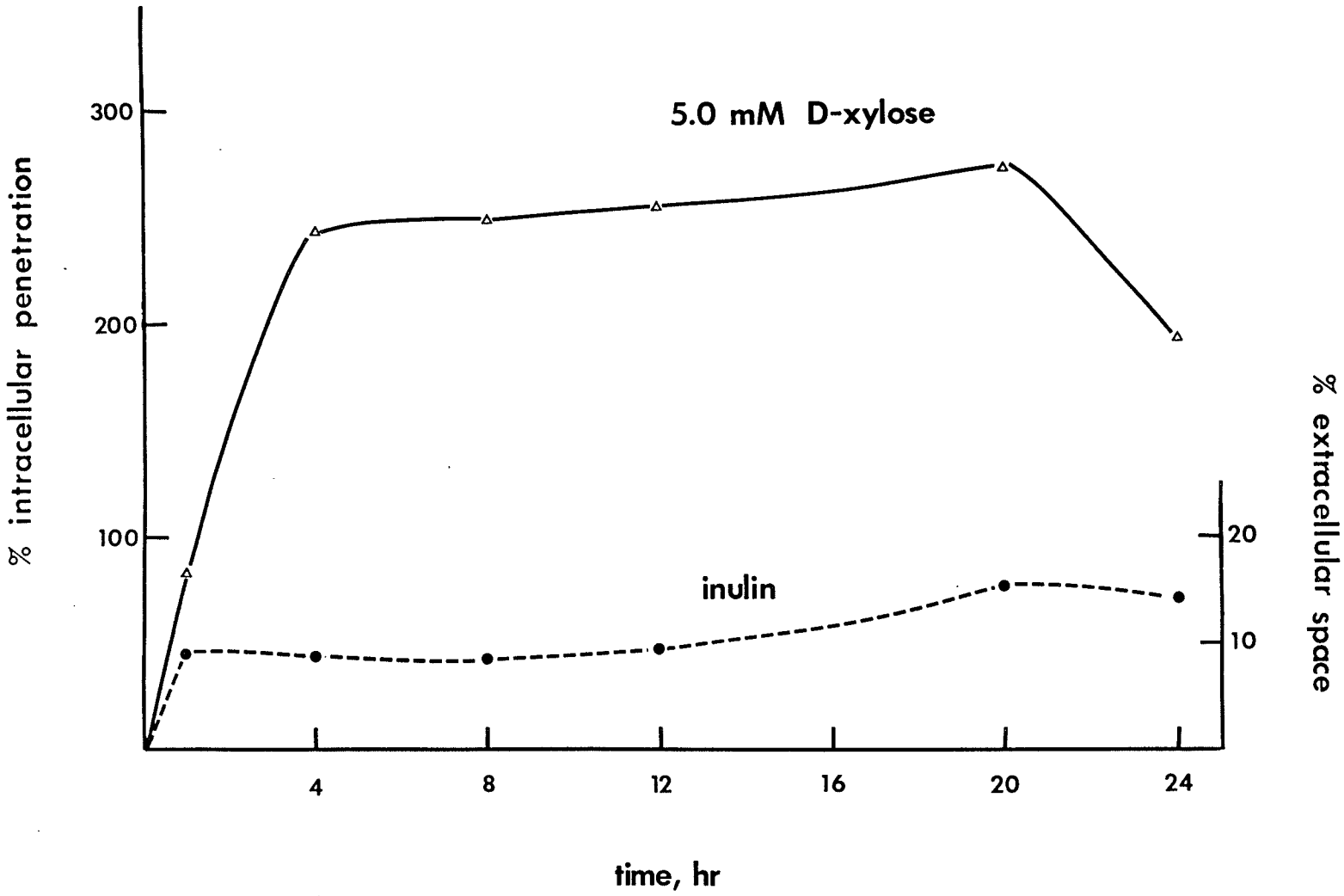


Fig. 6. The effect of incubation time on the intracellular penetration of 5.0 mM D-xylose and on the inulin space. Lenses which were incubated for more than four hours received additional D-glucose as metabolic substrate and fresh O_2 - CO_2 was blown into the flasks several times during the course of the experiment. For other details see METHODS. The standard errors have been omitted as they were smaller than the symbols of the data points.

steeper than was found with 3-O-MG and steady-state was already reached after four hours of incubation. At that time the percent penetration of D-xylose reached approximately 250% and stayed relatively constant for up to 20 hours. The extracellular space was also within normal limits during this time. After approximately 20 hours of incubation the lenses deteriorated, the ECS began to increase and the apparent intracellular concentration of D-xylose started to drop. D-xylose and D-galactose are known to be metabolized in lenses and the pathways for their conversion are well established (van Heyningen, 1959; Hayman and Kinoshita, 1965). It therefore appeared likely that the apparently active uptake of these sugars in the lens could be due to the accumulation of radioactive metabolites rather than of free sugar.

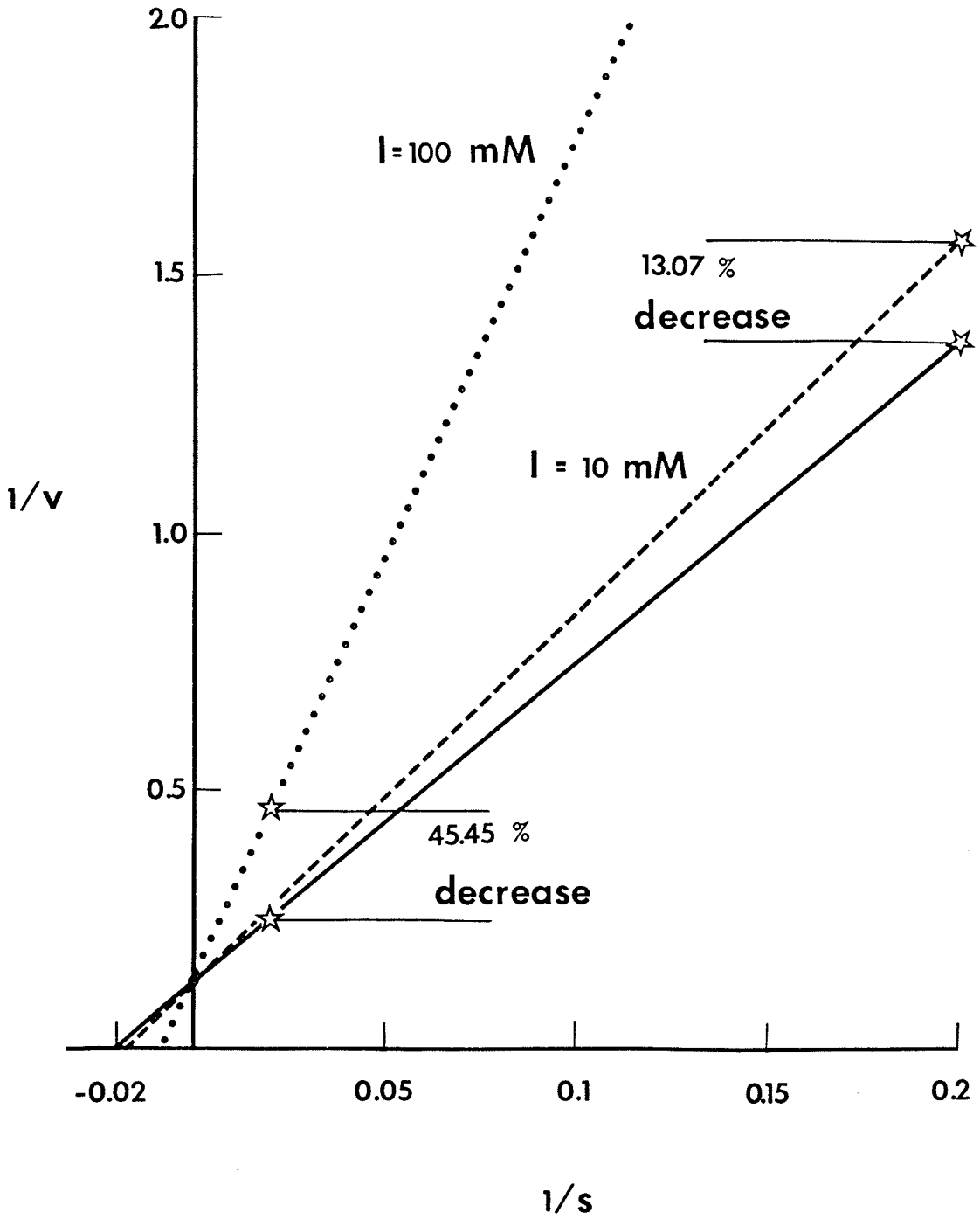
The contribution of metabolites to the total intracellular radioactivity was determined in two different ways. Using D-xylose as test sugar, lenses were incubated for four hours to reach the steady-state concentration. After incubation, the lenses were homogenized in distilled water using a glass-Teflon homogenizer. A comparison was then made between the value for penetration obtained when portions of the homogenate were counted before and after the addition of $Ba(OH)_2$-$ZnSO_4$. This procedure precipitates the phosphorylated intermediates but does not affect the concentration of free sugar in solution (Kipnis and Cori, 1959). The penetration as determined after $Ba(OH)_2$-$ZnSO_4$, $220.45 \pm 6.35\%$, was considerably less than the value of $304.85 \pm 2.25\%$ obtained without the precipitation. The high intracellular penetration in this particular experiment, over 300% compared to 250% at steady-state in the time-course experiment (Fig. 6), was probably due to the age of the rats used; the

younger the animals, the greater their metabolic activity. Since D-xylose is probably mainly oxidized to xylonic acid, the $Ba(OH)_2$-$ZnSO_4$ treatment cannot precipitate all the metabolites formed from xylose. The experiment was therefore repeated and D-xylose determined chemically by the spectrophotometric method of Roe and Rice (1948). The value for penetration now became 65.62 $\pm$ 3.13% which was very close to that found with 3-O-MG. Although not yet confirmed by chemical analysis, it is likely that the apparently active transport of D-galactose was similarly due to the accumulation of radioactive metabolites rather than of free sugar. Thus, the high values of penetration for D-xylose and D-galactose cannot be taken as evidence for active transport. On the other hand, the failure of the free sugars to exceed equilibrium cannot be taken to exclude active transport because their concentrations are determined by influx, efflux and metabolic disposal and, at steady-state, the intracellular sugar concentration could be less than that of the incubation medium. The data obtained with metabolic inhibitors (see below) do not support a requirement for energy, which would be characteristic for an active transport system. On balance, there is no reason to propose that active transport of these sugars does take place. The competition data (see below) also favour a common transport system for 3-O-MG and these metabolized sugars. L-arabinose also should be included in this group of metabolized sugars even though the accumulation of radioactivity was below 100%. Its penetration was appreciably higher than would be expected from data obtained in other tissues. By comparing disappearance from the medium with intracellular accumulation, Kern (1965) concluded that L-arabinose was metabolized in calf lenses. Patterson

(1955) has shown that this pentose, like other sugars which are metabolized in the lens, will accelerate the induction of galactose cataracts in rats. It is therefore likely that the high value obtained with this sugar may also be explained by the accumulation of radioactive metabolites in the lenses.

In the second series, the specificity of the transport system was further investigated by competition experiments. A decrease in the transport of 3-O-MG in the presence of another sugar was taken to mean that the sugars share a common pathway for entry. When lenses were incubated with 5.0 mM 3-O-MG, the addition of 10 mM of various other sugars did not significantly alter the penetration of the test sugar. When the concentration of test sugar was increased to 25 mM and that of the competitors to 50 mM, inhibition could be demonstrated but not consistently reproduced. A consideration of the kinetics of competitive inhibition indicated that the degree of inhibition at a given substrate/inhibitor concentration ratio will increase with the concentration of test sugar. This concept is explained in Fig. 7. In this idealized double reciprocal plot, the transport of a specific sugar is described by the solid line. The addition of a competitive inhibitor will give rise to another line with the same y-intercept, i.e. V_{max}, but the apparent K_m of the sugar will be increased. The increase in the slope and decrease in x-intercept is directly related to the concentration of the competitor. As illustrated in the Lineweaver-Burk plot, the degree of inhibition will not depend upon the ratio of test sugar to inhibitor but upon the absolute concentration of inhibitor used. The pairs of stars indicate the percent inhibition obtained with two different

Fig. 7. Idealized Lineweaver-Burk plot showing the effect of two different concentrations of a competitive inhibitor on the rate of penetration of test sugar. The solid line represents the kinetic characteristics of the substrate when alone; the dashed line indicates the situation in the presence of 10 mM competitor, and the dotted line with 100 mM competitor. The values used to calculate the lines were: V_{max}, 8; K_m, 50 and K_i, 60. The values between the stars indicate the percent decrease in the penetration of the substrate at the two concentrations where the concentration ratio of substrate to inhibitor equals two.


2.0
1.5
1.0
0.5
1/v
I=100 mM
13.07 %
decrease
I = 10 mM
45.45 %
decrease
-0.02
0.05
0.1
0.15
0.2
1/s

concentrations of inhibitor but with the same concentration ratio of test sugar to inhibitor.

In the light of these considerations, the inhibition experiments were repeated and the concentration of 3-O-MG increased to 50 mM and that of the competing sugar to 100 mM; the incubation time was either 30 minutes or one hour. With these high sugar concentrations, competition was clearly demonstrated with the shorter incubation time, while with the one hour incubation period the level of significance was further enhanced. These latter results are presented in Fig. 8. The sugars which inhibited the penetration of 3-O-MG are known to be metabolized in the lens, and include D-glucose as well as D-xylose, D-galactose and L-arabinose as discussed above. The degree of inhibition did not correlate with the degree of accumulation of these sugars; D-galactose was a more effective inhibitor than D-xylose. This was not unexpected since the accumulation of radioactivity is a reflection both of affinity and metabolism. The greatest inhibition was obtained with D-glucose which must have the lowest K_m of these metabolized sugars. D-arabinose and L-xylose did not compete for entry with 3-O-MG, while L-glucose apparently stimulated the penetration of the test sugar. Even though this was only significant at $p < 0.05$, it was consistently observed and is a still unexplained finding.

As determined by these experiments, the chemical specificity of the sugar transport system in the lens of the eye is very much the same as that found in other tissues where mediated transfer of sugars takes place. As a general rule, the transport site has an affinity for sugars of the D-configuration but not for the L-forms. However, for the

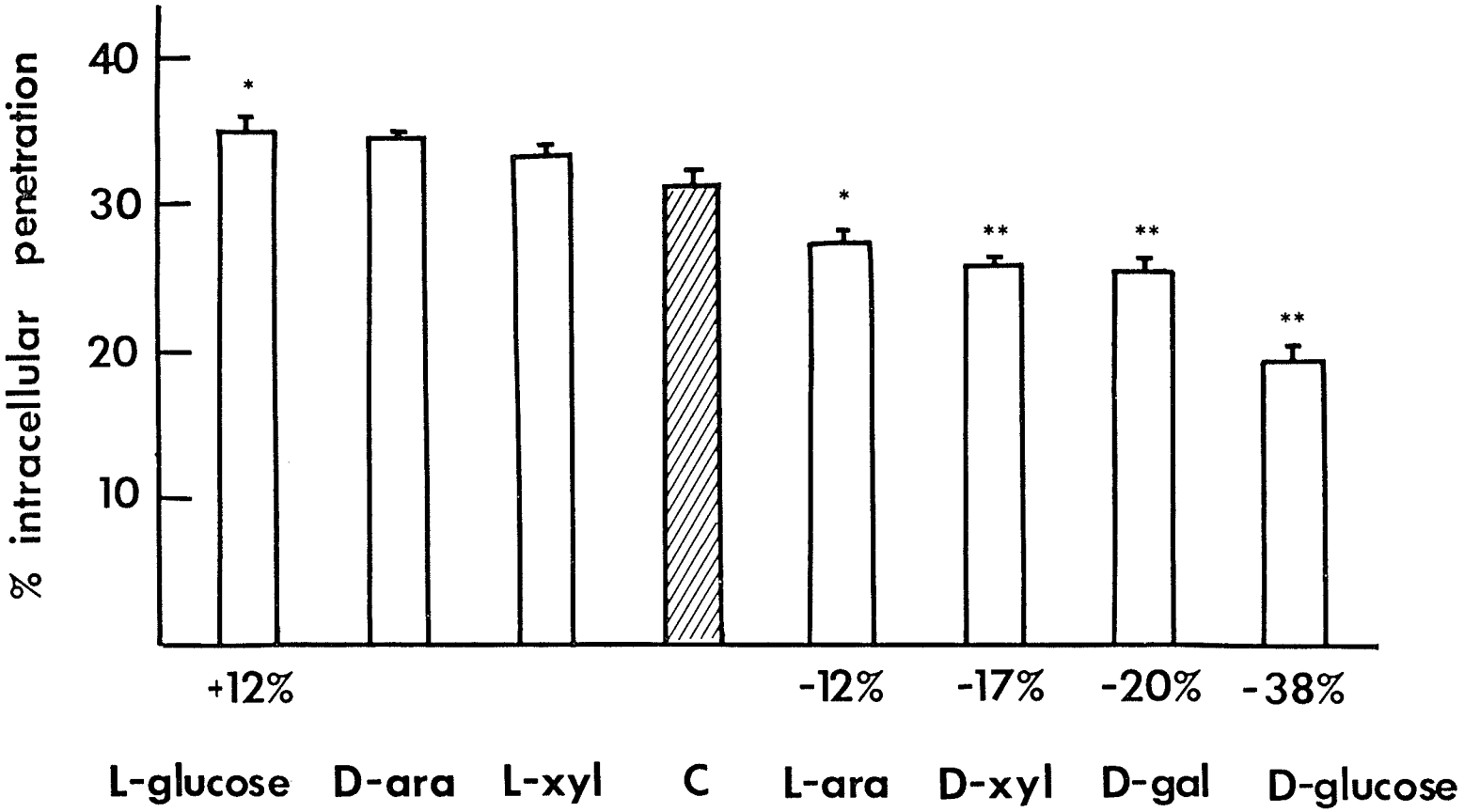


Fig. 8. The effect of competitors (100 mM) on the penetration of 50 mM 3-O-methyl-D-glucose. Incubation time, one hour. The vertical lines represent one standard error. Control is indicated by the hatched bar. An amount of NaCl, osmotically equivalent to the total sugar concentration was omitted to maintain the osmolarity of the buffer at 308 mOsmoles; in controls, sucrose was added instead of the competitor.
* $p < 0.05$, ** $p < 0.01$.

pentose arabinose, this rule is reversed (Stein, 1967).

An additional competition experiment was carried out under conditions in which the sugar transport system was inhibited with phloretin. It would be expected that if facilitated diffusion had been completely blocked, the presence of a competing sugar would not affect the penetration of the test sugar. The lenses were incubated with 50 mM 3-O-MG with or without 100 mM D-glucose as competing sugar (as described above) either in the presence or absence of 0.5 mM phloretin. The results indicate that this concentration of phloretin was insufficient to inhibit the transport system completely. After one hour of incubation, the penetration of test sugar, $36.84 \pm 0.41\%$, was significantly decreased by the addition of D-glucose to $24.62 \pm 1.23\%$ ($p < 0.01$). In the presence of phloretin, these values were $13.40 \pm 0.22\%$ and $7.30 \pm 0.08\%$ respectively, and the difference between them was significant at $p < 0.01$. These results are consistent with the conclusion that 0.5 mM phloretin does not completely block the transport system and that the residual penetration after phloretin treatment is still mediated by the specific transport system.

The effect of insulin and enzymes

The finding of Ross (1953) that insulin accelerated the uptake of glucose and galactose in rabbit lenses stimulated great interest in the action of this hormone on lenticular metabolism. At one time it was postulated that diabetic cataract formation was due to a deficiency of oxidizable substrate (Patterson, 1956). Studies comparing normal and diabetic lenses, however, indicated that the latter contained appreciable amounts of reducing sugar, suggesting that sufficient substrate

for energy production was available. It is therefore more likely that one of the steps involved in substrate utilization is responsible for cataract formation (Farkas et al., 1960). The effects of insulin in vitro on sugar penetration, as reported by various laboratories, were contradictory (Macintyre et al., 1956; Levari et al., 1961). The possible effect of this hormone on sugar transport in the lens was therefore studied under a variety of experimental conditions.

In a preliminary experiment, two concentrations of insulin, 1.0 and 10.0 milli-u/ml present during both preincubation and incubation failed to affect the penetration of the test sugar. 25 milli-u/ml of insulin was therefore used, a concentration which induces maximal stimulation of sugar transport in the rat hemidiaphragm in vitro (Bihler, 1968). To allow adequate time for insulin to reach its site of action, lenses were preincubated for 150 min with the hormone, followed by a 30 minute incubation period with test sugar and the same concentration of the hormone (Fig. 9, expt. A). The difference in penetration of the insulin-treated lenses compared to the controls, $+0.28 \pm 0.71\%$, was not significant. This absence of a stimulatory effect of insulin contradicts the results of Ross (1953) but confirms similar findings by Harris et al. (1955), Farkas and Patterson (1957) and Giles and Harris (1959).

Farkas and Roberson (1965) were able to demonstrate a stimulatory effect of insulin on glucose utilization in vivo only after pretreatment of animals with a specific concentration of trivalent chromium. Mertz et al. (1961) had previously reported that this ion enhanced the insulin-activated but not the basal glucose uptake in epididymal fat pads of chromium-deficient rats. However, in the lens 25 milli-u/ml of

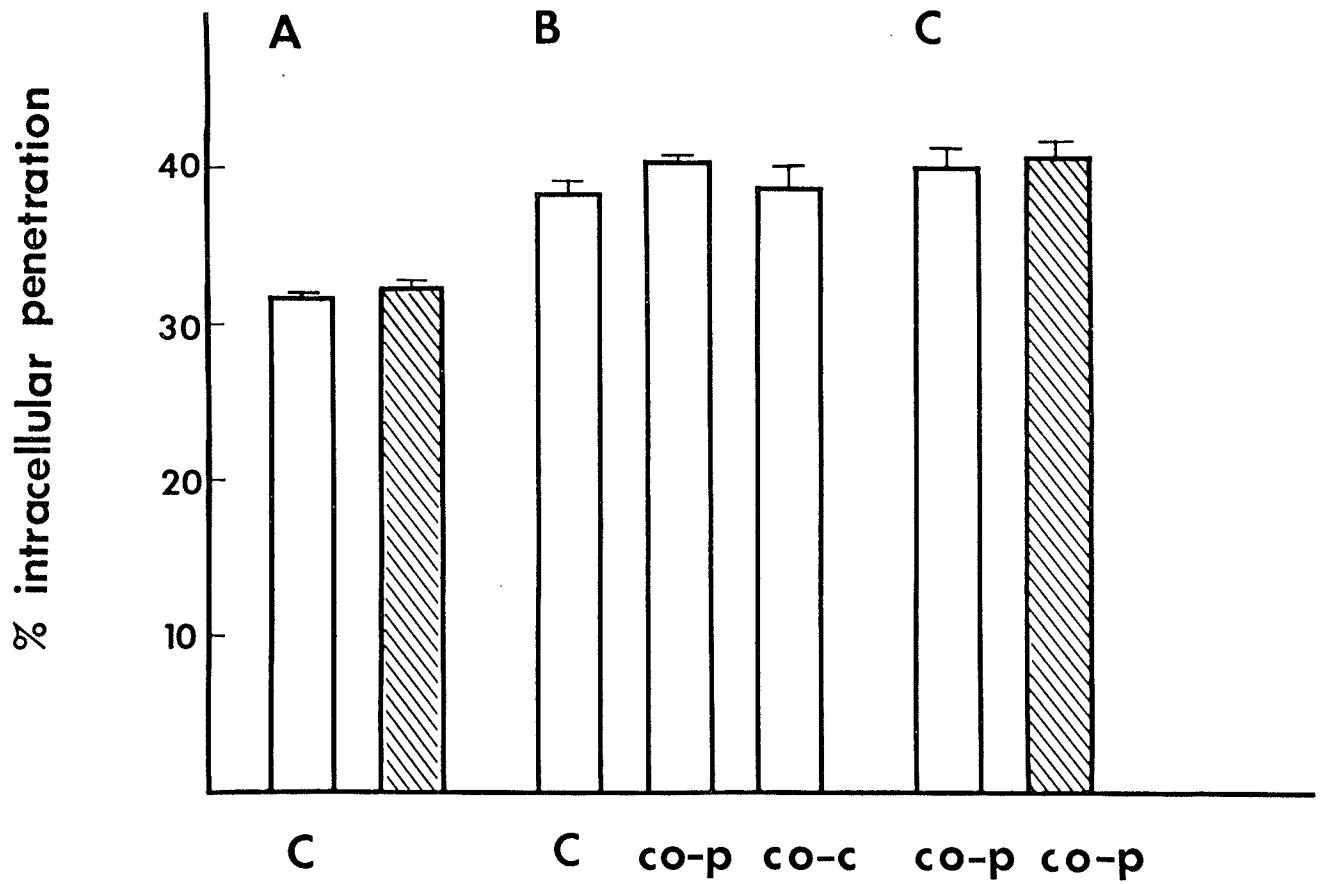


Fig. 9. The effect of insulin and collagenase on the intracellular penetration of 5.0 mM 3-O-methyl-D-glucose. For incubation times see text. The vertical lines represent one standard error. Abbreviations: C, control; co, collagenase, 1 mg/ml; p, pure and c, crude enzyme preparation. The hatched bars indicate the presence of insulin. The insulin concentration used in experiment A was 25 milli-u/ml and in experiment C, 100 milli-u/ml.

insulin had no effect on the penetration of 5.0 mM 3-O-MG in the presence of either 0.15 or 0.3 μg Cr^{+++}/ml. The differences from control were $-1.66 \pm 1.52\%$ and $+0.34 \pm 1.33\%$ respectively.

Next, the possibility was investigated that the capsule prevents insulin from reaching its site of action. Contradictory results have been obtained regarding the effect of injury or removal of the capsule. Gentle massage of the whole lens or incision of the capsule 'markedly alters its ability to concentrate the positive ions' (Harris et al., 1955). These workers also showed that these treatments did not affect the glucose 'accumulation' and that massage decreased lenticular glucose utilization only. Giles and Harris (1959) also found that glucose uptake was decreased in rabbit lenses from which the capsule had been removed by dissection. Decapsulation with collagenase, however, did not affect the intracellular sodium and potassium ion concentrations in toad lenses or interfere with the inhibitory action of ouabain on the sodium pump (Toyofuku and Bentley, 1970). Levari et al. (1961) also mentioned a decrease in sugar uptake in decapsulated rat lenses, and Kern (1965) found that V_{max} and K_m for 3-O-MG transport were greatly altered in collagenase-treated rat lenses; however, his results were difficult to interpret. Oxygen consumption has been shown to increase after decapsulation (Ely, 1949), while lactic acid production is decreased (Müller and Kleifeld, 1953, as quoted by Giles and Harris, 1959). Since the capsule consists of collagen or collagen-like material (Leeson and Leeson, 1966) decapsulation was first done with collagenase. Because the crude enzyme is contaminated with a peptidase as well as a trypsin-like proteinase, a chromatographically purified collagenase preparation

was also used for decapsulation. As described by Kern (1965), a two-hour treatment with 1 mg/ml of either of the two enzyme preparations proved to be sufficient to remove the capsule. The lenses became round and lost their elasticity and firmness. Microscopic examination confirmed that the capsule had been removed without visible damage to the epithelial layer or the lens fibers. In the present experiments, decapsulation did not affect the subsequent penetration of test sugar during a one hour incubation period (Fig. 9, expt. B). The difference from control using the pure collagenase was $+1.97 \pm 0.97\%$ and with the crude collagenase $+0.35 \pm 1.79\%$. These results suggest that the penetration of sugar through the lenticular capsule was not rate-limiting. Decapsulation did significantly decrease the extracellular space of the lens from the control of $11.40 \pm 0.61\%$ to $4.88 \pm 0.38\%$ ($p < 0.01$) with the crude collagenase and to $5.03 \pm 0.38\%$ ($p < 0.01$) with the purified preparation. This represents added support for the suggestion of Paterson (1970) that a large part of this space is due to the presence of the capsule itself.

After decapsulation with collagenase, the lenses were washed in a large volume of buffer and then incubated with test sugar and 100 milli-u/ml of insulin but no significant increase in the penetration of test sugar could be detected (Fig. 9, expt. C). These results indicate that the sugar transport system in the lens of the eye does not respond to insulin added <u>in vitro</u>.

Certain proteolytic enzymes such as trypsin and chymotrypsin may specifically stimulate the sugar transport system in skeletal muscle (Rieser and Rieser, 1964a). Trypsin, like collagenase, is often used

for cell separation and is also able to digest the capsule. When lenses were exposed during preincubation and incubation to 0.2 mg/ml trypsin obtained from the Nutritional Biochemical Cor. (NBC), the capsule was removed, as confirmed by microscopic examination. The lenses displayed the same physical characteristics as after collagenase treatment. As with collagenase, no effect on subsequent sugar penetration was seen, the difference from control being +1.02 $\pm$ 1.39% (Fig. 10, expt. A). However, when trypsin obtained from another supplier (Sigma Chemical Comp.) was used at the same concentration, penetration was significantly decreased from 38.23 $\pm$ 0.83% to 29.88 $\pm$ 4.40% ($p < 0.05$). The two preparations had the same trypsin activity. In other experiments, higher (0.4 mg/ml) or lower (0.1 mg/ml) concentrations of trypsin, while equally effective in removing the capsule, were also without effect on the rate of penetration of the test sugar. Surprisingly, trypsin did not consistently lower the extracellular space even though it was as effective as collagenase in removing the capsule. A decrease was found only with trypsin obtained from NBC (from 11.40 $\pm$ 0.61% to 7.10 $\pm$ 0.63%, $p < 0.05$) but not with the enzyme obtained from Sigma Chemical Comp. These observations suggest that trypsin may have been more effective than collagenase in separating the lens fibers and thereby increasing the ECS. This increase might have been roughly balanced by the decrease due to removal of the capsule. Total tissue water was slightly increased by trypsin-treatment from approximately 60 to 64%. The results obtained with trypsin are in contrast to findings in skeletal muscle where proteolytic enzymes may stimulate the activity of the sugar transport system. In the rat hemidiaphragm, these enzymes mimick some of the

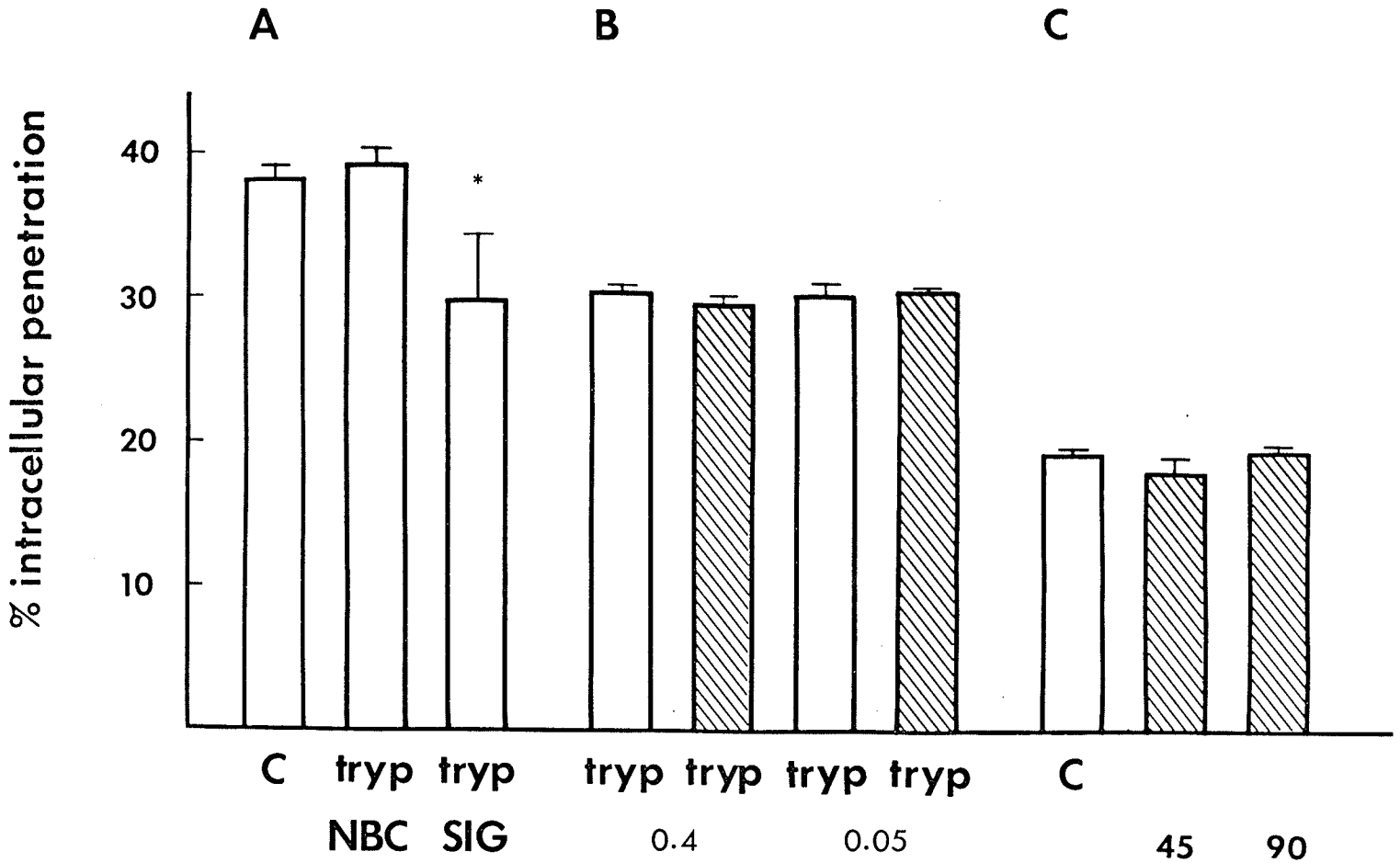


Fig. 10. The effect of trypsin and insulin on the intracellular penetration of 5.0 mM 3-O-methyl-D-glucose. For incubation times see text. The vertical lines represent one standard error. Abbreviations: C, control; tryp, trypsin; NBC, Nutritional Biochemical Corp.; SIG, Sigma Chemical Comp. The concentration of trypsin in experiment A was 0.2 mg/ml and those in experiment B are indicated (in mg/ml) on the figure. The hatched bars indicate the presence of insulin. The insulin concentration used in experiment B was 100 milli-u/ml. In the in vivo experiment (C) 10 u/100 g of insulin was injected while the controls received an equivalent volume of saline. In this experiment, the animals were killed either 45 min or 90 min after injection. * $p < 0.05$.

actions of insulin (Rieser and Rieser, 1964a); Kohn and Clausen (1971) have shown that trypsin enhanced the efflux of 3-O-MG from rat soleus muscle and that this increased efflux was sensitive to phlorhizin.

After decapsulation with trypsin (0.4 mg/ml or 0.05 mg/ml) the lenses were thoroughly washed by transfer to a large volume of medium to remove as much trypsin as possible, since the proteolytic action of this enzyme might inactivate insulin. After that, the tissues were incubated for 30 minutes with test sugar and 100 milli-u/ml insulin; appreciable sugar penetration took place but the high concentration of insulin was again ineffective in stimulating transport (Fig. 10, expt. B).

Since collagenase was as effective as trypsin in decapsulating the lenses, and insulin is not known to be attacked by collagenase, it is unlikely that the lack of an insulin effect was due to destruction of the hormone. Therefore, further experiments with trypsin inhibitors did not seem justified.

Some laboratories have reported an effect on the lens after insulin administration *in vivo*. Macintyre *et al*. (1956) described a significant increase in glucose uptake after injection of the hormone; this stimulating effect of insulin was said to be independent of the blood sugar level of the rats. Farkas and Patterson (1957) confirmed this finding. They also found that evisceration below the level of the diaphragm abolished this response and that the liver had to be present for the effect of insulin on glucose uptake. Our results failed to confirm these findings. Rats were injected intraperitoneally with 10 units of insulin per 100 g weight, the controls with equivalent volumes of 0.9% NaCl. The animals were killed 45 or 90 minutes after in-

jection and the lenses were then removed and sugar penetration was measured in the usual way. Insulin was not added to the media and the incubation time was 15 minutes. This experimental design was very similar to that of the investigators quoted above. Although blood glucose was not measured, the rats showed evidence of hypoglycemia such as drowsiness. As seen from Fig. 10, expt. C, appreciable penetration occurred during this short period but no effect of insulin was apparent. The differences in penetration from control were $-1.11 \pm 0.82\%$ and $+0.15 \pm 0.48\%$ for rats killed 45 minutes and 90 minutes after the administration of insulin, respectively. Further *in vivo* experiments therefore were not warranted.

In summary, insulin did not stimulate the transport of sugar in the lens of the eye *in vivo* or *in vitro*. Removal of the capsule by collagenase or trypsin did not allow insulin to have an effect, and this treatment by itself did not change the activity of the sugar transport system. These results with insulin were not unexpected in view of the fact that this hormone is probably not able to cross the blood-aqueous humour barrier in significant amounts. Giles and Harris (1958) have shown that after injection of radioiodinated insulin, the peak level in the aqueous humour was approximately one-hundredth of that present in the plasma; these authors concluded that the lens 'is always in a diabetic state'. Ross (1952) has reported that insulin increases the permeability of the blood-aqueous humour barrier to glucose and other transported sugars. Thus, the main anatomical location of insulin action in the eye may very well be at this site.

In certain insulin-sensitive tissues, some of the metabolic

effects of the hormone are separate from its action on sugar transport (see INTRODUCTION). It is not inconceivable, therefore, that the increase in lenticular lactate production (Levari et al., 1961) induced by insulin is mediated by a mechanism independent from an effect on transport.

In this connection, one report by Giles and Harris (1959) indicated that the drug phenformin could stimulate glucose uptake in rabbit lenses under conditions where insulin was ineffective. This hypoglycemic drug is used in the management of maturity-onset diabetes and apparently acts by potentiating the action of insulin. The concentration used in their experiments, 0.1 mg/ml, was about 20 times the required therapeutic plasma concentration. Such high concentrations of biguanides are known to uncouple oxidative phosphorylation and to inhibit other metabolic pathways (Williams, 1965). In our rat lenses, either phenformin (phenethylbiguanide) or methformin (dimethylbiguanide) were ineffective in increasing the penetration of 3-O-MG. With the drugs present during incubation only, the difference from control was $-2.13 \pm 1.13\%$ with 0.1 mg/ml and $+0.23 \pm 1.09\%$ with 0.2 mg/ml of phenformin and $+2.02 \pm 4.07\%$ with 0.1 mg/ml of methformin. With larger concentrations of the drugs, the lenses turned opaque and penetration was depressed.

The effect of ions

The dependence of sugar transport on extracellular sodium ions in the lens of the eye was determined by omitting one half or all of the sodium from the standard Krebs-Henseleit bicarbonate buffer. The osmolarity of the buffer was maintained by the addition of sucrose, D-mannitol or choline chloride. Of these three substituents, only choline

chloride is ionized. In one series of experiments, 50% of the total sodium was replaced by isosmotic amounts of these non-penetrating substituents. The lenses were both preincubated and incubated in the modified media. No statistically significant differences were found in the penetration of 5.0 mM 3-O-MG when either the ionizable or non-ionizable substituents for sodium were used (Fig. 11, expts. A and B With choline chloride but not with the non-ionized substituents, the extracellular space was slightly but significantly increased from $5.22 \pm 0.24\%$ to $6.35 \pm 0.22\%$ ($p < 0.01$). In a second series of experiments, the total sodium content of the medium was replaced by isosmotic amounts of the three substituents. Instead of sodium bicarbonate, 25 mM of tris(hydroxymethyl)-aminomethane (Tris), bubbled with O_2 : CO_2 (95% : 5%), was used as buffer. The lenses were preincubated and incubated in these modified buffers and the penetration of the test sugar was significantly decreased ($p < 0.01$) under these conditions (Fig. 11, expts. C and D). Concomittantly, the extracellular space increased from $5.22 \pm 0.24\%$ to $8.76 \pm 0.15\%$ with D-mannitol, to $7.76 \pm 0.29\%$ with choline chloride and from $5.63 \pm 0.69\%$ to $9.40 \pm 0.52\%$ with sucrose. In all cases this decrease was highly significant ($p < 0.01$). Since in these experiments bicarbonate was lower, there was a possibility that this ion as such had an effect on sugar transport. This was tested in the following experiment. Lenses were preincubated and incubated in media in which 50% of the sodium was replaced by sucrose, with either sodium bicarbonate or Tris as buffer. The difference in intracellular penetration between these two treatments, $1.43 \pm 2.14\%$, was not significant. These results do not support the hypothesis that extracellular

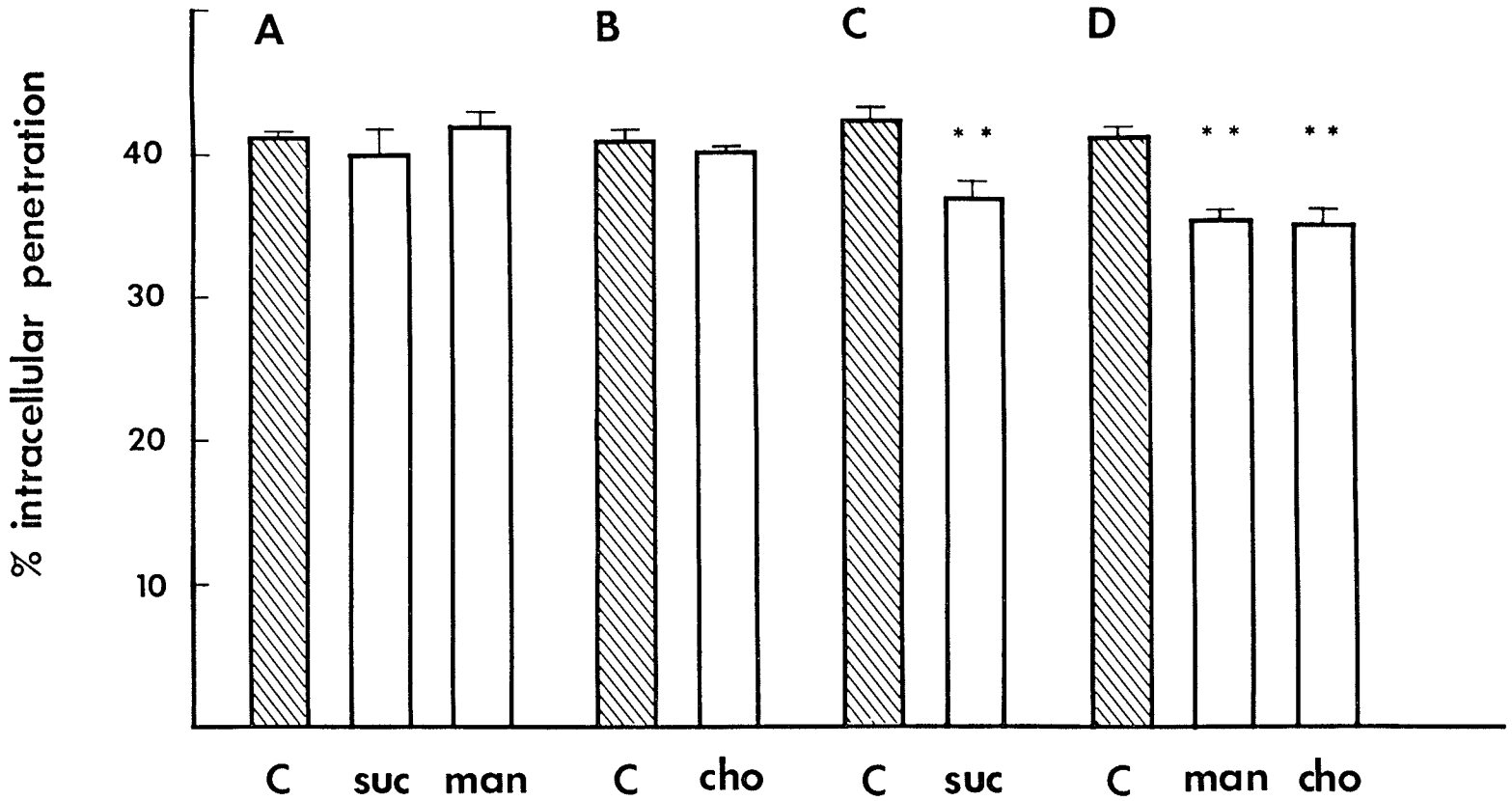


Fig. 11. The effect of sodium replacement on the intracellular penetration of 5.0 mM 3-O-methyl-D-glucose. Incubation time, one hour. The vertical lines represent one standard error. The controls are indicated by the hatched bars. Abbreviations: C, control; suc, sucrose; man, D-mannitol; cho, choline chloride. In experiments A and B, 50% of the total sodium has been replaced; in experiments C and D, 100%. In the latter experiments, Tris was used as buffer. ** $p < 0.01$.

sodium ions play a role in the penetration of sugars in the lens although such may be the case in rat hemidiaphragm (see INTRODUCTION). The lower intracellular penetration in the complete absence of this ion is probably due to nonspecific changes in the membrane. This suggestion is supported by the finding that the extracellular space was significantly increased under these conditions. Other changes in the membrane in complete absence of sodium include a significant inhibition of the Na^+-K^+-ATP-ase, as demonstrated for the lens epithelium by Bonting et al. (1963). Although Tris may inhibit some enzyme systems (Dayan and Wilson, 1964), it does not appear to affect the sugar transport system in the lens of the eye.

The effect of a change in intracellular ion levels on the activity of the sugar transport system was determined by inhibition of the sodium pump. Incubation of lenses in a potassium-free medium or inhibition by ouabain was used. A concentration of 10^{-6} M ouabain was reported to inhibit the Na^+-K^+-ATP-ase in rabbit lenses (Kern et al., 1962; Matuk et al., 1969) and a similar concentration (1.17×10^{-6} M = 10^{-6} g/ml) was tried in one experiment with rat lenses. The drug was added during both the preincubation and the incubation periods. The usual 5.5 mM D-glucose was present during preincubation to supply the required metabolic substrate for a possible binding or transport step of ouabain (Bihler, 1968). Sodium and potassium determinations were made in separate lenses incubated under identical conditions. Since the absolute sodium and potassium ion concentrations in the lens, although not their ratio, are a function of age (Hart et al., 1963), special care was taken in this experiment to use animals of the same

age. After either three or six hours of preincubation, the uptake of 5.0 mM 3-O-MG during one hour of incubation was not affected by ouabain. However, this concentration of ouabain was also insufficient to inhibit the sodium pump.

Since rats appear to be very insensitive to the action of cardiac glycosides on the heart (Chen, 1963), higher concentrations of these drugs may also be required to inhibit the sodium pump in the lens of these animals. The concentration of ouabain in the preincubation and incubation media was therefore increased to 10^{-4} g/ml. The usual concentration of 5.0 mM 3-O-MG was used. In the same experiment, lenses were also preincubated and incubated in a potassium-free medium for identical times. In this case one lens of a pair was used to obtain the percent penetration of 3-O-MG, the other for the ion determinations. The results are presented in Fig. 12. With ouabain sugar penetration was decreased slightly, but not significantly from $42.18 \pm 1.41\%$ to $39.62 \pm 1.17\%$. The decrease to $38.04 \pm 0.04\%$ after incubation in the potassium-free medium was significant ($p < 0.05$) but small. This decrease does not appear to be related to the induced ion shifts since both ouabain and incubation in the K^+-free medium effectively inhibited ion transport. In the absence of potassium, the ECS increased from $4.78 \pm 0.23\%$ to $7.52 \pm 0.76\%$ ($p < 0.01$) while with ouabain this space increased to a lesser extent ($6.64 \pm 0.51\%$, $p < 0.05$). The apparent decrease in sugar penetration in lenses incubated in a potassium-free medium would be explained by the small shrinkage of these lenses (see below). Indeed, if the appropriate correction was applied, this decrease was no longer significant. It is therefore more likely that potassium ions are required

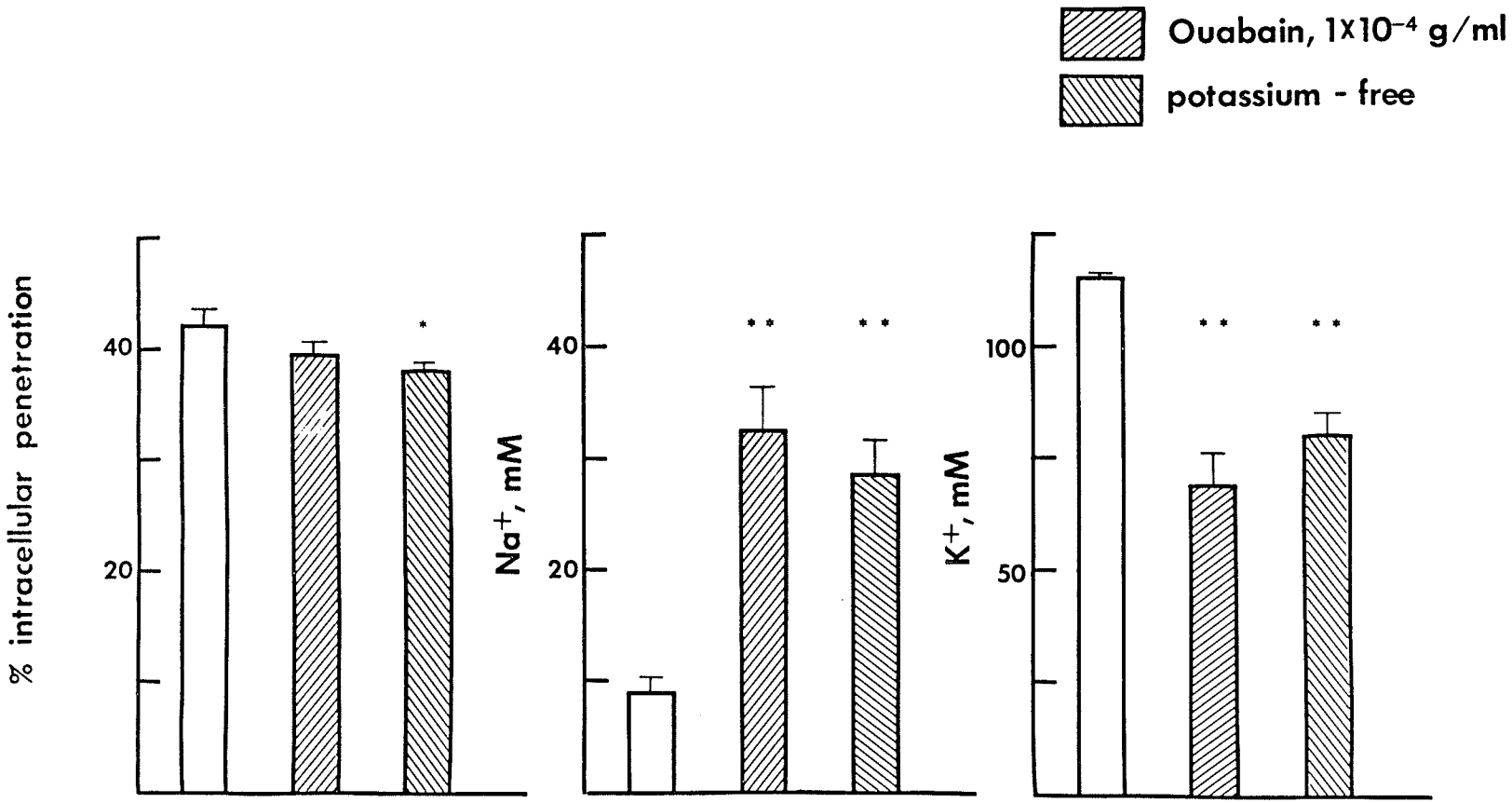


Fig. 12. The effect of ouabain(10^{-4} g/ml) and incubation in a potassium-free medium on the penetration of 5.0 mM 3-O-methyl-D-glucose and the concentrations of intracellular sodium and potassium ions. Incubation time, one hour. Controls are indicated by the open bars. The vertical lines represent one standard error. * $p < 0.05$, ** $p < 0.01$.

for maintenance of cell membrane integrity and that the observed decrease was an unspecific effect on the membrane rather than on the transport system.

The intracellular potassium concentration decreased from a control value of 115.78 $\pm$ 1.44 mM to 68.59 $\pm$ 6.59 mM with ouabain and to 80.10 $\pm$ 5.02 mM in the K^+-free medium. Intracellular sodium increased from 9.12 $\pm$ 1.29 mM to 32.93 $\pm$ 3.58 mM and to 28.85 $\pm$ 3.04 mM, respectively, with these treatments. After inhibition of the sodium pump, the sum total of the lenticular concentrations of sodium and potassium was significantly lower than in the controls. One explanation for this disparity could be the concurrent efflux of other ions, e.g. chloride as suggested by the data of Matuk et al. (1969). 10^{-4} g/ml ouabain appeared to be a more effective inhibitor than the absence of potassium but there was no statistical difference between the ion shifts induced by the two treatments. The slightly smaller inhibition of the sodium pump in the absence of potassium may have been due to the small amount of potassium (0.2 - 0.3 mM) leaked from the lens into the medium during incubation (Bonting et al., 1962). The results are expressed in mmoles/l intracellular water, and represent average values based on the assumption that all lens fibers have the same cationic composition. However, there is some evidence that the intracellular cation concentrations of the lens fibers may depend upon their position within the lens (Amoore et al., 1959). A further investigation of the actual ion distribution within the lens was unwarranted because no really significant effects on sugar penetration were observed upon inhibition of the sodium pump.

In another experiment, D-xylose was used as test sugar at a concentration of 5.0 mM. The lenses were exposed to ouabain (10^{-4} g/ml) during 45 minutes of preincubation and a four-hour incubation period. The accumulation of radioactivity in the ouabain-treated lenses, 218.48 $\pm$ 17.57% was not different from the control value of 215.13 $\pm$ 10.05%. In conjunction with the results obtained with 3-O-MG this would suggest that neither the transport nor the metabolism of D-xylose were affected by ouabain.

Because of the design of these experiments, lens wet weight could only be compared between the treatment groups and not between contralateral lenses from the same animal. With this comparison, no hydration of the lenses was observed during inhibition of the sodium pump. Lens wet weight of the ouabain treated group, 27.39 $\pm$ 0.24 mg, was not significantly different from the control value of 27.83 $\pm$ 0.38 mg. The wet weight of lenses incubated in the K^+-free medium was 26.95 $\pm$ 0.38 mg, which was significantly lower than the controls ($p < 0.05$). Therefore, under the present conditions inhibition of the sodium pump did not lead to lenticular swelling. This is consistent with the results of Matuk et al. (1969): In rabbit lenses incubated for 18 hours without glucose as metabolic substrate swelling did not occur while ion shifts had taken place; in this respect, lenses appear to differ from erythrocytes. In sheep erythrocytes it has been shown that the cell volume, as well as the cation composition, is controlled by the action of the sodium pump (Tosteson and Hoffman, 1960).

The possible role of calcium ions in the regulation of the activity of the sugar transport system in skeletal and cardiac muscle

has been referred to in the INTRODUCTION. To determine whether in the lens a similar link exists between this ion and sugar penetration experiments were carried out in which the calcium concentration in the media was varied. It appears likely that, as in other tissues, such treatment would influence the cellular and membrane-bound calcium. Incubations were carried out in a high-calcium medium, containing twice the normal ion concentration (5.08 mEq.Ca^{++}/l), in a calcium-free medium and in a Ca^{++}-free medium to which ethyleneglycol-bis(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA) had been added. This chelating agent is very effective in depleting tissue calcium in muscle. The lenses were preincubated and incubated in these different media; 5.0 mM 3-O-MG was present during the one-hour incubation period. Statistical analysis showed no significant effects either on sugar penetration or on the extracellular space. The differences from control were $-1.68 \pm 2.16\%$ for lenses incubated in a Ca^{++}-free medium, $+0.67 \pm 1.51\%$ in the Ca^{++}-free medium with the chelating agent and $-0.90 \pm 0.94\%$ in the high-calcium medium. The respective differences for the extracellular space were $+1.23 \pm 2.48\%$, $-0.40 \pm 1.72\%$ and $-1.15 \pm 1.61\%$.

These results would indicate that the presence or absence of extracellular calcium has no effect on sugar penetration in the lens. However, this conclusion may be erroneous. Thoft and Kinoshita (1965) have found that the extracellular space of lenses incubated in a calcium-free medium was greatly increased when sucrose was used as the extracellular marker, but was unchanged when inulin was used. The reasons for using inulin as the marker for the ECS have been discussed in the METHODS section. Accepting the data of Thoft and Kinoshita (1965), the

results were recalculated on the basis that the ECS had doubled in size. In the calcium-free medium, the penetration of the test sugar would have been 35.10% instead of the original 40.50 $\pm$ 2.12%. For lenses incubated in the calcium-free medium to which disodium EGTA had been added, this figure would have been 38.88% instead of 42.85 $\pm$ 1.46%. The recalculated results would suggest that sugar transport is decreased in the absence of extracellular calcium. On the other hand, the change in the size of the extracellular space would also suggest alterations in the cell membrane unrelated to specific effects on sites involved in transport.

In summary, extracellular sodium, potassium or calcium ions do not appear to be required for the functioning of the sugar transport system in the lens of the eye. The effects observed with lenses incubated in the complete absence of these ions were most probably due to non-specific alterations in membrane properties and not to a specific effect on the transport system. Since the concentrations of these ions in the extracellular fluid change very little under normal conditions, regulation of transport activity by sodium, potassium or calcium would not be a useful feature in the lens. The activity of the transport system for sugars also does not appear to be regulated by the intracellular concentrations of certain ions, as has been postulated for skeletal muscle. The lens is a non-excitable tissue and ionic gradients across the cell membranes are stable under normal physiological conditions. Therefore, changes in intracellular ion concentrations would not appear to be a functionally useful mechanism in this tissue.

The effect of metabolic inhibitors

To determine if sugar transport was regulated by metabolism

in the lens of the eye, the penetration of test sugar was determined under conditions in which specific biochemical pathways were inhibited. With 2,4-dinitrophenol (DNP) as in anoxia, ATP cannot be supplied by oxidative phosphorylation and the tissues rely entirely on anaerobic glycolysis for energy production. A concentration of 0.5 mM DNP present during both preincubation as well as incubation failed to increase the penetration of 3-O-MG (Fig. 13, expt. A). This high concentration of inhibitor did not change the extracellular space; the difference from control was $+0.65 \pm 1.15\%$. Since the lens is entirely surrounded by the capsule, the inhibitor may not have been able to diffuse to its site of action. The above experiment was therefore repeated with lenses in which the capsule had been removed by collagenase as described previously (Fig. 13, expt. B). A much lower concentration of DNP, 0.1 mM, present only during the incubation period, now decreased the penetration significantly ($p < 0.01$). Another uncoupler of oxidative phosphorylation, 2,4-dinitro-o-cresol (DNOC) under the same conditions also decreased penetration significantly. Only with DNP and not with DNOC was the extracellular space significantly increased; from $3.55 \pm 0.25\%$ to $7.93 \pm 1.12\%$ ($p < 0.05$).

Incubation of the lenses with iodoacetate (IAA), an inhibitor of glycolysis, gave similar results. Although the penetration of 3-O-MG was significantly decreased by 2.5 mM IAA in normal lenses ($p < 0.01$), decapsulation further enhanced this effect. This increase in inhibition was statistically significant at $p < 0.05$ (Fig. 13, expt. C).

The effect of these metabolic inhibitors on the penetration of D-xylose was also determined. The penetration of this sugar is

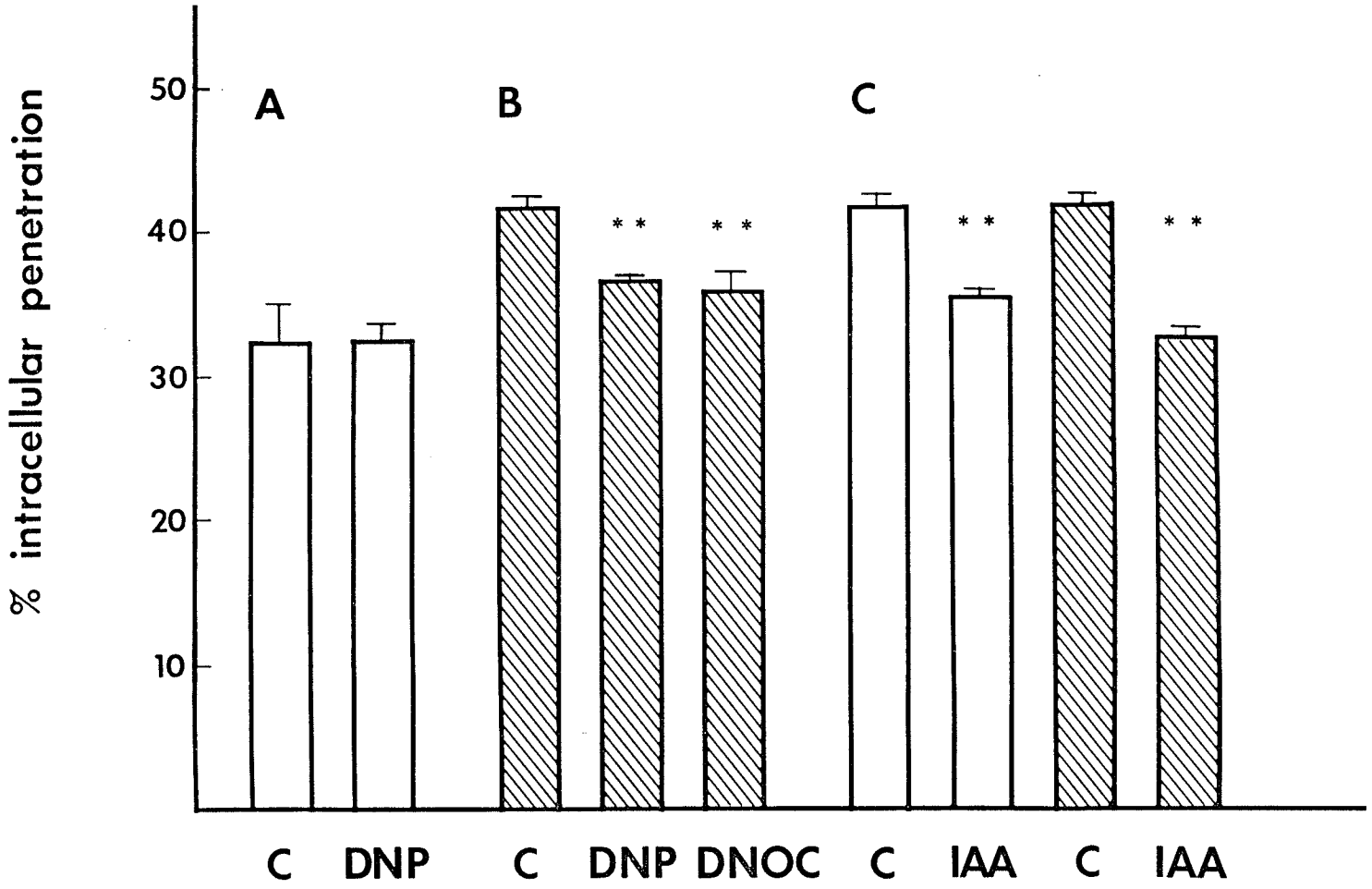


Fig. 13. The effect of metabolic inhibitors on the intracellular penetration of 5.0 mM 3-O-methyl-D-glucose. Incubation time, one hour. Abbreviations: C, control; DNP, 2,4-dinitrophenol; DNOC, 2,4-dinitro-o-cresol; IAA, iodoacetate. The concentration of DNP in experiment A was 0.5 mM, in experiment B, 0.1 mM. The concentration of DNOC was 0.1 mM and of IAA 2.5 mM. The hatched bars indicate lenses decapsulated by preincubation for two hours with 1 mg/ml collagenase. The vertical lines represent one standard error. ** $p < 0.01$.

followed by metabolic transformation so that the accumulated radioactivity reflects both transport and metabolism. The lenses were incubated for four hours with 0.5 mM DNP or with 1.25 mM IAA. The decrease in the distribution of radioactivity from a control of 215.13 $\pm$ 10.05% to 125.08 $\pm$ 4.29% with DNP and to 152.63 $\pm$ 10.96% with IAA was highly significant ($p < 0.01$). These effects are much greater than on the penetration of 3-O-MG, and other evidence also suggests that the metabolism and not the penetration of D-xylose was mainly inhibited. Although IAA did decrease the penetration of 3-O-MG, (Fig. 13, expt. C) this agent also effectively inhibits the oxidation of D-xylose to D-xylonic acid in calf lens homogenates (van Heyningen, 1958). The greater decrease in the accumulation of radioactivity with DNP is more difficult to explain. DNP did not decrease sugar penetration in intact lenses, and while its effect on D-xylose metabolism has not been determined, it is known that incubation under nitrogen does not affect the oxidation of D-xylose in the calf lens homogenates. Since our rat lenses were incubated for four hours, the uncoupling agent may have been able to interfere with D-xylose metabolism during this long incubation time. However, nonspecific depression of membrane function cannot be ruled out. The above results on the lens of the eye and similar findings in mature mammalian erythrocytes (LeFevre, 1961), suggest that in both these tissues inhibition of biochemical pathways involved in energy production does not increase the activity of the transport system.

This is in contrast to findings in tissues such as skeletal and cardiac muscle where inhibition of oxidative phosphorylation or the absence of oxygen increase the rate of sugar penetration. In these

tissues the operation of the Pasteur effect requires an increased supply of glucose to the glycolytic pathway. In the lens, there does not appear to be a link from metabolism to the sugar transport system. While the Pasteur effect exists in this tissue, there appears to be no need for an increase in sugar penetration since free intracellular glucose is present.

The effect of phlorhizin and phloretin

The glycoside phlorhizin and its aglycone phloretin are specific blockers of sugar transport in many tissues (Stein, 1967). Phlorhizin has been shown to inhibit sugar active transport in the intestine and the kidney but is less effective in tissues such as skeletal muscle. On the other hand, phloretin strongly inhibits sugar penetration in erythrocytes (LeFevre, 1961) and fibroblasts (Stein, 1967) but has less effect on intestinal sugar transport. The effect of two concentrations of these agents on the penetration of 5.0 mM 3-O-MG in the lens was studied. As seen in Fig. 14, expt. A, 0.5 mM phlorhizin, present during incubation only, inhibited the penetration of 3-O-MG significantly ($p < 0.05$) but this effect could not be increased by doubling the concentration of the inhibitor. On the other hand, the inhibitory effect of phloretin was concentration-dependent, and this agent inhibited sugar penetration to a much greater degree than the glycoside. In an additional experiment, the effect of a longer exposure to 1.0 mM phlorhizin was also determined by adding the glycoside during preincubation but this did not increase its inhibitory effect (Fig. 14, expt. B). These results are consistent with earlier conclusions that phloretin is the more potent inhibitor of sugar transport by facilitated diffusion.

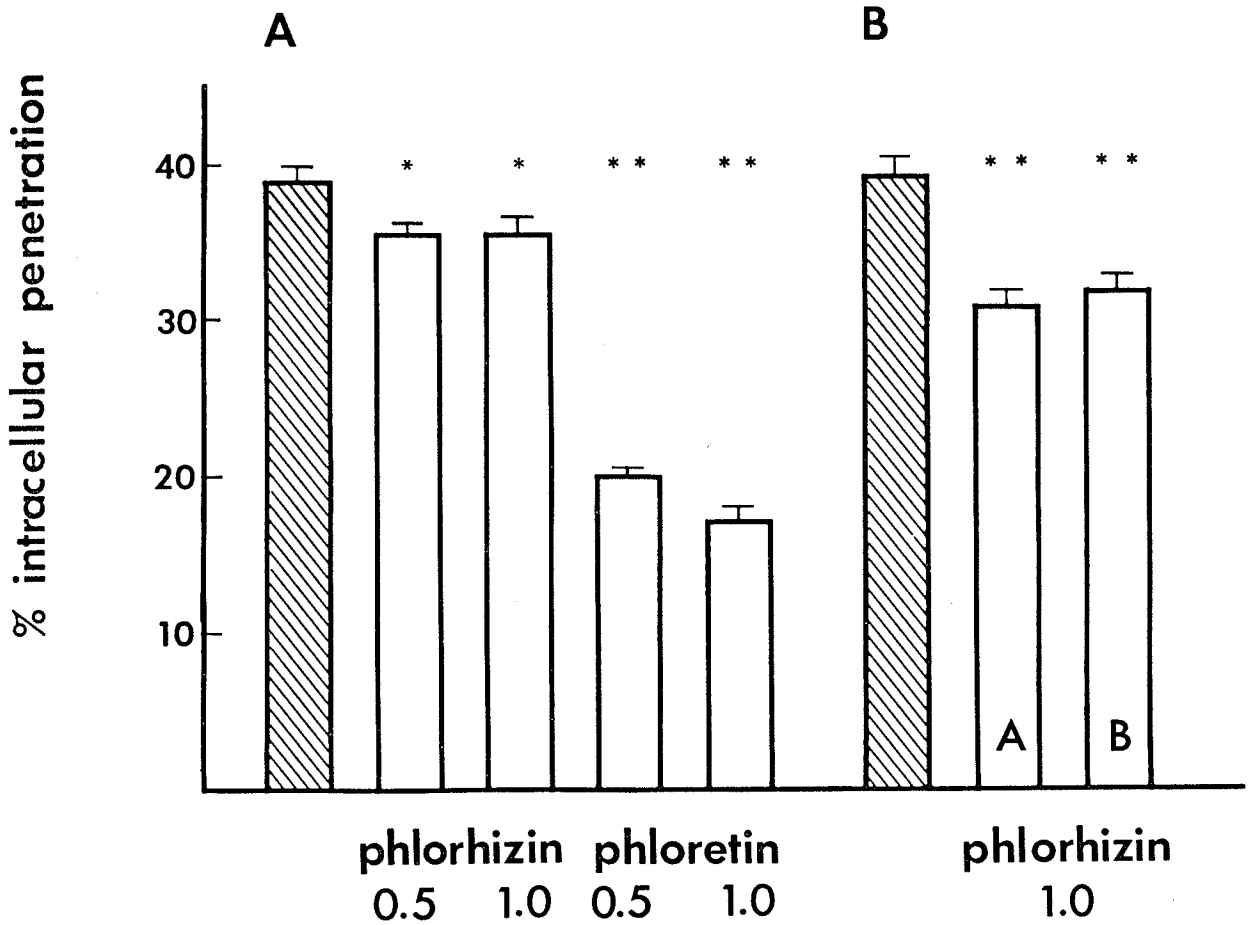


Fig. 14. The effect of phlorhizin and phloretin on the intracellular penetration of 5.0 mM 3-O-methyl-D-glucose. Incubation time, one hour. The hatched bars indicate controls. The vertical lines represent one standard error. The concentrations of inhibitors (in mM) are indicated on the figure. In experiment A, the agents were only present during incubation; in experiment B, phlorhizin was present during incubation (A) or during preincubation as well as incubation (B). * $p < 0.05$, ** $p < 0.01$.

Since phloretin was used to specifically block the sugar transport system in the lens, it was of some interest to determine the nature of its inhibitory effect in this tissue. The experiments were designed in such a way that the results could be interpreted by the method of Dixon (Dixon and Webb, 1964). The initial velocity is determined in the presence of increasing inhibitor concentrations at two different substrate concentrations. When 1/v is plotted against I, the point where the two straight lines cross will determine whether the inhibition is competitive or non-competitive and will also give an approximate value for the K_i. The substrate concentrations were 1.0 and 5.0 mM 3-O-MG and the phloretin concentrations ranged from 0.025 to 0.5 mM; the lenses were incubated for 15 minutes. The results are shown in Fig. 15. Straight lines could be fitted by eye through the data points obtained with the two substrate concentrations. The lines intersected each other and the abscissa at approximately the same point, indicating that the inhibition by phloretin was non-competitive. The point at which the two lines cut across the abscissa equals $-K_i$. The intercepts and slopes of the lines were calculated by least square regression analysis; the values for K_i were 0.58 and 0.62 mM for 1.0 and 5.0 mM 3-O-MG respectively. The concentration of phloretin required to inhibit sugar penetration in the lens of the eye by 50% is therefore approximately 0.6 mM. In human erythrocytes, LeFevre (1954) showed that the inhibition of sugar transport by phloretin could be reversed and probably was competitive. It was found subsequently that the inhibition is not entirely of the competitive type and appears to include a non-competitive component (LeFevre, 1961). Our results indicate that

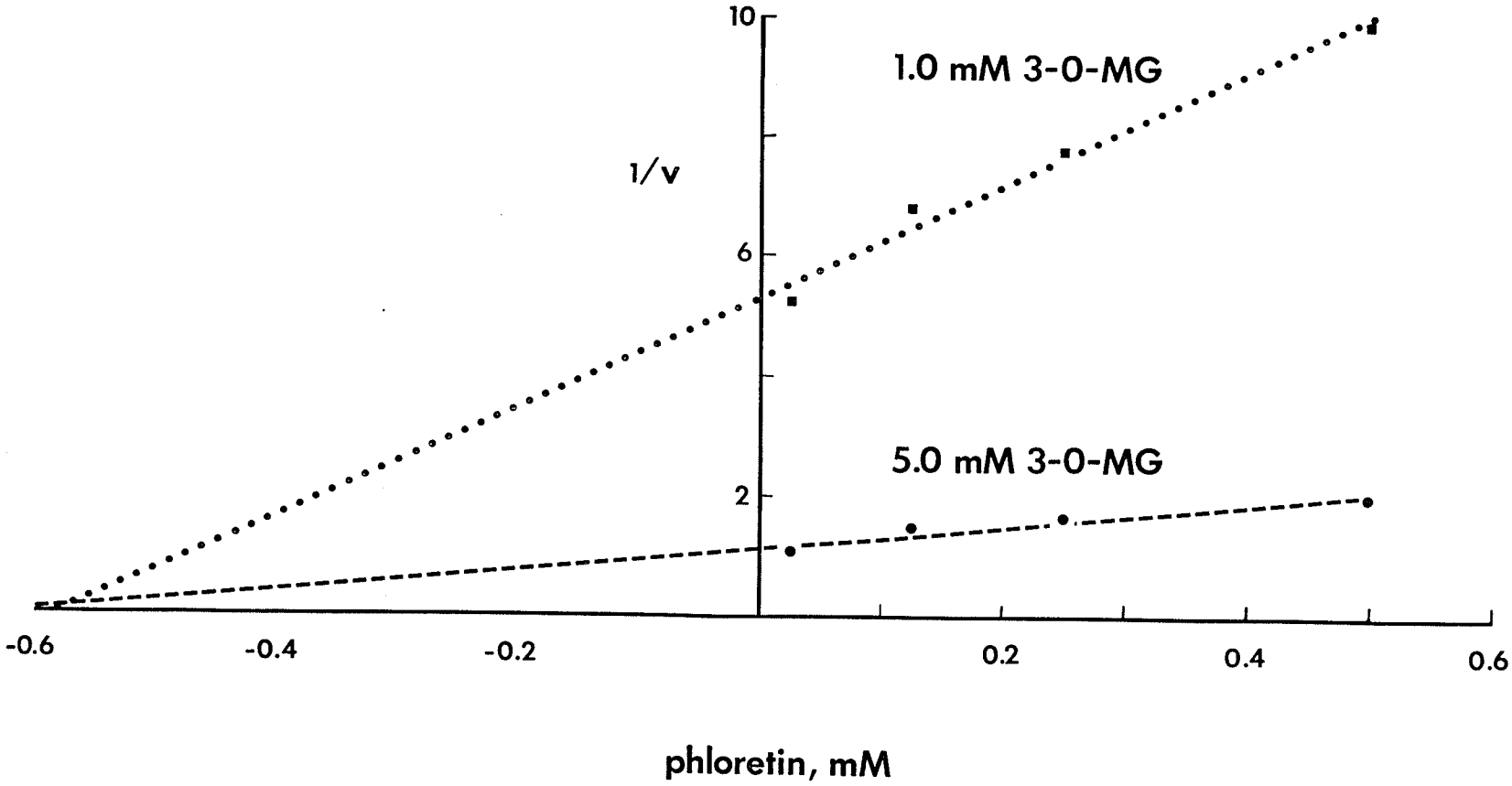


Fig. 15. Determination of the K_i of phloretin by the Dixon plot. Two concentrations of 3-O-methyl-D-glucose, 1.0 and 5.0 mM, were incubated with various concentrations of phloretin. Incubation time, 15 minutes. The straight lines were determined by regression analysis.

in the lens of the eye, phloretin inhibits sugar transport in a non-competitive manner.

The effect of sympathomimetic drugs

It is generally believed that the hyperglycemic effect of sympathomimetics in man is partly mediated via α-receptors, through inhibition of insulin release while glycogenolysis in muscle and liver appears to be stimulated through interaction with β-receptors. To determine whether sympathomimetics altered the rate of sugar penetration in the lens, experiments were done with adrenaline, the α-agonist phenylephrine, and an α-antagonist, phentolamine. After one hour of incubation the penetration of 3-O-MG remained unchanged (Table III). In order to ensure that these drugs reached the lenticular fiber membranes, the experiment was also carried out on lenses decapsulated by collagenase treatment as described above. As shown in Table III, various concentrations of phenylephrine and a β-agonist, isoproterenol, were without effect.

These results are consistent with all the other data, indicating that sugar transport in the lens is not susceptible to regulation.

The effect of sulfhydryl reagents

To determine the possible involvement of thiol groups in sugar transport in various tissues, the sulfhydryl group inhibitors, N-ethylmaleimide (NEM) and p-chloromercuribenzoate have been used. In skeletal muscle, conditions can be chosen under which NEM acts only to prevent stimulation of sugar transport by insulin or other factors but longer exposure to the drug also blocks basal sugar transport (Eboue-Bonis et al., 1967; Bihler, 1968). Instead of p-chloromercuribenzoate,

TABLE III

The effect of sympathomimetics on the intracellular penetration of 5.0 mM 3-O-methyl-D-glucose in normal and decapsulated lenses incubated for one hour.

Drug	Concentration	Effect
Normal lenses		
phenylephrine	1.4×10^{-4} M	-0.45 ± 2.12*
phentolamine	8.34×10^{-5} M	-1.28 ± 2.07
adrenaline	1.28×10^{-4} M	-1.41 ± 2.25
Decapsulated lenses		
phenylephrine	1×10^{-3} M	-2.00 ± 1.98
	1×10^{-4} M	-3.00 ± 2.44
	1×10^{-5} M	$+0.83 \pm 2.48$
isoproterenol	1×10^{-5} M	-3.27 ± 2.45
	1×10^{-6} M	-2.97 ± 2.28
	1×10^{-7} M	-2.05 ± 2.67

*Difference from control $\pm$ standard error of the difference.

its sulfonic acid derivative, p-chloromercuriphenylsulfonic acid (CMPS) was used in the present experiments because the cell membrane is very little permeable to this charged molecule. Its effect is, therefore, restricted to sulfhydryl groups on the outer membrane surface (VanStevenick et al., 1965). NEM (1.0 mM) was present during the last 100 seconds of preincubation, while CMPS (0.125 mM) was added to the incubation medium. As shown in Fig. 16, expt. A, neither of these agents inhibited the penetration of 3-O-MG and the extracellular space also remained unchanged. With longer exposure of the lens to these agents, sugar penetration was significantly decreased (Fig. 16, expt. B). When CMPS was present during preincubation and incubation, the percent penetration decreased from $39.20 \pm 1.05\%$ to $35.03 \pm 1.15\%$ ($p < 0.01$); with NEM present during the entire preincubation period, this value became $24.78 \pm 0.84\%$. Again, the extracellular space remained unchanged. To make access of these agents to the lenticular fiber membranes easier, the experiments were repeated in lenses decapsulated with collagenase. When the same concentration of CMPS was present during the incubation period only, significant inhibition of sugar penetration was again observed (Fig. 16, expt. C). Unexpectedly, the decrease in penetration with NEM in the decapsulated lenses, although statistically significant, was much less than found in the intact lenses.

The specificity of different sulfhydryl group reagents for the various types of cellular thiol groups is not the same and an interpretation of their effect is difficult. These agents may also react with other functional groups to a varying extent (Webb, 1966). Virtually all cellular proteins contain thiol groups and the decreased

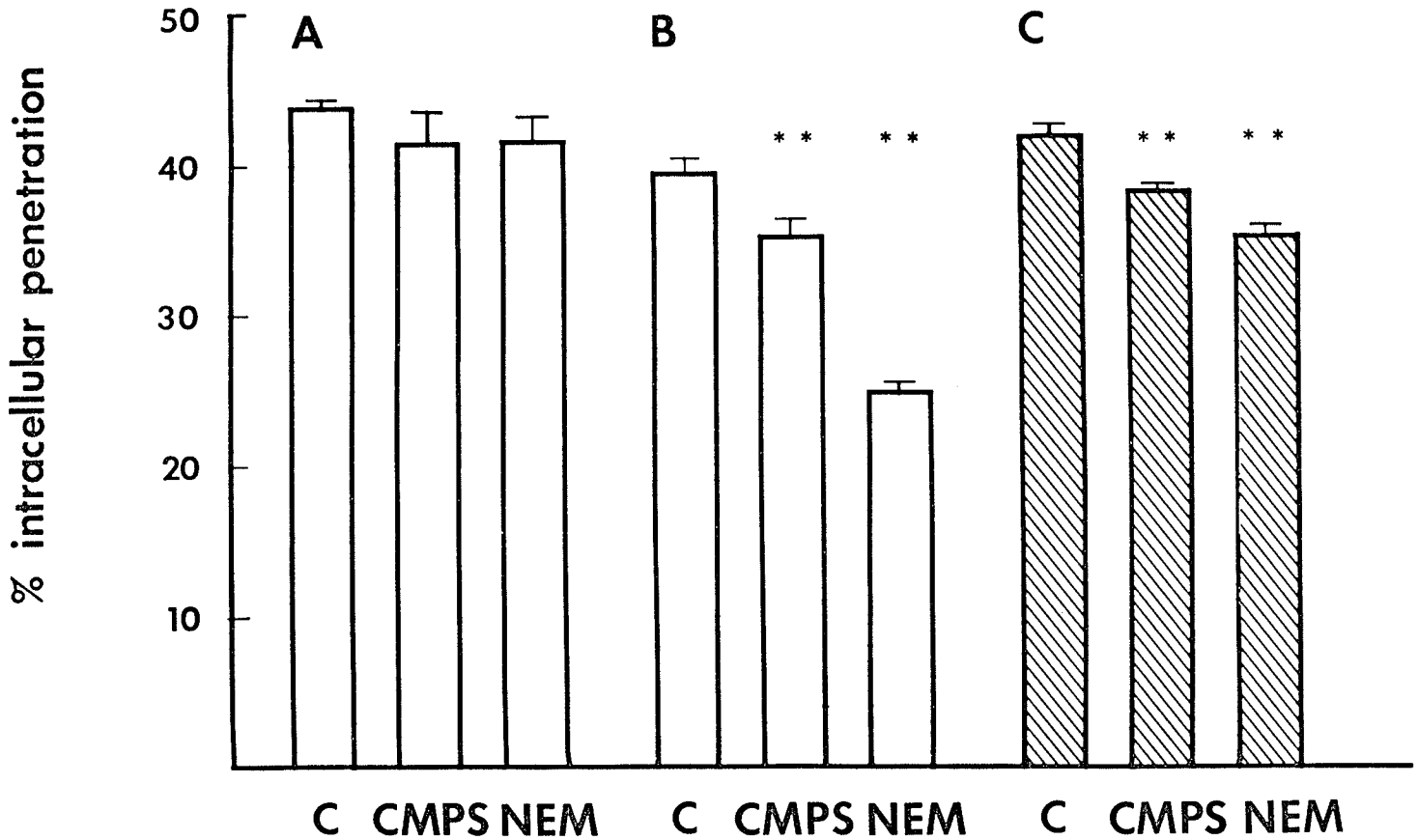


Fig. 16. The effect of sulfhydryl reagents on the intracellular penetration of 5.0 mM 3-O-methyl-D-glucose. Incubation time, one hour. The vertical lines represent one standard error. The hatched bars indicate lenses decapsulated by preincubation for two hours with 1 mg/ml collagenase. Abbreviations: C, control; CMPS, p-chloromercuriphenylsulfonic acid; NEM, N-ethylmaleimide. The concentration of CMPS was 0.125 mM; in experiments A and C, this agent was only present during incubation and in experiment B during preincubation and incubation. The concentration of NEM was 1.0 mM, present for the last 100 seconds of preincubation in experiment A, and during the whole preincubation period in experiments B and C. ** $p < 0.01$.

penetration obtained in lenses incubated in the presence of sulfhydryl group reagents cannot be interpreted therefore as evidence that thiol groups modify or directly affect the transport sites. Since the structure and conformation of the cell membrane depends on sulfhydryl groups, the effect of the inhibitors may be nonspecific.

Other tissues

Sugar transport has been extensively studied in skeletal, cardiac and very recently, smooth muscle. Studies on the penetration of monosaccharides in atrial muscle have so far not been carried out but would be of great benefit. Since this tissue has often been used in the study of calcium fluxes (Winegrad and Shanes, 1962; Reuter, 1970), it would be ideal to test the possible relationship between this ion and the activity of the sugar transport system. A number of preliminary experiments were performed which suggest that sugars enter this tissue by facilitated diffusion. In rat left atria, insulin increased the penetration of 3-O-MG but, like smooth muscle, this tissue was less sensitive to the hormone than ventricular and skeletal muscle. An effect of insulin could be demonstrated only with prolonged exposure to the hormone but not when high concentrations of hormone were used. It was also found that the transport of 5.0 mM 3-O-MG was inhibited by 10 mM D-glucose. However, good quantitative data could not be obtained with this tissue because of difficulties in obtaining stable conditions and responses. Although the left atrium should not be spontaneously active, contractions occurred. In an attempt to stabilize the tissue, the atria were stimulated electrically at a constant rate. Several experiments were performed in which sugar transport was measured at

various resting tensions of these driven atria. Unfortunately, the preparation still proved unsatisfactory, probably because of its extreme sensitivity to hypoxia. These studies were, therefore, not pursued further.

In another series of experiments in this laboratory, sugar transport in the detrusor muscle of the rat was studied by methods similar to those used with the lens. The author did not take part in the experimental work but was involved in its planning and interpretation. The results are discussed in Section IV.

Summary

The above data suggest that sugars penetrate across the lenticular cell membranes by facilitated diffusion. This energy-independent, equilibrating system displays saturation kinetics although under the conditions of the experiments complete saturation could not be demonstrated. The chemical specificity, competition between pairs of sugars and the inhibition by phloretin and phlorhizin are also consistent with mediated transport.

Regulation of the sugar transport system does not appear to take place. The hormone insulin, *in vivo* or *in vitro* and after decapsulation with collagenase and trypsin, failed to change the penetration of test sugar. These enzymes by themselves were also ineffective. Metabolic inhibitors and sulfhydryl group reagents had some depressant effects, presumably of nonspecific nature. Inhibition of the sodium pump or a 50% decrease in the extracellular sodium ion concentration was also without any effect on sugar penetration. The observed decrease in the absence of sodium could best be explained as a nonspecific effect.

The kinetics, chemical specificity and absence of regulation suggest that the sugar transport system in the lens of the eye is very similar to the system of the mature mammalian erythrocyte.

SECTION IV

INTERPRETATION AND CONCLUSIONS

Integration of sugar transport with cellular metabolism

One of the basic features common to all cells is the function of the cell membrane which serves as a barrier separating the intracellular environment from the extracellular space and allows selective transport of biologically important substances into and out of the cell. The membrane is also an excellent electrical insulator and contains, at least in certain tissues, specific receptor sites for various hormones and neurotransmitter substances which regulate cellular activity. The organization of the lipid and protein components of the membrane is such that all of these functions can be carried out effectively. The original model of membrane structure, the unit membrane of Danielli and Davson (1935) consisted of a bimolecular lipid layer to which proteins (and mucopolysaccharides) were attached on both surfaces. By physicochemical criteria this was the most stable structure of the membrane constituents. Newer work on the possible arrangement of the constituents of the cell membrane led to the concept that the structure is probably less rigid than a continuous lipid leaflet. Membrane proteins are not necessarily stretched out over the surface only but may penetrate into the lipid layer to form globular structures and even channels crossing the membrane. The structure of the membrane is probably not static and may vary all the way from a bimolecular lipid leaflet to a globular arrangement of lipid and protein (Stein, 1967; Korn, 1968; Dewey and Barr, 1970). Protein lined, aqueous channels of some kind are now widely believed to serve as location or components of transport systems mediating the passage across the cell membrane of various hydrophilic substances such as ions, nutrients such as simple monosaccharides, amino

acids, purines and pyrimidines and others. Since the membrane is functionally a lipid structure, no specialized transport systems are required for lipid soluble materials, and gaseous oxygen and carbon-dioxide can also cross the membrane quickly by physical diffusion. The evidence for the existence of a specialized transport mechanism for sugars is reviewed in the INTRODUCTION.

This discussion will be concerned with the integration of the sugar transport systems and the metabolic requirements in various tissues. The discovery by Levine and his associates (Levine et al., 1949, 1950) that insulin increased the penetration of sugars in skeletal muscle has led to extensive investigations into the effect of this hormone in a number of different tissues. As discussed in the INTRODUCTION, insulin is an anabolic hormone which stimulates the deposition of energy reserves and enhances other synthetic processes. The stimulation of sugar transport across cell membranes is a prominent feature of its action. It soon became apparent that the various tissues of higher organisms could be divided into two groups with respect to their response to the action of insulin on sugar transport. The hormone stimulated the activity of the sugar transport system in skeletal and cardiac muscle and adipose tissue but proved ineffective in tissues such as erythrocytes. The physiological significance of this observation has so far not been explained. The present results obtained with the lens of the eye, together with data published for other tissues, suggest that the regulation of the activity of the sugar transport system is integrated with the function and metabolic pattern of the tissues. According to this concept, in tissues where glucose utilization is stable, the sugar

transport system has a sufficient capacity to supply substrate at a constant rate and no regulatory mechanisms appear to be required. The liver is an exception to this generalization and will be discussed below. In some other tissues the rate of glucose utilization undergoes changes because of varying functional requirements and the activity of their sugar transport system may be adjusted to supply sufficient substrate under these variable conditions. It is significant that the same factors which induce activity associated with increased glucose utilization also stimulate sugar penetration. For example, insulin stimulates both sugar transport and synthesis of energy reserves in muscle and adipose tissue. Likewise, muscular contraction increases glucose transport and utilization. A scheme of the properties of sugar transport systems in various tissues and their metabolic patterns is presented in Table IV.

The activity of the sugar transport system is not only integrated with the energy requirements of the tissues but also with the supply of substrate in the bloodstream. At high blood sugar levels, insulin secretion by the pancreas is stimulated and consequently the transport of sugars and synthesis of glycogen and triglycerides is enhanced. On the other hand, when conditions are not favorable for the deposition of energy reserves, the plasma insulin level drops and the activity of the sugar transport systems is reduced.

Even though certain tissues use non-carbohydrate substrate preferentially, the separate consideration of glucose utilization and the sugar transport system is justified. The plasma concentration of glucose is well maintained within narrow limits so that this substrate is constantly available for energy production. In certain tissues,

TABLE IV

Properties of glucose transport and metabolism in various tissues

Tissue	Glucose metabolism	Transport
Lens Mature mammalian erythrocyte Central nervous system	stable	not rate-limiting
Liver	variable; energy reserves present	not rate-limiting; efflux of glucose is functionally important
Avian erythrocytes	variable	rate-limiting, regulated
Adipose tissue	variable; energy reserves present	rate-limiting, regulated
Cardiac) Skeletal) muscle Smooth)	variable; energy reserves present	rate-limiting, regulated
Polymorphonuclear leukocytes	variable; energy reserves present (?)	rate-limiting, regulated

glucose uptake is functionally related to the use of non-carbohydrate substrates. For example, in starvation or intensive exercise some types of muscle will preferentially oxidize free fatty acids, ketone bodies or lactic acid; the oxidation of free fatty acids has been shown to inhibit the metabolism and transport of sugars in muscle under certain experimental conditions (Randle et al., 1964; Neely et al., 1969; Bihler and Sawh, unpublished results). This glucose-sparing effect is of great physiological importance because it will tend to preserve this substrate for use by the central nervous system, where it is the only source for energy. The tissues whose transport characteristics will be discussed are those in which sugar penetration takes place by facilitated diffusion, i.e. by equilibrating, energy-independent transport; the intestine and kidney in which sugar transport is active are excluded from this discussion. The term regulation will be used exclusively to describe stimulation of transport activity. With the exception of the effect of free fatty acids (which is as yet little understood) all the other known factors which affect the activity of the sugar transport system are stimulatory.

Tissues in which glucose penetration is not rate-limiting for utilization

LENS

Metabolism in the lens of the eye serves primarily for the continuing turnover of cellular constituents and maintenance of cellular integrity but not directly for its function. Accommodation, i.e. a change in the curvature of the lens, is achieved entirely by the action of the ciliary muscles. The only contribution of the lens itself is its

passive recoil due to its surrounding elastic capsule. The metabolic rate of the lens, as measured by its oxygen consumption, is quite low compared to other tissues, but the epithelial layer at the anterior surface appears to respire as rapidly as other comparable tissues (Matuk et al., 1969). This lenticular surface is in contact with the aqueous humour from which it can extract oxygen; the oxygen tension in this humour is, however, much less than in the blood. Not surprisingly, anaerobic glycolysis is therefore the primary pathway in energy production and its contribution to total metabolism has been estimated as 70 - 80% of the total (Kuck, 1970). Although all the enzymes of the Krebs cycle are present, their activity is low (Ely, 1951) and less mitochondria and respiratory enzymes are present in the lens than in more rapidly respiring tissues (Kuck, 1970). Kinoshita et al. (1961) have shown that in the presence of glucose, the lens can maintain its transparency under a nitrogen atmosphere for up to 20 hours. Some glucose is oxidized by the hexose monophosphate shunt, and other pathways, such as the reduction of glucose to sorbitol, also play a role in the initial conversion of this substrate. Nevertheless, the main feature of energy production in the lens is its nearly total dependence upon glycolysis.

Transport is not rate-limiting for glucose utilization and lenses contain an intracellular pool of glucose and other reducing sugars. The capacity of the sugar transport system is more than sufficient for the low and stable rate of glucose utilization. As anaerobic glycolysis by itself is able to support normal metabolic function, it is believed that the Pasteur effect is operative in this tissue. Since

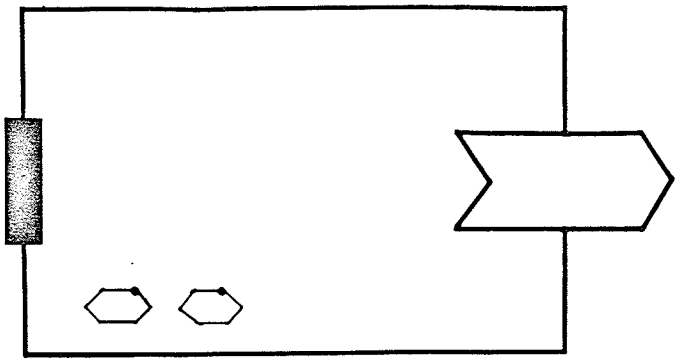
free intracellular glucose is present, an increase in its utilization does not necessarily require enhanced transport activity.

The experiments reported here and those from other laboratories strongly suggest that regulation of the sugar transport system in the lens of the eye does not take place. This implies that various factors such as the rate and pattern of metabolism or hormones do not stimulate sugar penetration. Of course, sugar transport will depend upon the substrate concentration in the aqueous humour. For example, in galactosemia, the concentration of galactose in the aqueous humour is increased, leading to a greater penetration of this sugar into the lens, increased metabolic conversion to dulcitol and, eventually, cataract formation. The lack of insulin sensitivity in the lens is consistent with the absence of appreciable glycogen reserves in the lenses of most vertebrates, with the exception of certain birds (Rabaey, 1963).

The characteristics of the transport system and the metabolic features of the lens of the eye are outlined in the diagram on Fig. 17A. The stable rate of glucose utilization is indicated by the solid lines of the arrow and the free intracellular glucose pool by the hexagonal symbols in the lower left hand corner. Since no appreciable glycogen reserves are generally present, the upper left hand corner is left blank. The sugar transport system is indicated by the 'black box' at the left hand side of the diagram. Its size, which exceeds that of the arrow symbolizing glucose utilization indicates that the capacity of this system is more than sufficient to supply the required substrate. The absence of regulation of the activity of the transport system is indicated by the solid rectangle.

Fig. 17. Features of sugar transport and metabolism in tissues where sugar penetration is not rate-limiting for its utilization. The large arrows on the right hand side represent glucose utilization; the solid lines indicate that the rate is stable, the broken lines that the rate is variable. Energy reserves (G, glycogen) are indicated in the top left hand corner and free intracellular glucose (hexose symbols) in the bottom left hand corner. The sugar transport system is represented by the rectangle on the left side; the solid black box indicates that stimulation of the sugar transport system does not take place. The two arrows through the rectangle in diagram C suggest that influx and efflux of sugar are equally important functionally.

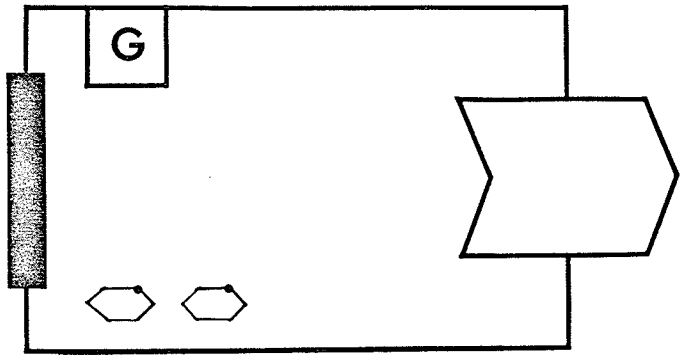
A



lens

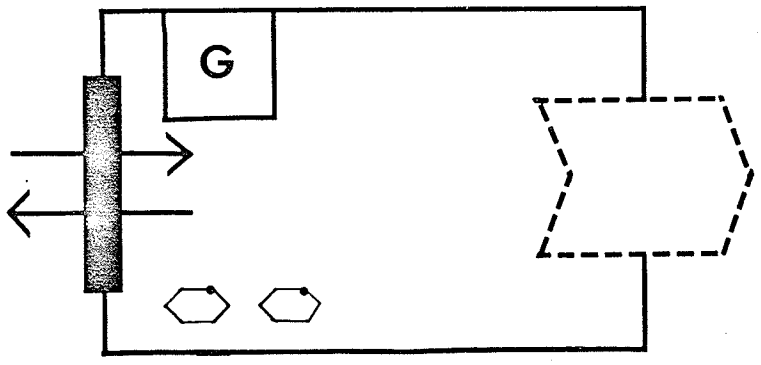
mature mammalian erythrocyte

B



central nervous system

C



liver

ERYTHROCYTES

The main energy-providing biochemical pathway in mature mammalian erythrocytes is also anaerobic glycolysis. The mechanism for the oxidative phosphorylation is not present in these cells (Harris and Kellermeyer, 1970) and energy is provided mainly by glycolysis. The hexose monophosphate shunt accounts for about 10% of glucose metabolism (De Verdier, 1962, in Williamson, 1970). The erythrocytes are another tissue in which energy is only required for maintenance and continuing metabolism but not for the functional activity. The association and dissociation of oxygen with hemoglobin is independent of metabolic energy. There are great differences between species in the permeability of their erythrocytes to monosaccharides as was already reported by Kozawa (1914). Compared to other tissues, the capacity of the sugar transport system is very large (Stein, 1967) and in the erythrocytes of most species glucose penetration is not rate-limiting for its utilization. For example, in human erythrocytes incubated in 10 mM glucose, the rate of glucose entry is about 250 times greater than the rate of its utilization (Widdas, 1954). In these erythrocytes, which are the most extensively studied tissue, no evidence for regulation of sugar transport has been obtained (LeFevre, 1961; Stein, 1967). More than sufficient substrate can be supplied for the stable energy requirements and an appreciable free intracellular glucose pool is present in these cells. The lack of insulin sensitivity (Park et al., 1956) in this tissue is correlated with the absence of substantial glycogen reserves.

One divergent observation should be mentioned. Rieser and

Rieser (1964b) were able to induce insulin-sensitivity to human erythrocyte membranes by trypsin treatment. If this is substantiated, it would suggest that insulin receptors are present but not functional in cell membranes of certain tissues. This study with lenses did not show an insulin effect after trypsin treatment, and would not tend to support this view.

The metabolic pattern and characteristics of the sugar transport system are thus similar to those of the lens (Fig. 17A). Glucose utilization is stable, the transport system has a more than sufficient capacity to supply the required substrate, free intracellular sugar is present, energy reserves are absent and regulation of the activity of the transport system does not appear to take place.

In contrast to human and ape red cells, penetration of glucose in horse, ox and pig erythrocytes is so slow that these cells have been described as 'impermeable' (Whittam, 1964). Little is known about the details of these impermeable cells but, in general, a good correlation has been found between the activity of the sugar transport system and the rate of glucose utilization from various species (Laris, 1958).

One exception to the lack of regulation of sugar transport in mature red cells is the erythrocytes of birds. In these nucleated cells oxidative phosphorylation as well as anaerobic glycolysis contributes to energy production. Glucose transport is rate-limiting for its utilization and no measurable amount of free intracellular glucose is present. As in other tissues, in which glucose penetration is increased under anoxic conditions, inhibition of oxidative phosphorylation by agents such as cyanide or incubation in the absence of oxygen stimulates the activity

of sugar transport in these cells (Morgan et al., 1965; Wood and Morgan, 1969). As far as known, the nucleated erythrocytes do not contain appreciable glycogen deposits and their sugar transport system is not sensitive to insulin (Morgan, private communication). The reason why the metabolic pattern in erythrocytes of birds should be different from those of mammals is unknown but would suggest that their metabolic rate undergoes changes in activity. With respect to integration of the sugar transport system with the metabolic requirements, the avian erythrocyte appears to be intermediate between the lens of the eye and muscle.

CENTRAL NERVOUS SYSTEM

Oxidative phosphorylation is the major pathway for energy production in the central nervous system (CNS). The oxygen consumption of the brain is one of the highest of all tissues in the body and remains remarkably stable under a variety of conditions. The metabolic rate of the brain as a whole is quite constant but it has been suggested that localized changes in energy production may occur which would not be reflected in the overall oxygen consumption (Quastel and Quastel, 1961). Experimental evidence for the presence of mediated transfer of sugar in the brain has been obtained only fairly recently (Crone, 1965; LeFevre and Peters, 1966; Buschiazzo et al., 1970; Cutler and Sipe, 1971); the transport system is probably situated at the level of the endothelial cells. Whether facilitated diffusion of sugar also occurs at the level of the nerve and glial cell membranes is at present not known. Facilitated diffusion has also been shown to occur between ventricular fluid and the brain substance (Brøndsted, 1970a, 1970b) but its importance has

not yet been evaluated. In the brain, sugar penetration is not rate-limiting for its utilization and free intracellular glucose has been demonstrated (Regen et al., 1969; Buschiazzo et al., 1970). As mentioned, the rate of glucose utilization in the brain as a whole is very constant and the activity of the sugar transport system does not appear to change under various conditions. Uncoupling of oxidative phosphorylation by DNP failed to affect the penetration of L-arabinose in cat brain in vivo (Eidelberg et al., 1967). Both pathways for aerobic and anaerobic energy production are present and the Pasteur effect is possible; because of the presence of free cellular glucose it is not necessarily dependent on an increase in transport activity. Anaerobic glycolysis, however, cannot support function in the CNS. The early work of Sacks and Bakshy (1957) suggested that insulin increased the activity of the transport system in the CNS in vivo. The more recent experiments by Buschiazzo et al. (1970), however, indicate that the reported insulin effect was probably due to the induced hypoglycemia and the consequent decrease in the competitive effect of glucose on the transport of the test sugar in the in vivo experiments. The role of glycogen as reserve fuel for energy production in the CNS is not clear. The concentration of this polysaccharide is much less than in tissues such as muscle but it has a faster turnover, roughly equivalent to that found in liver. It has been suggested that glycogen plays a role during development and under extreme metabolic conditions such as insulin-induced hypoglycemia and circulatory shock (Coxon, 1970). An effect of insulin on these glycogen deposits has not been investigated and since this hormone is a large hydrophilic molecule, it is doubtful if it

would pass through the blood-brain barrier (Haugaard et al., 1954).

The features of sugar transport in the CNS are presented in Fig. 17B. The diagram is similar to the one which characterizes the lens and the mature mammalian erythrocyte, except that in the top left hand corner, the presence of glycogen reserves is indicated. However, as in the other tissues in this group, insulin and other factors do not affect the activity of the sugar transport system.

In these three tissues there appears to be no mechanism modifying the activity of the sugar transport systems. Since glucose penetration is not rate-limiting for its utilization and since the energy requirements are stable, a transport system with a fixed capacity is adequate to supply the needs of these tissues. Reserve fuel in the form of glycogen or triglycerides is not an important source of energy and adaptations in transport activity are not needed for the accumulation of such reserves; indeed, these tissues are not sensitive to the action of insulin. Consequently, it may be concluded that these tissues would not derive any evolutionary advantage from a transport system subject to regulation.

Other tissues which might belong to this group include bone and skin; in the latter, an appreciable free intracellular glucose pool is present (Kahlenberg and Kalant, 1966). Carbohydrate transport in bone has not yet been investigated by modern methods but a similar integration of metabolism and the transport system as discussed above may be expected.

LIVER

The liver has a key function in glucose homeostasis; glucose

can be stored as glycogen for future release and in addition can be produced by gluconeogenesis. Many tissues can store glycogen as reserve fuel but only the liver, and to a much lesser extent the kidney, contain glycogen for subsequent release as glucose into the bloodstream. Other functions of the liver in carbohydrate metabolism are the conversion of fructose and galactose to glucose. Glucose is metabolized both by glycolysis and oxidative phosphorylation; the hexose monophosphate shunt is also of great importance in this tissue and provides many intermediates for synthetic processes. The relative contributions of these pathways to the total metabolic activity of the liver depend upon conditions and are integrated with the overall requirements of the organism. Liver cells are in general more 'permeable' than cells of most other tissues. Earlier studies suggested that there was no specific sugar transport system in the membranes of liver cells (Cahill et al., 1958) but newer work has shown that in the liver simple monosaccharides also enter by mediated transport (Williams et al., 1968). The liver contains substantial amounts of free glucose and penetration is not rate-limiting for its utilization. As opposed to other tissues, efflux of free glucose from liver cells is of major physiological importance. In the post-prandial state, the blood glucose levels are high and glycogen synthesis will occur; this process is stimulated by insulin which is then present in relatively high concentrations. At a later stage, when the blood glucose levels decrease, glucose derived from glycogenolysis and gluconeogenesis, will be discharged from the liver to maintain a constant plasma concentration. Thus, in the liver both sugar influx and efflux have to occur rapidly under entirely different conditions and two different

regulatory mechanisms would be required. Alternatively, the transport system should have a sufficient capacity without stimulation to transport the required large amounts of sugar in both directions across the cell membrane; this appears to be the case and the sugar transport system in the liver is indeed not sensitive to insulin (Hetenyi and Studney, 1967). In other words, even though glucose utilization in the liver is variable, its penetration across the cell membrane is not rate-limiting. The sugar transport system has a sufficiently large capacity for the high rates of both influx and efflux of glucose without any regulatory mechanisms.

The diagram in Fig. 17C represents the above described characteristics of sugar transport in the liver. The variable rate of glucose utilization is indicated by the dashed arrow. Free intracellular glucose and the glycogen reserves are marked in the bottom and top left hand corners respectively. The large size of the 'black box' representing the sugar transport system indicates that regardless of the substrate requirement, sugar penetration is not rate-limiting for utilization. The two arrows through the 'black box' point out that both influx and efflux of glucose are of physiological importance.

Tissues in which glucose penetration is rate-limiting for utilization

ADIPOSE TISSUE

The glucose which enters adipocytes is used for energy production and lipogenesis. In this tissue, glucose penetration is rate-limiting for utilization and facilitated diffusion of sugars has been demonstrated (Crofford and Renold, 1965). No significant free glucose

pool is present in these cells. When oxidative metabolism is inhibited sugar penetration is stimulated and anaerobic glycolysis enhanced. Inhibition of the sodium pump by ouabain or incubation in a potassium-free medium has been shown to enhance the efflux of 3-O-MG (Clausen, 1969); in addition ouabain, like insulin, increased glucose uptake and its oxidation through the hexose monophosphate shunt (Ho and Jeanrenaud, 1967). The metabolic pattern of adipose tissue will depend upon the plasma levels of substrates and the various factors which govern the synthesis or hydrolysis of the triglycerides. The main function of adipose tissue is to store energy reserves, mainly in the form of triglycerides. The sugar transport system is sensitive to the action of insulin, and so is the synthesis of triglycerides which depends on the availability of sufficient glucose for the formation of glycerol phosphate.

In the postprandial state, when blood glucose levels are high and insulin is present, conditions are favourable for the deposition of triglycerides and the activity of the sugar transport system is increased. Conversely, when blood glucose levels are normal or low and free fatty acids are released for oxidation in various tissues, the stimulus for increased transport activity is no longer present; in addition, free fatty acids have a glucose-sparing effect and are used in certain tissues in preference to carbohydrate. Blood glucose will therefore be maintained at a fairly constant level so that this substrate remains available for the CNS.

In adipocytes, the sugar transport system is stimulated when an increased supply of substrate is required for its functional activity. In other tissues such as muscle, a distinction can be made between

transport stimulation linked to activity or to storage of reserves. Since in adipocytes these two are the same, this classification is inapplicable in this tissue.

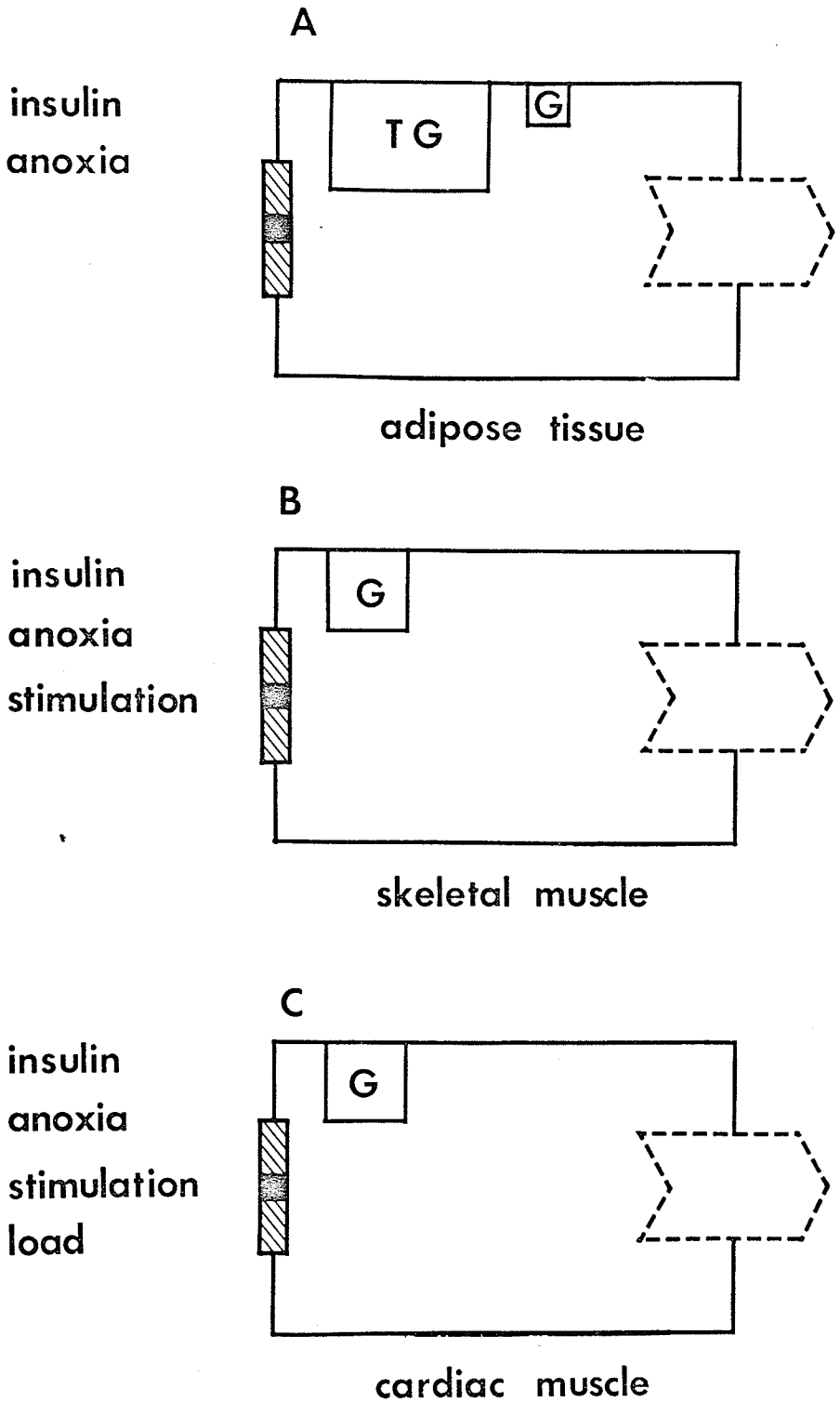
The characteristics of metabolism and sugar transport in adipocytes are shown in diagram A, Fig. 18. The broken lines of the arrow depicting glucose utilization indicate that the metabolic rate is variable. The stored triglycerides and the small amount of glycogen are presented at the top left hand corner. The small solid block of the rectangle at the left side indicates that under basal conditions the rate of sugar penetration is low while the hatched areas indicate that the activity of the sugar transport system can be increased; the stimuli which enhance sugar penetration are listed.

Skeletal, cardiac and smooth muscle are excitable contractile tissues in which the mechanical activity and therefore the energy requirements may vary suddenly. Since in these tissues sugar transport is rate-limiting for its utilization the activity of their sugar transport systems must be integrated with their metabolism. These muscles characteristically contain glycogen stores although the physiological significance of this reserve fuel is not clear for either cardiac or smooth muscle. The differences which exist between these three types of muscle may also be reflected in the regulation of the activity of their sugar transport systems.

SKELETAL MUSCLE

The common characteristics of muscles with respect to energy production have already been outlined. In skeletal muscle energy is supplied both by aerobic and anaerobic metabolism and, in contrast to

Fig. 18. Features of sugar transport and metabolism in tissues where sugar penetration is rate-limiting for its utilization. The large arrows on the right hand side represent glucose utilization; the broken lines indicate that the rate is variable. Energy reserves (G, glycogen; TG, triglycerides) are indicated in the top left hand corner. No free glucose is present in these cells. The sugar transport system is represented by the rectangle on the left side; the small solid black area indicates that at rest, the rate of sugar penetration is low. The hatched areas of the rectangle indicate that the activity of the sugar transport system can be increased. The various agents and conditions which stimulate sugar penetration in these tissues are listed on the left hand side of the diagrams.


A
insulin
anoxia
TG
G
adipose tissue
B
insulin
anoxia
stimulation
G
skeletal muscle
C
insulin
anoxia
stimulation
load
G
cardiac muscle

cardiac muscle, the latter pathway plays an important role under normal physiological conditions. The metabolic rate in this tissue undergoes changes in activity depending upon the energy required for contraction. Skeletal muscle cells do not contain measurable amounts of free glucose, and penetration is the rate-limiting step in its utilization. Even though the oxygen consumption of contracting skeletal muscle increases during heavy exercise, aerobic oxidation is insufficient to supply all the energy required; skeletal muscle has the ability to obtain additional amounts of energy anaerobically and to accumulate an oxygen debt. Glucose is therefore very valuable for providing energy in skeletal muscle since it is the only substrate which can be catabolyzed without oxygen. After exercise has ceased, the oxygen consumption remains elevated for some time until creatine phosphate and glycogen return to their original levels. Some of the lactic acid produced by the muscle is converted to glucose in the liver, released into the bloodstream and is again available for metabolism in the muscle (Cori cycle); in addition, some glucose is obtained by gluconeogenesis from amino acids such as alanine and glutamine which are released from skeletal muscle and are subsequently captured by the liver (Ruderman, 1972). This appears to be of importance in starvation only.

Sugar penetration increases in exercise <u>in vivo</u> and <u>in vitro</u> and the stimulation of the sugar transport system induced by functional activity in muscle will be called activity regulation. An increase in the frequency of stimulation <u>in vitro</u> was found to enhance the activity of the transport system. The bulk of the evidence presented in the INTRODUCTION suggests that one or more of the events associated with

excitation-contraction coupling is probably responsible for this stimulation since when driven at the same frequency, sugar penetration in muscles with different work loads remains the same.

Anoxia will also increase sugar penetration and in vivo this is probably an important mechanism by which the supply of substrate to contracting muscle is increased. Apart from activity regulation, the sugar transport system is also stimulated by insulin, the hormone involved in the synthesis of energy reserves; this will be called storage regulation. The importance of glycogen as a source of energy for contraction has already been referred to. Even though the effect of insulin on the activity of glycogen synthetase is independent from its effect on sugar transport, the two events are coordinated in skeletal muscle. In contrast to adipose tissue, in skeletal and cardiac muscle the stimuli which enhance sugar transport to provide energy required for functional activity or for the deposition of energy reserves are well separated. The characteristics of sugar transport and metabolism in skeletal muscle are outlined in Fig. 18, diagram B. The dashed arrow indicates that the rate of glucose utilization is variable, depending upon the metabolic requirements of the muscle; the glycogen reserves are represented in the top left hand corner. As in adipose tissue, the rate of sugar penetration is quite low when the muscle is not working (as indicated by the small black area in the rectangle) while an increase in the activity of the sugar transport system can occur both with activity regulation or with storage regulation. This is symbolized by hatched areas. The various stimuli involved in activity and storage regulation are listed.

CARDIAC MUSCLE

Cardiac muscle is nearly entirely dependent upon oxidative phosphorylation for energy production and under normal conditions aerobic glycolysis does not take place to any significant extent. The Pasteur effect is present and under conditions which inhibit oxidative phosphorylation, glycolysis is much increased but is unable to support sustained function in mammalian heart (for review see Opie, 1968). Theoretically, the maximal activities of the glycolytic enzymes are high enough to provide ATP at the same rate as oxidative phosphorylation. Nevertheless, in isolated perfused rat hearts a high glycolytic rate lasts only for a few minutes and is not maintained even though glycogen stores have not yet been depleted. In certain amphibians such as turtles, anaerobic energy production may be of greater importance and cardiac function may depend upon glycolysis to a much larger extent.

The rate-limiting step in glucose utilization in the heart is its penetration across the cell membrane and this process is regulated by several mechanisms which ensure an adequate substrate supply under different conditions. When isolated hearts are perfused with an oxygen-deficient medium, the activity of the sugar transport system is greatly increased. As discussed above, the Pasteur effect is probably of minor importance <u>in vivo</u> in most warm blooded species, and in anoxia or severe hypoxia, cardiac function will be impaired. It has been suggested instead that the heart may adjust to increased energy requirements by extracting more oxygen from the coronary circulation. However, the significance of an increased substrate supply under mild hypoxic conditions remains to be evaluated.

The mechanical activity of the heart is subject to continual changes and represents an important regulator of sugar transport. As in skeletal muscle, there is a direct relationship between the rate of sugar penetration and the frequency of stimulation, as mentioned in the INTRODUCTION. However, in contrast to voluntary muscles, the activity of the sugar transport system in the heart is also directly dependent upon the load. These observations suggest that the activity of the sugar transport system is integrated with those factors which regulate the strength of contraction and thereby affect the metabolic requirements of the muscle. In cardiac muscle activity regulation is of great importance and affords a sensitive mechanism for supplying substrate as required by the work load on the heart.

The role of glycogen as reserve fuel in the heart is not well established but probably is only of minor importance. As summarized by Opie (1968), under certain experimental conditions, glycogenolysis takes place in isolated working heart preparations. Glycogen will only be degraded and used for cardiac work when no other substrates are available. In isolated working rat hearts, glycogenolysis is related to the work load in the absence of external substrate. In fasted rats cardiac glycogen is higher than in fed rats and is further increased during exercise. It has been concluded that there is no good evidence for the use of this reserve fuel <u>in vivo</u>. Even though the role of glycogen as reserve fuel is probably not of major physiological significance, the activity of the sugar transport system is stimulated by insulin and storage regulation takes place.

Diagram C of Fig. 18 outlines the characteristics of cardiac

muscle. In essence, these are the same as those of skeletal muscle. The rate of glucose utilization is variable, glycogen is present as reserve fuel and the sugar transport system exhibits activity and storage regulation. In addition to the stimuli listed for skeletal muscle, load on the contracting muscle also acts as a regulator.

SMOOTH MUSCLE

Compared to skeletal and cardiac muscle, much less is known about the metabolic requirements of smooth muscle. In this tissue both anaerobic and aerobic pathways are involved in ATP production but their relative contributions in providing energy for contraction under various experimental conditions remain to be established. It has been suggested that anaerobic glycolysis is of greater importance in smooth than in skeletal muscle (Stephens and Wrogemann, 1970). Sugar penetration is rate-limiting for its utilization and no free glucose has been demonstrated in this muscle. Early work by R-Candela et al. (1962) demonstrated that in rat uteri, glucose uptake was enhanced when the frequency of contraction and the contractile force were increased by edrophonium. In contrast, during an acetylcholine-induced contracture there was a significant reduction in the uptake of this substrate. High concentrations of insulin stimulated glucose utilization under certain conditions. These studies did not provide any direct evidence for mediated sugar transport in this tissue. In the toad urinary bladder glucose uptake could also be stimulated by insulin (Bower and Grodsky, 1963), and Timms et al. (1966) demonstrated countertransport of sugars in guinea-pig taenia coli muscle. Roskoski and Steiner (1967) have shown that 3-O-MG enters rat uterus by facilitated diffusion.

In the detrusor muscle of the urinary bladder, the most important characteristics for mediated transport also have been demonstrated (Bihler et al., 1971): In this tissue sugar penetration displayed saturation kinetics, chemical specificity, competition between pairs of sugars and could be inhibited by phlorhizin and phloretin. As in the other types of muscle, the Pasteur effect was operative and inhibition of aerobic metabolism increased the transport of 3-O-MG. The addition of ouabain or incubation in a potassium-free medium, conditions which affected the normal sodium and potassium gradients across the cell membranes, also increased sugar penetration. It appears that many of the agents and conditions which stimulate the activity of the sugar transport system in cardiac and skeletal muscle are equally effective in smooth muscle. Sugar transport was also stimulated by insulin although the concentration required in vitro was much higher than in either cardiac or skeletal muscle. The presence of glycogen has been established in uterine and intestinal smooth muscle (Boettiger, 1946) but little is known about the physiological role of this energy reserve. One might speculate that, if anaerobic glycolysis is indeed of major importance in smooth muscle, its metabolic pattern may somewhat resemble that of skeletal muscle and glycogen would be used as a substrate. The high concentration of insulin required to stimulate the sugar transport system could be due to the experimental conditions used as well as to differences in the sensitivity of smooth muscle in vivo and in vitro.

From the foregoing discussion it seems probable that many, if not all, of the agents and conditions which stimulate the activity

of the sugar transport system in skeletal and cardiac muscle are equally effective in smooth muscle.

The factors and conditions which stimulate the activity of the sugar transport system in cardiac, skeletal, and as far as investigated, smooth muscle, appear to be almost identical. One interesting difference is the absence of a load-dependent stimulation in skeletal as compared to cardiac muscle. This difference in activity regulation may perhaps be related to their contractile properties. Cardiac muscle 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. In in vitro experiments with skeletal muscle usually a supramaximal stimulus is given and all individual muscle fibers will be depolarized. A load-dependent regulation might be found if the stimulus strength were to be gradually increased from the threshold of the most easily excitable fibers to supramaximal. On the other hand, the load-dependence of sugar transport in cardiac muscle would appear to be related to the graded response of its fibers to stimulation. Thus, there is an important difference in activity regulation between these two types of muscle.

LEUKOCYTES

Sugar penetration in leukocytes has not been studied in any great detail and it is uncertain whether glucose transport is rate-limiting for its utilization. There is, however, some evidence consistent with the existence of a link between transport and metabolism. Glucose uptake is increased during phagocytosis in polymorphonuclear leukocytes where this activity is linked to an increase in glucose

oxidation (Karnovsky et al., 1966). In human leukocytes both glucose utilization and xylose transport have been reported to be increased by insulin (Luzzatto, 1960; Kalant and Schucher, 1962). Glycogen is present in these cells.

General conclusions

Our own results with the lens of the eye and published data for other tissues have been used in an attempt to correlate the regulation of facilitated diffusion of sugars with the metabolic activity in various tissues. This has led to the concept that the activity of the sugar transport system is integrated with the metabolic requirements of tissues. As a general rule, in tissues where glucose utilization is stable, free intracellular sugar is present and the transport system is not regulated. On the other hand, in tissues where glucose utilization is variable, its penetration across the cell membrane is rate-limiting and provides an additional means of controlling metabolism. In these tissues regulation of the transport system is achieved by the same factors which modulate activity (e.g. muscular contraction, phagocytosis) and deposition of energy reserves (e.g. insulin). The liver does not fit neatly into this classification because of its exceptional function to release glucose.

SECTION V

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